Australian Network for Plant Conservation Inc

Plant Germplasm Conservation in Australia

strategies and guidelines for developing, managing and utilising ex situ collections in Australia

Third Edition

Edited by Amelia J. Martyn Yenson, Catherine A. Offord, Patricia F. Meagher, Tony D. Auld, David Bush, David J. Coates, Lucy E. Commander, Lydia K. Guja, Sally L. Norton, R.O. (Bob) Makinson, Rebecca Stanley, Neville Walsh, Damian Wrigley, Linda Broadhurst.

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The Australian Network for Plant Conservation (ANPC) in partnership with the Australian Seed Bank Partnership (ASBP)

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Dedication

With gratitude to all those who introduced us to the beauty and complexity of Australia's flora, and to the children and grandchildren who inspire our efforts to conserve Australia's biodiversity.

About the Australian Network for Plant Conservation (ANPC)

The ANPC is a national not-for-profit, non-government organisation established in 1991. Our mission is to *Promote and develop plant conservation in Australia* and our vision is *that Australia's native plant diversity and habitats are valued and safeguarded*. We support people and organisations dedicated to the conservation of Australia's native plant species and vegetation communities. This has been achieved over the past 30 years through facilitating the open exchange of information and ideas amongst researchers, policy makers, government agencies, practitioners, private organisations and the community through best-practice guidelines, conferences, newsletters, our quarterly bulletin *Australasian Plant Conservation*, workshops and training. The ANPC is sustained via memberships, grants, fundraising and donations, and relies heavily on volunteer contributions.

For more information about the Australian Network for Plant Conservation and to find out how you can support us through membership, sponsorship and donations:

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About the Australian Seed Bank Partnership (ASBP)

The Australian Seed Bank Partnership is an alliance of 13 organisations, bringing together expertise from Australia's leading botanic gardens, state environment agencies and NGOs. The Partnership's vision is *a future where native plant diversity is valued, understood and conserved for the benefit of all*. Governed by The Council of Heads of Australian Botanic Gardens Inc., the Partnership works on strategic solutions to deal with the multitude of threats facing Australia's biodiversity. The Partnership is committed to building our shared knowledge in seed science through our national network of conservation seed banks and associate organisations across Australia. Collectively we are working to ensure future access to Australia's diverse native botanical resources under both present and changing climates and in response to major disasters.

For more information about the Australian Seed Bank Partnership:

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Foreword

Having had a long professional association with the Australian Network for Plant Conservation, and gained a deep appreciation of their knowledge and experience in plant conservation over many years, I am delighted to write this foreword for the latest iteration of ANPC's Plant Germplasm Guidelines.

This third edition and update of the Guidelines is timely, with significant advances in knowledge gained through research and experience at a time when threats to plant diversity are increasing. The emergence of new pests and diseases, such as Myrtle Rust, the increasing frequency and intensity of bushfires, and recurring extreme weather trends and events are not just confined to Australia. They are a worldwide phenomenon. It was only a decade ago that the Stern Review warned us of the consequences of climate change, and those predictions have come to pass far more quickly than we could have imagined. This gives added urgency to our mission to conserve and manage plants for future generations. Fortunately, we continue to learn from what does and doesn't work and – crucially – to share that learning through publications like this.

In this volume, ANPC's collaborators, including the ASBP, document their experience in planning, collecting and managing collections of both common and threatened species. There is also increased emphasis on the role of genetics in capturing and maintaining ex situ plant collections. Genetics is covered comprehensively in Chapter 3 but is a theme throughout. In addition, the ex situ conservation techniques covered in this volume have been refined and applied to a wider range of plant species than ever before. This will help us restore ecosystems with a greater diversity of plant species, which confers resilience at a landscape scale. There is significant new content to guide readers in identifying seed storage behaviour (Chapter 6) and conserve species that are difficult to store using conventional seed banking techniques, including rainforest species, and orchids (Chapter 12), as well as non-seed germplasm such as spores and pollen (Chapter 13). The Guidelines also include updated information on cryopreservation (Chapter 10), which provides the potential to store collections for much longer periods. We have more information on alleviating dormancy in seed collections (Chapter 7), and new information on how to identify short-lived species (Chapter 5). Readers will also get an insight into why particular plant species might need to be re-collected or regenerated, and why ongoing curation of collections is so important (Chapter 15).

Of course, storing and germinating seed are important first steps but, often much more difficult to do is establishing plants in ex situ living collections or in situ back in the wild. In Chapters 8 and 11, readers can find guidance on the horticulture of threatened species. Chapter 8 on the importance of the nursery in maintaining ex situ collections is new, and there are updates to Chapter 11 on managing and maintaining living collections. Both detail why strict hygiene measures are so critical to protecting the Australian flora from threats such as Myrtle Rust, which has emerged as a threat since the last edition of the Guidelines was published in 2009. Throughout the Guidelines, there are tips for maintaining meticulous record keeping, including managing the data and images that go with plant collections. This element is key to learning how to carry out plant conservation better, and is one of the things that differentiates botanic garden collections from those of other kinds of gardens. Ultimately, we want to make the most of every single germplasm collection to safeguard Australia's national plant treasures. We share our collections, data and expertise so that these species are available for ecological restoration, translocation, agriculture, horticulture and other uses into the future.

Finally, the challenges in plant conservation are so huge (there are more threatened plant species than all of the world's mammal and bird species put together) that we cannot work in isolation. Working in partnership, and the importance of collaboration, comes through very strongly in these Guidelines, particularly in the case studies. I hope that you will not only find this volume useful but inspiring as well.

Dr Paul Smith, Secretary General, Botanic Gardens Conservation International

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The Ian Potter Foundation for funding the revision of these 'Germplasm Guidelines' to support conservation of Australia's national plant treasures, and particularly Louise Arkles for her support throughout the project.

Australian Network for Plant Conservation: Jo Lynch, Christine Fernance, fellow staff Lucy Commander and Martin Driver, and office volunteers, as well as ANPC committee members.

Australian Seed Bank Partnership National Co-ordinator Damian Wrigley and project officer Bradley Desmond.

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The authors of each chapter and case study, and the organisations that supported them. Despite the distractions imposed by an extraordinary year (Black Summer fires, bushfire recovery efforts and the COVID-19 pandemic), all were generous in sharing their time and expertise.

The authors of the Guidelines second edition, many of whom have been involved again in this third edition, and others who provided input to earlier editions.

The original ANPC Germplasm Working Group: David Coates, Kingsley Dixon, Peter Lawrence, Jock Morse, Mark Richardson, Dale Tonkinson, Anne Cochrane and Darren Touchell.

The reviewers who provided valuable comments and feedback to improve this third edition and are acknowledged by name in each chapter.

The project manager, Amelia Martyn Yenson, would particularly like to thank the Germplasm Guidelines steering committee for their support – Jo Lynch, Lucy Commander, Bob Makinson, David Coates, Linda Broadhurst, Damian Wrigley, Sally Norton, Cathy Offord, Lydia Guja, Neville Walsh, David Bush, Rebecca Stanley, Tony Auld – and her colleagues at the Australian Institute of Botanical Science for their encouragement and feedback during the revision process. It is a pleasure and an inspiration to work with people who love plants and do so much to save species from extinction.

Organisation logos of steering committee and lead authors:

Australian Network for Plant Conservation Inc

Australian Grains Genebank

Australian Institute of Botanical Science

The Royal Botanic Gardens & Domain Trust

Australian National Botanic Gardens

Australian Seedbank Partnership

Auckland Botanic Gardens Where ideas grow

Botanic Gardens Conservation International

Centre for Australian National Biodiversity Research

CSIRO

Government of Western Australia

Department of **Biodiversity, Conservation and Attractions**

NSW Government

Australian Government

Parks Australia

Royal Botanic Gardens Victoria

Contributors to the third edition

John Arnott has held the role of Manager of Horticulture at the Royal Botanic Gardens Victoria, Cranbourne Gardens since 2008. John has provided inspiration and leadership to the growing team of horticultural staff, with a key focus on the horticultural management of the Australian Garden and facilitation of a range of conservation horticulture activities. Prior to this, he worked in significant public gardens including Melbourne Zoo for over 20 years, and the Geelong Botanic Gardens. In 2015, John was awarded the Australian Plants Award by the Australian Native Plant Societies (Australia) in recognition of his outstanding contribution to the knowledge of Australian plants.

Tony Auld is a Professorial Fellow at the University of New South Wales and the University of Wollongong, and an Honorary Scientific Fellow at NSW Department of Planning, Industry and Environment. He has worked on a broad range of Australian plants, with a particular focus on extinction risk, impacts of threats (such as altered fire regimes and feral animals) and the role of soil seed banks in maintaining plant populations.

Christine Bach wrote her PhD in monsoon rainforest ecology investigating the interactions between frugivores and monsoon rainforest patches in the Top End of the Northern Territory. This study involved seed germination studies, seed dispersal and predation. She was also involved in developing a monitoring program for the Mary River Floodplains. She has been a High School Teacher for the last 15 years.

Daniel Ballesteros has been a research fellow since 2015 in the Comparative Seed Biology Group at the Royal Botanic Gardens, Kew, UK. Daniel has over 15 years of experience in diverse international centres focused on the science and preservation of plant genetic resources in USA, South Africa, Spain and Italy. He is known for his fundamental work on the physicochemical and structural basis of seed and spore desiccation, freezing tolerance and longevity. Daniel's research also has an applied component focused on the development and optimization of seed, spore and pollen storage protocols, including cryopreservation.

Sarah Barrett is a Threatened Flora Conservation Officer with the WA Department of Biodiversity Conservation and Attractions, based in Albany on the South Coast of WA. Her work is largely focussed on the conservation of flora threatened by plant disease, in particular Phytophthora Dieback, as well as fire, herbivory and a drying climate, and involves both in situ and ex situ recovery actions.

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Linda Broadhurst has almost 20 years' experience applying molecular and ecological tools to enhance the long term persistence of vegetation. Her research has helped Natural Resource Managers and Non-Government Organisations to redefine their collecting practices to maximise genetic diversity in the seed being used for restoration projects.

Eric Bunn is a Research Scientist at Kings Park Science, Biodiversity Conservation Science, DBCA Western Australia. Eric manages the Conservation Biotechnology program at Kings Park, curating the tissue culture and cryopreservation collections. Eric's research has focused on endangered Australian native plants for ex situ conservation and translocation, establishing new in vitro propagation and cryopreservation methods for these species.

David Bush is the Director of the Australian Tree Seed Centre. His research interests include conservation, domestication and genetic improvement of tree and shrub species in Australia and the Pacific.

Stephanie Chen is a PhD Candidate at the University of New South Wales and the Royal Botanic Gardens and Domain Trust, Sydney. She develops and optimises genomic resources, such as reference genomes, for native plants. A focus of Stephanie's research is the conservation of species threatened by Myrtle Rust.

Caroline Chong has a background in plant biodiversity research to understand the drivers of population variation and support decision-making for species conservation and management. As part of the National Seed Bank team at the Australian National Botanic Gardens, Caroline undertook research on seed biology and ex situ plant conservation, including seed banking, to achieve conservation outcomes, with a focus on threatened and significant flora of the Commonwealth National Parks. Currently, she is a Threatened Species Botanist with the Northern Territory Government, where she leads the conservation assessment and listing of threatened and significant flora in the NT, strategic species conservation management planning and collaborative research to improve our knowledge about threats to plant persistence and biodiversity values from population to regional scales.

David Coates is a Research Associate at the Department of Biodiversity, Conservation and Attractions, and adjunct Professor at the University of Western Australia and Murdoch University, Perth. His research activities cover conservation genetics, restoration genetics, population biology and evolutionary biology with particular interests in the recovery of rare and threatened plant species, plant translocations, plant mating systems and managing threatening processes such as Phytophthora Dieback and habitat fragmentation.

Anne Cochrane is currently an Honorary Research Associate with the Department of Biodiversity, Conservation and Attractions, Western Australia, and previously a senior research scientist and manager of the Department's seedbank for threatened species. Her research focussed on the seed biology of threatened WA plant species and the influence of a changing climate on seed germination of native WA species.

Lucy Commander is a Project Manager with the Australian Network for Plant Conservation. She has been responsible for co-ordinating the updates of the Florabank Guidelines – best practice guidelines for native seed collection and use – and the Guidelines for the Translocation of Threatened Plants in Australia, as well as running seminars and workshops. Lucy's background is in restoration seed ecology, and she has undertaken research projects in collaboration with mining companies in Western Australia to help them improve their seed-based restoration, with a particular focus on seed germination and dormancy, and restoring plant communities.

Andrew Crawford is the manager of the Department of Biodiversity, Conservation and Attractions, Western Australian Seed Centre in Kensington (Perth). Andrew has been involved with the collection of Australian native plant species for over 30 years, with 20 years spent collecting and conserving seed of Western Australia's conservation significant plant species. Andrew's work covers all aspects of the seed conservation process; from seed collection and processing, to seed storage and testing, generating plants for translocations, as well as seed focused research aiding in threatened species management and recovery.

Adam Cross is recipient of the Research Fellowship in Restoration Ecology at Curtin University and is Science Director for international restoration initiative the EcoHealth Network. Adam is a seed biologist and restoration ecologist regarded as an expert on the ecology and reproductive biology of carnivorous plants. Adam's research encompasses the ecological requirements for seed dormancy alleviation and the stimulation of germination, as well as the collection, storage, and use of seeds in ecological restoration.

Peter Cuneo is Manager, Seedbank & Restoration Research at the Australian PlantBank, part of the Australian Institute of Botanical Science at the Australian Botanic Garden, Mount Annan, where he leads the statewide threatened species seed conservation program. Peter's main research interest is restoration ecology including threatened plant translocations, management of invasive species and restoration techniques for grassy woodland ecosystems.

Emma Dalziell is a Postdoctoral Research Scientist at Kings Park and Botanic Garden (Perth) and the University of Western Australia. She has been working with seeds for 10 years, across a range of restoration and conservation projects. Her current research focusses on the ex situ conservation of seeds of wild species; specifically, the need to find non-destructive technologies/ methodologies for assessing seed viability, and for predicting seed longevity in storage.

Belinda Davis is the orchid research scientist based at Kings Park and Botanic Garden, for Kings Park Science/Department of Biodiversity, Conservation and Attractions. Her research is focused primarily on the conservation of threatened orchid species. Belinda's research encompasses orchid mycorrhizal nutrition and ecology, pollination ecology, ex situ conservation, population dynamics, propagation, and translocations.

Rebecca Dillon is a Research Scientist at the Department of Biodiversity, Conservation and Attractions, Western Australia. Rebecca's research encompasses techniques for the establishment, management and measures of success for threatened plant translocations.

Richard Dimon is a Research Assistant with the Royal Botanic Gardens Victoria Orchid Conservation Project. He helps propagate and maintain the extensive terrestrial orchid collection situated at Cranbourne Gardens. This includes research on symbiotic propagation, ex situ cultivation, pollination and reintroduction studies in order to facilitate conservation efforts for Australia's most threatened orchid species.

Nathan Emery is a Restoration Biology Officer at the Royal Botanic Gardens and Domain Trust, based at the Australian PlantBank, part of the Australian Institute of Botanical Science. Nathan's research is a combination of plant ecology, seed biology and restoration. He has worked with difficult to propagate species, such as *Actinotus* and threatened *Persoonia* species, and has developed several threatened plant translocation programs. He also works on the seed biology of threatened ecological communities in northwest NSW.

Graeme Errington is the Seed Bank Curator for the Royal Botanic Gardens and Domain Trust, Sydney. He is responsible for managing the large and diverse collection of wild sourced seed held at the Australian PlantBank, part of the Australian Institute of Botanical Science. Graeme has an extensive background in collecting seeds of Australian native plants from a diverse range of habitats as well as processing, viability assessment and germination testing of seed collections.

Marc Freestone is a conservation assessment officer with the Department of Agriculture, Water and Environment. He has recently finished a PhD with the Australian National University and Royal Botanic Gardens Victoria on the propagation of *Prasophyllum* (Orchidaceae). He successfully researched how to grow this difficult genus of threatened orchids in cultivation, paving the way for future ex situ conservation programs for this genus.

Bryn Funnekotter is a Research Scientist at Curtin University and Kings Park Science, Biodiversity Conservation Science, DBCA, Western Australia. Bryn's research focuses on cryopreserving rare and threatened Australian native plants, with a focus on understanding the stresses imposed by the cryopreservation process.

Paul Gibson-Roy is a restoration ecologist specialising in re-establishing species-rich native grasslands and grassy woodlands. In 2004, he instigated the Victorian Grassy Groundcover Research Project (Melbourne University and Greening Australia), a state-wide, field-scale grassland restoration program. In 2011, the project expanded to focus on EPBC-listed Cumberland Plain Grassy Woodland and Paul developed advanced seed production facilities growing wildflowers and grasses for restoration across the region. Following the award of a Churchill Fellowship in 2016, Paul co-developed an ANPC-led national native seed sector survey. He now oversees rehabilitation with Kalbar Resources for their Fingerboards project in east Gippsland (Victoria), which has ambitious goals to restore nationally listed grassy woodland to ~200 ha of a post-mined landscape.

Toby Golson is currently a Senior Horticulturalist at the Australian National Botanic Garden (ANBG) and has been the custodian of the rainforest living collection since 2000. Between 2018–20, he coordinated the security and development of the broader ANBG living collection.

Joachim Gratzfeld is Regional Programmes Director at Botanic Gardens Conservation International (BGCI). In this capacity he manages BGCI's integrated ex situ and in situ conservation programmes at regional and in-country levels, working with botanic gardens and other partner organisations around the world. Joachim's main research and practical conservation interest lies at the interface between people's dependence on plant resources and conservation in times of rapid global change.

Alasdair Grigg is the manager of the Christmas Island Minesite to Forest Rehabilitation (CIMFR) Program. His background in ecological restoration and PhD in plant ecophysiology are applied in his role designing and implementing rehabilitation earthworks and reafforestation of mined areas on Christmas Island. He works with a team of dedicated staff, and oversees the collection, propagation and cultivation of a biodiverse assemblage of about 40 local flora species. Each year the CIMFR team plants ~20,000 trees (on average), and species selection is tailored according to the ecophysiological tolerances of each species matched with the edaphic and microclimatic conditions present at each site. Alasdair is passionate about Christmas Island's unique flora and fauna and their conservation management.

Lydia Guja is the Manager of the National Seed Bank in the Australian National Botanic Gardens and a Seed Conservation Biologist at the Centre for Australian National Biodiversity Research (a joint venture between CSIRO and Parks Australia). Lydia's research focuses on achieving conservation outcomes for native Australian plants, through an understanding of the biology, ecology and stress tolerance of seeds and plants.

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Robert (Bob) Makinson is a conservation botanist and plant taxonomist. He was Curator of the Australian National Herbarium (1992–2001), Conservation Botanist at the Royal Botanic Gardens and Domain Trust (2001–2016), and is a former president of the Australian Network for Plant Conservation.

Amelia Martyn Yenson is a Project Manager at the Australian Network for Plant Conservation and coordinated updates for this third edition of 'Plant Germplasm Conservation in Australia'. She has research interests in seed lifespan and germination, with a focus on improving seed quality for conservation storage and utilisation of Australian native plant species. Amelia's research is based at the Australian PlantBank, part of the Australian Institute of Botanical Science, Royal Botanic Gardens and Domain Trust.

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Stig Pederson has many years' experience in cultivation of Australian native flora, specialising in and being actively involved in ex situ conservation of several NSW South Coast threatened species. He has recently retired after more than 30 years employment with botanic gardens, initially at the Australian National Botanic Gardens in Canberra, then largely at the Booderee Botanic Gardens at Jervis Bay where he was the curator of living collections. Stig is currently applying his knowledge of local South Coast flora, engaging as a volunteer with the local community in assessing native vegetation associated with proposed development applications.

Valerie C. Pence is the Director of Plant Research at the Center for Conservation and Research of Endangered Wildlife (CREW) at the Cincinnati Zoo & Botanical Garden. Her work focuses on applying in vitro methods and cryobiotechnologies to the conservation of exceptional species. Her research has included developing protocols for the cryo-conservation of shoot tips, embryos, gametophytes, spores, seeds and pollen of species from across the U.S., many critically endangered.

Julie Percival is a horticulturalist who has specialised in conservation horticulture throughout her career at the Australian National Botanic Gardens. She is currently the acting Curator at Booderee Botanic Gardens in Booderee National Park, where she is responsible for the management of the gardens' living collection and nursery. Julie has a strong background in the cultivation and management of threatened species collections for ex situ conservation, research and translocation projects.

Lorraine Perrins is the Curator, Conservation Collections and Subantarctic Flora, at the Royal Tasmanian Botanical Gardens (RTBG) where she oversees ex situ potted collections and plant propagation for the RTBG collections, as well as supporting the Tasmanian Seed Conservation Centre by managing the seed orchards for this program. Lorraine also co-ordinates regular meetings of the Threatened Species Networking Group to encourage communication, information exchange and engagement regarding threatened flora in Tasmania.

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Karen Sommerville is a Research Scientist with the Royal Botanic Gardens and Domain Trust. Her research experience ranges from the reproductive biology and genetics of saltmarsh plants to symbiotic culture of orchids and the biology of rainforest seeds. She currently leads the Rainforest Seed Conservation Project at The Australian PlantBank, part of the Australian Institute of Botanical Science, working to determine which Australian rainforest species are suitable for seed banking and which require alternative methods of ex situ conservation.

Rebecca (Bec) Stanley is a threatened plant field botanist and a former curator of Auckland Botanic Gardens. Bec's most recent work focusses on collaborations to achieve plant conservation, revegetation and biosecurity in restoration and plant translocation. Bec also represented BGANZ on the Germplasm Guidelines steering committee.

Amelia (Millie) Stevens is a Research Technician at the National Seed Bank in the Australian National Botanic Gardens. She has a background in seed biology and horticulture. Millie's duties involve managing the Seed Bank Laboratory, overseeing a germination testing program of seed collections and conducting research on the seed biology of species from across Australia's diverse landscapes. Millie's interests lie at the intersection of horticulture and science, bringing together her knowledge and experience from both fields to support the conservation of Australia's flora.

Nigel Swarts is currently Centre Leader for Horticulture at the Tasmanian Institute of Agriculture. Nigel's research focuses on supporting sustainable productivity of Tasmania's horticulture sector. Nigel continues to be involved in conservation of Tasmania's orchids with the Royal Tasmanian Botanical Gardens.

David Taylor was the Curator of the living collections at the Australian National Botanic Gardens for more than 12 years. During this period, he prioritised collaborative work aimed at improving trajectories for biodiversity and plant conservation. This included work with many partners to secure threatened species as ex situ collections, with a particular focus on non-seed methods for use in research and restoration.

Vidushi Thusithana recently completed her PhD, "Seed biology, establishment ecology and comparison of community assembly models for rehabilitation of coastal dry seasonal rainforest near Darwin, Australia." She completed her Bachelor of Science (Honours) majoring in botany at the University of Peradeniya, Sri Lanka. Her honours project investigated seed dormancy in the medicinal weed *Cardiospermum halicacabum*.

Shane Turner is a Curtin University Research Scientist where he has been since 2020. During his 20-year career Dr Turner has produced over 80-refereed scientific papers and book chapters working across a range of disciplines including seed biology and ecology, threatened flora conservation, minesite restoration and tissue culture and cryogenics.

Marlien van der Merwe is a Research Scientist at the Research Centre for Ecosystem Resilience at the Australian Institute of Botanical Science, Royal Botanic Gardens and Domain Trust. Currently, the focus of her research is on using genomic and ecological data to inform restoration and conservation management. With a deep interest in biodiversity, she has applied a range of molecular tools to evolutionary studies of plants and fungi. She is equally happy in the field, in the glass house or at the computer.

Karin van der Walt is the Conservation and Science Advisor at Ōtari Native Botanic Garden, Wellington, New Zealand. She works on the ex situ conservation of New Zealand plant species and her work encompasses conventional seed banking, tissue culture and cryopreservation as well as long term pollen storage for key threatened species.

Mark Viler is the Nursery Coordinator for Penrith City Council. He manages the production nursery which provides all plants to support council's numerous green initiatives, Bushcare programs and projects. Mark is a Horticultural Nurseryman who has extensive experience with NSW threatened species in the areas of wild source collecting, specialist propagation techniques and the curation of clonal collections. He previously worked for the Royal Botanic Gardens and Domain Trust, and was based at The Australian Botanic Garden, Mount Annan.

Veronica Viler is a Horticultural Research Assistant in Germplasm Conservation at The Australian Plantbank, Australian Institute of Botanical Science, Royal Botanic Gardens and Domain Trust. Veronica's work is primarily cultivation of threatened species for research, translocation and conservation with a focus on terrestrial orchids, rainforest and Myrtle Rust susceptible species.

Neville Walsh is the Senior Conservation Botanist at the Royal Botanic Gardens Victoria. Neville instigated the Victorian Conservation Seedbank in 2005 and continues to contribute to that program and other work with threatened species. He is co-editor of the 4-volume *Flora of Victoria* and undertakes taxonomic research into various plant groups.

Katherine Whitehouse is a Research Scientist at the Australian Grains Genebank (AGG). She is one of the few international experts in applied seed longevity research, with many years of real-world experience of long-term seed storage for crop genetic resources conservation where the effective utilisation of the germplasm is the principle goal. Katherine's research focuses on optimising species longevity under long-term dry storage and improving efficiencies within genebank operations.

James Wood is the manager of the Tasmanian Seed Conservation Centre based at the Royal Tasmanian Botanical Gardens, Hobart. He has worked in and around seed conservation since 1989, starting at the Royal Botanic Gardens, Kew, Wakehurst Place before moving to Tasmania in 2005. He has experience working in the fields of horticulture, seed curation, seed collection, databasing and germination testing.

Warren Worboys is the Curator of Horticulture at the Royal Botanic Gardens Victoria Cranbourne Gardens. He has worked in a diversity of roles managing the nurseries, garden development, and indigenous bushland at the Melbourne and Cranbourne Gardens. He has travelled extensively across Australia, studying the flora and collecting and propagating plants to extend the RBGV's collections of wild provenance plant species.

Magali Wright is an environmental consultant with Enviro-dynamics and a project leader for the Landscapes Recovery Foundation. She supervises the Tasmanian Orchid Conservation Program based at the Royal Tasmanian Botanical Gardens, where she works with volunteers to grow Tasmania's threatened orchid flora to support conservation efforts. She is also involved in Tasmanian threatened eucalypt conservation with a focus on seed conservation to support restoration efforts.

Damian Wrigley is the National Coordinator of the Australian Seed Bank Partnership; Australia's national focal point for the Global Strategy for Plant Conservation under the CBD; and the Oceania Representative to the CITES Plants Committee. Damian is hosted by the Australian National Botanic Gardens.

Heidi Zimmer is a Research Scientist at the Centre for Australian National Biodiversity Research (a joint venture between CSIRO and Parks Australia). Heidi's research focuses on the ecology and conservation management of Australia's threatened plants and ecosystems. After completing her doctoral research on the Wollemi Pine, Heidi worked on extinction risk assessment for NSW's threatened flora. Heidi's research is currently focused on orchid taxonomy and conservation.

Chapter 1
Introduction

Amelia J. Martyn Yenson, Lucy E. Commander, Catherine A. Offord and R.O. Makinson

This chapter is a revision of the previous edition chapter by Catherine A. Offord and R.O. Makinson, 2nd edition, pp. 1-10.

1.1 Background

There are more than 21,000 terrestrial plant species known in Australia[1] occurring across many different bioregions (Figure 1.1). Internationally, Australia's flora is considered megadiverse, and demonstrates a high degree of **adaptation**[2], specialisation and **endemism**, as about 84 % of vascular plant species occur nowhere else in the world (Chapman 2009). Endemism is particularly high in **biodiversity hotspots** such as the Southwest Australia Ecoregion and the Forests of East Australia (Broadhurst and Coates 2017). This diverse native flora faces a multitude of **anthropogenic threats**, especially **habitat** loss, fragmentation and degradation.

Globally, and in Australia, the rate of biodiversity loss is increasing, and more species are threatened with **extinction** than ever before (IPBES 2019). While around 6 % of Australian plant species are listed as threatened nationally (DAWE 2020a), there are other species that are listed by States and overall, more than 10 % are considered threatened. The number of post-European plant extinctions in Australia is uncertain, with estimates ranging from 71 (aggregated current legislative lists), to 12 **taxa** almost certainly or probably extinct and a further 21 taxa possibly extinct in a significant recent reappraisal by Silcock *et al*. (2020). Discrepancies exist because of the difficulty of verifying plant extinctions in general, but particularly for those species that are poorly known through dubious taxonomy or vague occurrence information, difficulty in surveying due to their remote occurrence, or those that may have cryptic life form stages (Silcock *et al.* 2020). High numbers of threatened species are concentrated in regions where 'hotspots' of endemism coincide with highly modified agricultural and urban landscapes (Broadhurst and Coates 2017; Silcock and Fensham 2018).

Most of the threatened plant taxa are vulnerable to numerous threatening processes, with past and ongoing habitat loss, inappropriate fire regimes, invasive weeds, and disease having the most impact (Silcock and Fensham 2018) (Figure 1.2). The two diseases with the biggest impact are Phytophthora Dieback (*Phytophthora cinnamomi*) and Myrtle Rust (*Austropuccinia psidii*). Myrtle Rust was first detected in Australia in 2010 and is impacting restricted, threatened and hitherto common species (Makinson 2018). This disease is currently affecting rainforest, and some heathland and wetland flora along much of the east coast of Australia (Box 1.1).

1 Vascular plants including ferns and bryophytes numbered 21,171 species in Chapman (2009); more recent figures obtained from the CHAH Australian Plant Census (2021) database and experts (Monro A. pers.comm; Field, 2020; Klazenga N. pers.comm.) indicate a figure closer to 23,224 for data accessed April 2021.

2 Key terms are highlighted in bold on their first occurrence in each chapter and defined in the glossary.

Changed and inappropriate fire regimes are also a driver of biodiversity decline, such as fires that are too frequent or not frequent enough, too intense and not in the ideal season. There is an emerging trend to more extensive, hotter, and more frequent fires as the legacy of past and present land management practices intersects with changing climate (CSIRO 2020). The extreme bushfires of 2019–20 burned more than 10,000,000 ha of land in south-eastern Australia alone and impacted vegetation across 11 Australian bioregions and 17 major native vegetation groups (Godfree *et al.* 2021). Almost 600 species had more than 50 % of their range burnt, and up to 153 species had more than 90 % burnt (Gallagher *et al*. 2021). Although many of these species are resilient to fire, the biogeographic, demographic and taxonomic breadth of impacts are likely to have ongoing **conservation** management implications (Godfree *et al.* 2021). Fires at this scale can override the natural resilience of already fragmented and stressed species and vegetation systems.

Climate change is one of the drivers of change in nature (IPBES 2019) and is likely to significantly increase the magnitude of threatening processes (Broadhurst and Coates 2017). Climate change is likely to exacerbate issues associated with deforestation, fire, urban expansion, water extraction and tourism, as well as the spread of exotic pests and diseases (Australian National University 2009). Complex interactions, for example, between fire and climate change, may be even more important for the persistence of plant **populations** and individual species than direct impacts (Enright *et al*. 2015), such as the effect of drought followed by fire. Estimates vary as to the proportion of species subject to the effects of climate change now and in the future (see Silcock and Fensham 2018 and Gallagher *et al*. 2019 for discussion).

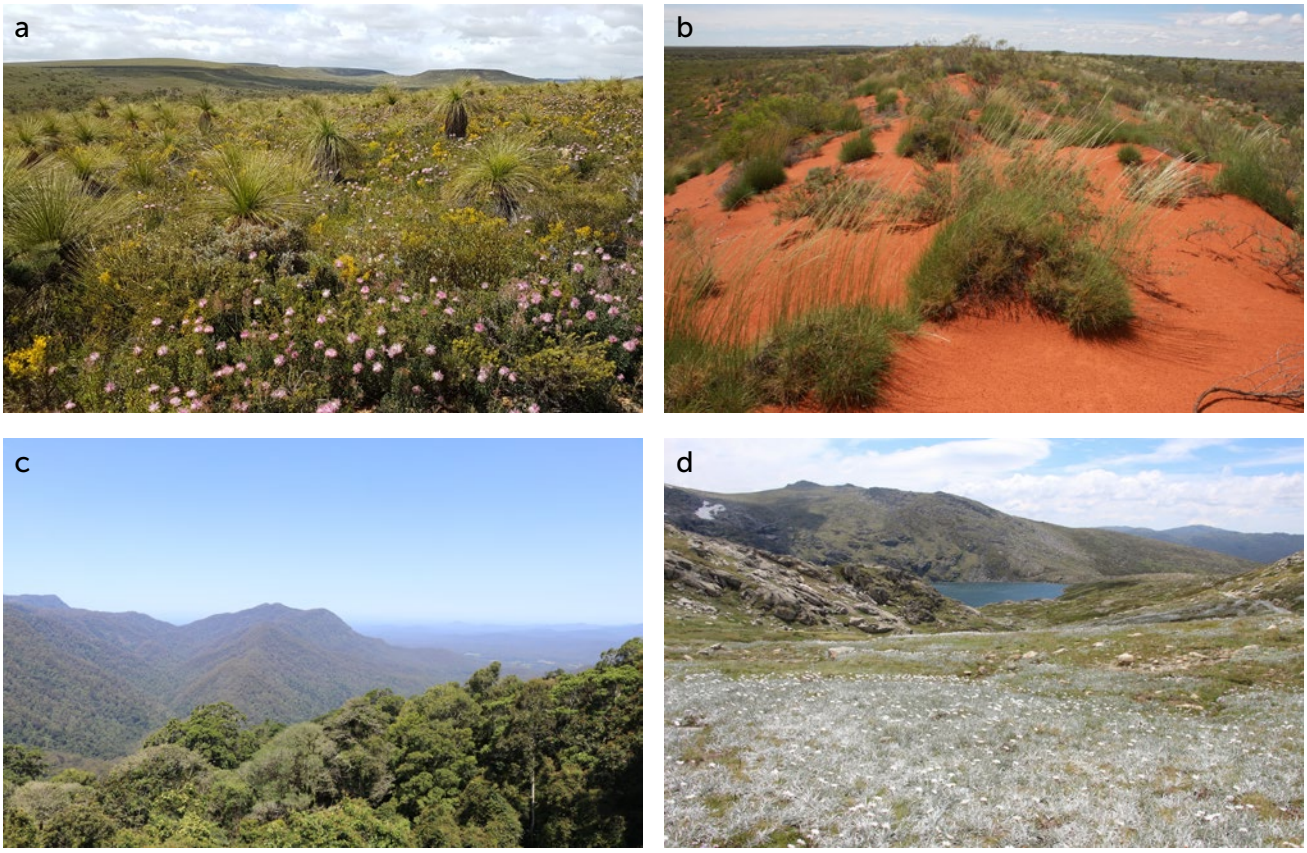

Figure 1.1: Australia has one of the most biodiverse floras on the planet; there are more than 21,000 known plant species. **(a)** Wildflowers at Mount Lesueur in south west Western Australia (Image: Lucy Commander). **(b)** Vegetation in the Great Sandy Desert in north west Western Australia (Image: Lucy Commander). **(c)** Rainforest vegetation viewed from the Skywalk lookout in Dorrigo National Park on the north coast of New South Wales (Image: Gavin Phillips). **(d)** Alpine vegetation around Blue Lake in Kosciuszko National Park in New South Wales. (Image: Gavin Phillips)

These complex and interactive factors are a threat to a large proportion of Australia's native vegetation and **ecosystems**, not only via extinction but through the loss of biological and genetic resources, adaptive potential, and landscape services vital for both natural and human needs. This underscores the need for consistent, coordinated action to conserve germplasm **ex situ**, alongside action to protect and restore plant species and communities **in situ**.

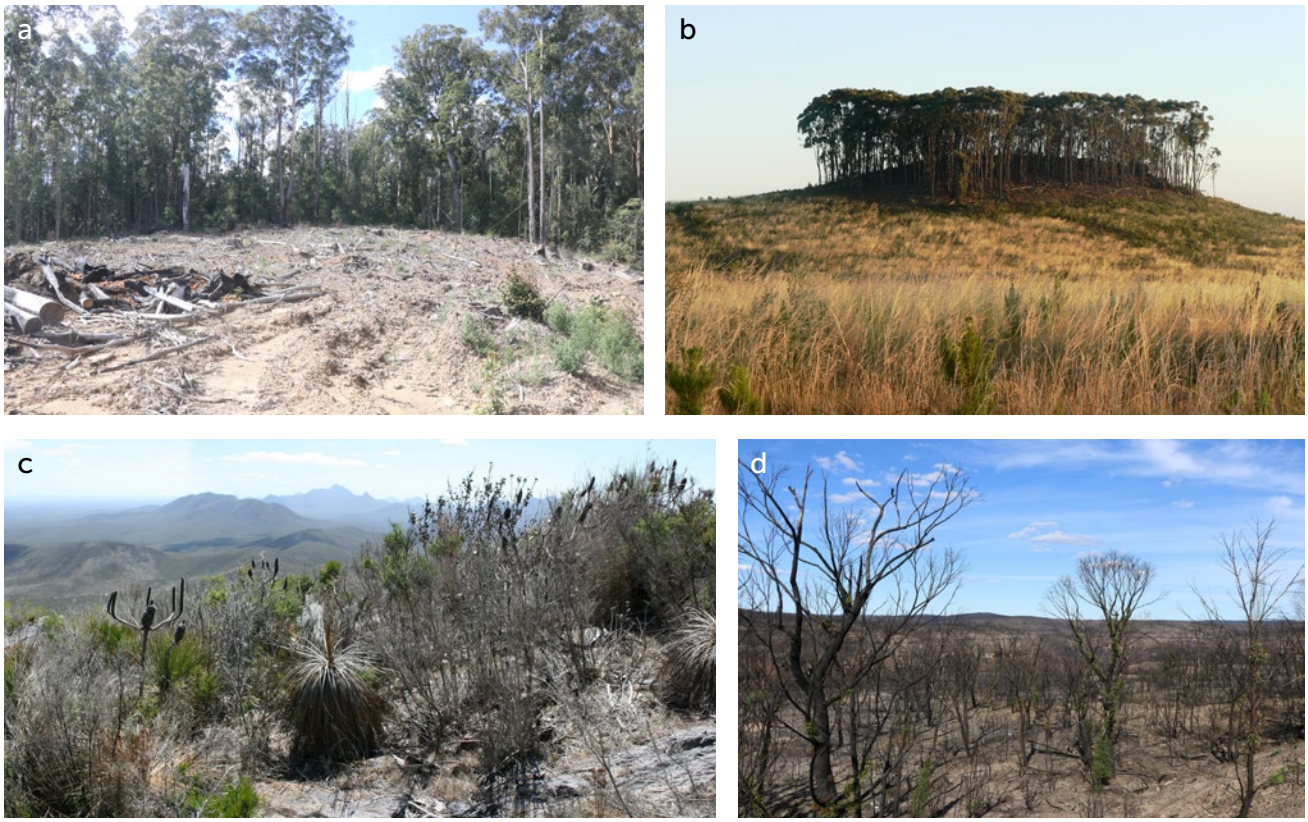

Figure 1.2: Threats to biodiversity in Australia include habitat loss and fragmentation, diseases such as Phytophthora Dieback and inappropriate fire regimes. **(a)** Land clearing (Image: Gavin Phillips). **(b)** Fragmentation in the landscape: a remnant of *Eucalyptus sieberi* near Penrose, New South Wales (Image: Bob Makinson). **(c)** Phytophthora Dieback at Mount Success, Western Australia (Image: D. Rathbone). **(d)** Vegetation burnt in the Black Summer fires, near Goulburn NSW. (Image: Gavin Phillips)

In the mid-1990s, the Australian Network for Plant Conservation (ANPC) initiated a Germplasm Working Group to prepare national guidelines for the conservation of plant germplasm, building on the work of the CSIRO Australian Tree Seed Centre and the very limited range of other Australian and international work available at that time. This resulted in the publication of *Germplasm Conservation Guidelines for Australia* (Touchell *et al*. 1997), supported by the Australian and New Zealand Environment and Conservation Council (ANZECC), which became a *de facto* national standard for germplasm conservation.

A second edition, *Plant Germplasm Conservation in Australia: strategies and guidelines for developing, managing and utilising ex situ collections* (Offord and Meagher 2009), was published 12 years later by the ANPC in collaboration with AuSCaR (now known as the Australian Seed Bank Partnership[3], ASBP, see Case Study 2.6). The second edition reflected the substantial increase, in Australia and globally, in knowledge of **seed** biology, plant reproductive biology, and technical expertise for germplasm collection, storage, and end uses (both for general **revegetation** and targeted conservation).

This third edition of *Plant Germplasm Conservation in Australia*, hereafter referred to as the 'Germplasm Guidelines', builds on those previous editions, reflecting rapid advances in science and practice. Increasingly, we see the available information synthesised and evaluated for application, supported by global advances in the technology associated with germplasm use and conservation (Li and Pritchard 2009; Hay and Probert 2013). The third edition provides updates on seed banking, **germination** and dormancy, **tissue culture** and **cryopreservation**, orchids and their **symbionts**, and living collections. New chapters have been added on **non-orthodox** seeds, special collections (mosses, ferns, pollen, carnivorous and **parasitic** plants), the role of the nursery, genetic guidelines for acquiring and maintaining collections, maintenance and utilisation of collections, and risk management.

In this introductory chapter, we define plant **germplasm**, discuss the scope and objectives of germplasm conservation, discuss ex situ plant conservation in the international and Australian contexts, and outline the purpose and content of the chapters that follow.

3 https://www.seedpartnership.org.au/

Box 1.1: Myrtle Rust

R.O. Makinson

Myrtle Rust disease is caused by the introduced fungal pathogen *Austropuccinia psidii*. It attacks new aerial tissues of a wide variety of species in the family Myrtaceae. First detected in Australia in 2010, Myrtle Rust disease is now established all along Australia's eastern seaboard, and in New Zealand. Australian and NZ species of this family are 'naive' to this pathogen and have no specifically co-evolved defences against it, although they show a wide range of susceptibility from zero ('immune' thanks to other characteristics), to extremely susceptible. Myrtle Rust spores are airborne, and the disease cannot be managed in the wild. It can be managed in cultivation.

Several species, some of them previously widespread, have near 100 % susceptibility and are declining catastrophically due to Myrtle Rust; these are likely to become extremely rare or extinct in the very near future. As of May 2021, three species – *Rhodamnia rubescens*, *Rhodomyrtus psidioides*, and *Lenwebbia* sp. 'Main Range' – have been listed as Critically Endangered in NSW and Queensland as a direct result of this pathogen; the first two were formerly widespread and common and of no conservation concern. Most or all of the genetic variation in these species, and the species themselves, will soon be lost except for whatever proportion of germplasm is captured and maintained ex situ. Rust-tolerant **genotypes** may exist but have not yet been detected. Searches for these, and the capture of a very wide range of genotypes followed by screening trials and selection of any rust-resistance traits, is the only path to recovery.

Up to 40 to 50 other species may also be faced with serious decline but many have variable susceptibility to Myrtle Rust *within* each species, from medium to extreme, sometimes patterned on a geographical or populational basis. These species face a more complex pattern of widespread local decline or local extinction, reduced reproductive capacity, and contraction or fragmentation of range and gene-flow. The pace of decline far outstrips the potential for natural selection to compensate in many cases. Again, much local genetic variation will be lost unless captured, but it is also vital to capture the full range of more rust-tolerant genotypes, to enable augmentation of seriously declining populations with stronger and regionally appropriate genotypes, and to enable selective breeding and trait-transfer where warranted.

For both these response-types, germplasm capture and its rigorous maintenance and development ex situ are of critical importance. The scale and pace of decline, and the number of species involved, present qualitatively new challenges to the ex situ capabilities of the plant conservation sector. Only some of these challenges are technical; many are 'human', relating to the design, resourcing, and continuity of a collaborative national recovery effort for Myrtle Rust-affected species.

Rhodamnia rubescens, direct infection of ripening fruits by Myrtle Rust; the fruits are undersized and contain no viable seed. This, and some other species, have virtually ceased to produce seed in the wild as a result of this pathogen. (Image: Craig Stehn)

1.2 Definition and scope of plant germplasm conservation

'Germplasm is living tissue from which new plants can be grown. It can be a seed or another plant part – a leaf, a piece of stem, pollen or even just a few cells that can be turned into a whole plant. Germplasm contains the information for a species' genetic makeup, a valuable natural resource of plant diversity.' (UC Davis Seed Biotechnology Center 2020). Figure 1.3 includes examples of a diverse range of germplasm. Germplasm conservation involves more than just **genes** – it may also encompass **epigenetic** and **somatic** components of the living organism, and even symbiotic partner organisms.

Figure 1.3: Examples of different types of germplasm. **(a)** Seeds of *Acacia brachystachya* (Image: Simone Cottrell). **(b)** Branches of *Wollemia nobilis* (Image: Amelia Martyn Yenson). **(c)** Tissue cultures of *Actinotus helianthi* (Image: Royal Botanic Gardens and Domain Trust). **(d)** Spores in sporangia on a fern frond (Image: Daniel Ballesteros). **(e)** Pollen grains of *Metrosideros bartlettii* (Image: Karin van der Walt).

Ex situ conservation strategies (Figure 1.4) preserve plants or plant germplasm away from the site of natural occurrence. Methods of ex situ conservation include 'stored' collections (seed, pollen, and tissue banks) and 'whole plant' or 'living plant' collections (**clonal** and seedling orchards, botanic gardens, and arboreta); and any collection of plant material not intended to regenerate itself naturally (Potter *et al*. 2017 and references therein).

In situ conservation (Figure 1.4) refers to conservation actions that occur 'on site' in ecosystems and natural habitats and incorporates the maintenance and recovery of **viable populations** of species in their natural surroundings (Secretariat on the Convention of Biological Diversity 2005). In the case of domesticated and cultivated species, this refers to the surroundings where they have developed their distinctive properties.

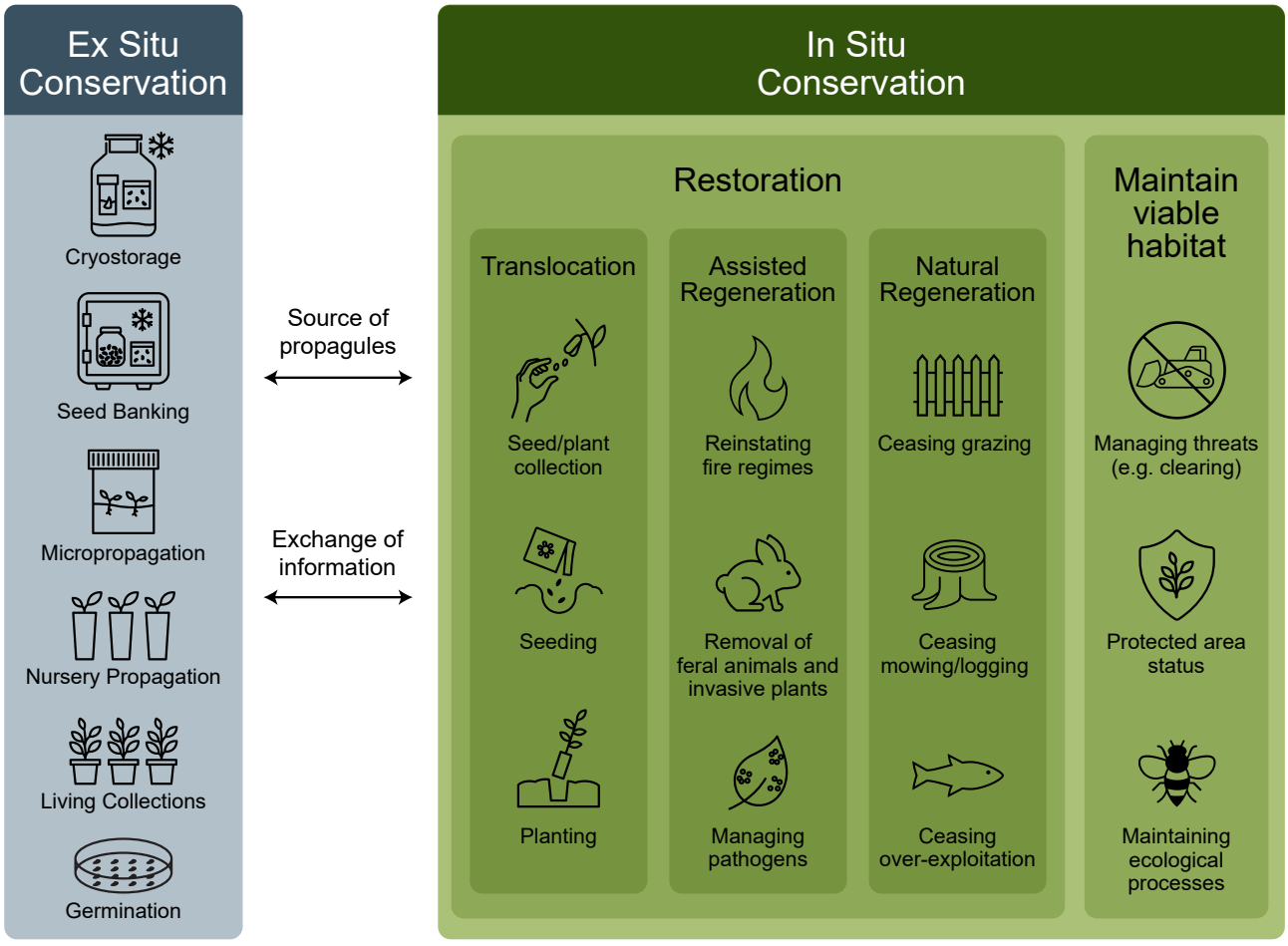

Figure 1.4: Links between various types of ex situ collections, and their role in supporting in situ ecological restoration approaches and actions through provision of **propagules** and knowledge transfer. Ex situ collections can contribute to the maintenance of viable populations through reintroductions, alongside in situ management activities such as undertaking assisted regeneration and facilitating natural regeneration. The capture of genetic variation, the scale of seed collection and the time frame for curating ex situ collections depends on the project goals. Source: Commander *et al*. (2021) Florabank Guidelines Module 1.

An ex situ conservation strategy should aim to capture genetic variation at the species, population, individual and allelic levels as **genetic diversity** provides the basis for adaptation of an organism to its existing environment and its potential for adaptation to future environmental changes. Ex situ germplasm conservation enables the use of this captured diversity in a variety of ways, primarily to enhance in situ conservation efforts.

Ex situ plant conservation activities, such as seed banking, do not by themselves constitute the protection of wild species; but complement in situ conservation efforts, especially for rare and threatened species and also when in situ reproduction and survival is not adequate to meet conservation goals (Smith 2006; Potter *et al*. 2017). Germplasm conserved ex situ, for example, may serve as insurance against the loss of populations or species and may be used to support the retention and **restoration** of wild plant diversity, as shown in Figure 1.5 (Potter *et al*. 2017). It may also be used to re-establish extinct populations in the wild or to supplement populations on the verge of extinction (Cochrane *et al*. 2007); if it has been collected with that purpose in mind (see Box 1.2). Conservation **translocation** (Commander *et al*. 2018) is an example of the complementary nature of ex situ and in situ conservation strategies (see Figure 1.4), as it typically utilises plants produced ex situ for in situ planting (Zimmer *et al*. 2019).

1.3 Objectives of germplasm conservation

The ultimate objective of ex situ germplasm conservation is to support the survival or restoration and continued natural evolution of species in **self-sustaining populations** in the wild. Specifically, ex situ germplasm conservation contributes to in situ conservation by:

- Providing material for propagation to remove or reduce pressure from wild collecting;
- The selection and provision of appropriate plant material for conservation actions;
- Providing material for translocation of rare, threatened or key species or ecosystem restoration and management;
- Enabling the rescue of threatened or 'condemned' germplasm, where destruction of a population is imminent and unavoidable (Bragg *et al.* 2021);
- Providing material for conservation biology research;
- Generating essential biological information on the species and its interactions with the environment and other biota;
- Providing material for selection and/or breeding for improved genetic diversity and/or enhanced characteristics for potential **reintroduction** (e.g., disease resistance, improved reproductive capacity);
- Generating skills and knowledge to support wider conservation aims;
- Contributing to education and raising public awareness about plant conservation.

(After Smith 2006; BGCI 2020)

Figure 1.5: Ex situ germplasm collections of *Persoonia* species (Proteaceae) are used to support in situ populations. **(a)** Cross section of *Persoonia hirsuta* drupe. **(b)** Germination of *Persoonia hirsuta* on water agar in a 24-well plate. **(c)** *Persoonia hirsuta* in tissue culture at the Australian PlantBank. **(d)** *Persoonia hirsuta* germinants potted up into soil. **(e)** *Persoonia hirsuta* plants in the nursery ready for planting out. **(f)** *Persoonia hirsuta* seedling in situ. **(g)** Nursery-grown *Persoonia pauciflora* plants ready for planting in situ. **(h)** *Persoonia pauciflora* translocation conducted as a local community planting day. (Images: Nathan Emery, Amanda Rollason)

The Australian Network for Plant Conservation, from its founding in 1991, has strongly advocated the integration of in situ and ex situ techniques – that is, making ex situ actions serve in situ conservation of plant biodiversity to the maximum extent possible, while recognising that ex situ germplasm maintenance has a variety of purposes.

Research required for effective ecosystem restoration includes sourcing biological material and optimising plant establishment, growth and survival (Miller *et al*. 2017). Ex situ germplasm is often the starting point for research in areas such as seed germination and dormancy, which are important to seed utilisation in restoration and translocation. Ex situ material can also provide a reference against which subsequent morphological, physiological or genetic changes in a population may be measured (Everingham *et al.* 2020). Like herbarium collections, these germplasm collections, such as Project Baseline[4] provide a rich resource for plant scientists to study long-term change and adaptation in plant populations.

Ex situ conservation is important because in situ management may not be sufficient to prevent a decline in threatened plant species, particularly under climate change (Mackey *et al*. 2008; for discussion see Broadhurst and Coates 2017) or with novel disease threats such as Myrtle Rust, even with landscape scale connectivity as a priority (Objective 5, Commonwealth of Australia 2019). At present, it is estimated that 20–25 % of Australian land is managed for conservation in the National Reserve System, which includes Commonwealth, state and territory reserves, Indigenous lands and protected areas run by not-for-profit organisations (Broadhurst and Coates 2017; DAWE 2020b). However, many imperilled species exist in small, fragmented remnants such as roadsides or rail reserves, rather than in National Parks or large remnants (Silcock and Fensham 2018). The importance of ex situ conservation has been recognised in the Convention on Biological Diversity as an activity that may be necessary to complement in situ measures (CBD 1992) and by the Australian Government to meet biodiversity conservation goals (Commonwealth of Australia 2019).

1.3.1 Actively conserving plant diversity

In the previous edition (Offord and Meagher 2009 p. 4), it was noted that '...even traditional habitat-based conservation strategies are moving from hands-off approaches to more active and interventionist methods. This trend toward recovery and reintroduction creates a strategic opportunity for ex situ institutions to serve as active partners in species-based research and recovery projects' (Maunder *et al*. 2004).

The national (Standards Reference Group SERA 2017) and international (Gann *et al.* 2019) standards for **ecological restoration** acknowledge the importance of choosing the appropriate ecological restoration approach depending on the capacity of each species to regenerate and the constraints to recovery. The capacity may depend on whether or not there is a soil or canopy seedbank, or whether the species is able to migrate from surrounding areas. The constraints include both the anthropogenic causes of degradation and their consequences. In situations where damage is relatively low, a natural regeneration approach, involving removal of threatening processes to allow recolonisation, resprouting or germination from the soil seedbank, is sufficient. With higher levels of degradation, an assisted regeneration approach may be more appropriate, in which greater intervention is required. At the highest levels of degradation, a reconstruction approach is needed, in which most of the biota need to be reintroduced. A combination of approaches may be needed in many circumstances.

4 http://baselineseedbank.org/

In particular, translocation (the deliberate transfer of plants or regenerative material from an ex situ collection or natural population to a new location, usually in the wild; Commander *et al*. 2018) has been recognised as 'an increasingly viable option for the conservation management of threatened plants, provided best practice guidelines are followed' (Zimmer *et al*. 2019 p. 501). Translocation includes reintroductions, introductions, reinforcements and assisted migration (Commander *et al*. 2018). Coordination of ex situ and in situ conservation efforts provides an important insurance measure against extinction (Potter *et al*. 2017; Zimmer *et al*. 2019).

1.4 Ex situ plant conservation in Australia and across the world

Recognition of the need to protect our remaining flora has led to Australia's participation in several international conservation initiatives which specifically include the ex situ conservation of plant germplasm. In 1993, Australia became a signatory to the Convention on Biological Diversity (CBD 1992). In response to the recognition that plants are an essential resource for the planet, the *Global Strategy for Plant Conservation* (GSPC) was developed (Sharrock 2020). Target 8 of the GSPC (2011–20) relates specifically to germplasm conservation with the aim to establish by 2020 'At least 75 % of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20 % available for recovery and restoration programmes.' (CBD 2010). In Australia, the Sixth National Report to the Convention of Biological Diversity 2014–18 (Commonwealth of Australia 2020) notes that a total of 67.7 % of nationally listed threatened flora are represented in Australia's ex situ conservation seed banks, with many already accessed to support recovery and restoration programs (Figure 1.6). Attainment of GSPC germplasm-related targets is ably supported by the Australian Seed Bank Partnership and it is notable that the next phase of the Partnership will focus on securing multiple collections that increase the genetic representation of the target species across their range (Commonwealth of Australia 2020; Sharrock 2020).

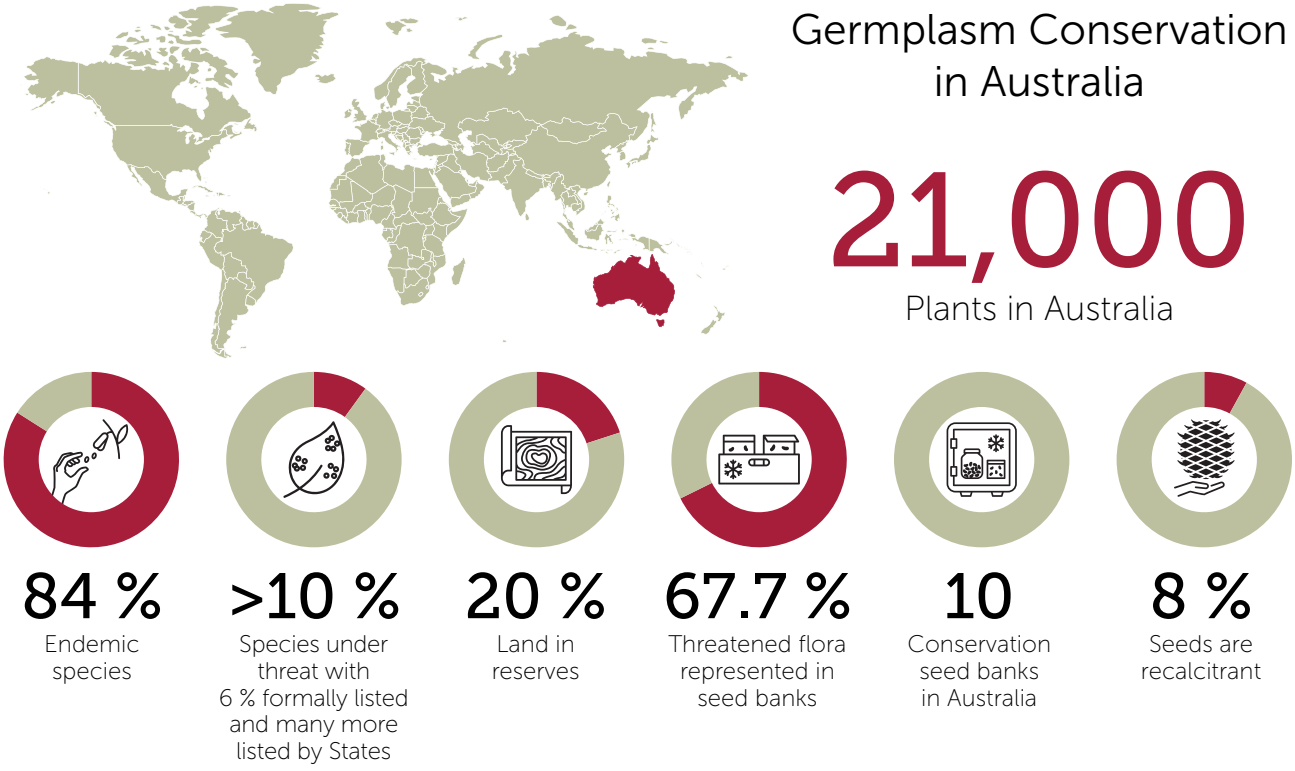

Figure 1.6: Germplasm conservation in Australia: an overview, as at April 2021. The proportion of recalcitrant species is a global estimate (Hay and Probert 2013) though prevalence is higher in some habitats (see Chapter 6).

The *National Strategy for the Conservation of Australia's Biological Diversity* (DEST 1996) was prepared in response to the CBD and reflected, at Commonwealth level, the widening community support for biodiversity conservation. The National Strategy called for States and Territories to strengthen ex situ conservation, and to integrate ex situ and other measures for the conservation of threatened species.

Australia's Strategy for Nature 2019–2030 (Commonwealth of Australia 2019) is the overarching national document for biodiversity targets set by the CBD, as well as those under other international agreements such as the Aichi Targets[5] and the Sustainable Development Goals[6]. Goals such as 'Care for nature in all its diversity' include both in situ actions and ex situ conservation measures. Action to support biodiversity also assists in interdisciplinary collaborations such as those of the WHO-IUCN Expert Working Group on Biodiversity, Climate, One Health and Nature-based Solutions. The *Strategy for Nature* complements other Commonwealth instruments such as the Threatened Species Strategy[7] and legislation including the *Environment Protection and Biodiversity Conservation Act* (1999) as well as State and Territory legislation (Table 1.1) and local government policies, programs and legislation. In addition, laws and policies in domains such as biosecurity, cultural heritage, and biological property rights may necessitate adaptations to the traditional practice of ex situ conservation.

Table 1.1: Principal primary legislation protecting flora in each jurisdiction and the authorities responsible for administering the legislation (Cuneo *et al*. 2021). Other legislation and authorities may also be relevant, for example, in NSW marine vegetation is protected under the *Fisheries Management Act* 1994 and the F*isheries Management (General) Regulation* 2010, administered by the NSW Department of Primary Industries.

Jurisdiction	Key Legislation*	Licensing Authority
Commonwealth lands	*Environment Protection and Biodiversity Conservation Act* 1999	Australian Government Department of the Environment
ACT	*Nature Conservation Act* 2014	Environment, Planning and Sustainable Development Directorate - Environment
NSW	*Biodiversity Conservation Act* 2016 *National Parks and Wildlife Act* 1974	Department of Planning, Industry and Environment (Environment, Energy and Science Group)
NT	*Territory Parks and Wildlife Act* 1999 *Biological Resources Act* 2006	Parks and Wildlife Commission of the Northern Territory
QLD	*Nature Conservation Act* 1992	Department of Environment and Science
SA	*National Parks and Wildlife Act* 1972	Department for Environment and Water
TAS	*National Parks and Reserves Management Act* 2002 *Crown Lands Act* 1976 *Threatened Species Protection Act* 1995 *Nature Conservation Act* 2002	Department of Primary Industries, Parks, Water and Environment
VIC	*Flora and Fauna Guarantee Act* 1988	Department of Environment, Land, Water and Planning
WA	*Environmental Protection Act* 1986 *Biodiversity Conservation Act* 2016	Department of Biodiversity Conservation and Attractions

**Parent Acts listed. Subsidiary legislation (e.g., regulations or guidelines) apply.*

5 https://www.cbd.int/sp/targets/
6 https://sustainabledevelopment.un.org/topics/sustainabledevelopmentgoals
7 https://www.environment.gov.au/biodiversity/threatened/publications/strategy-home

1.4.1 The role of botanic gardens

Botanic gardens, herbaria and museums, as well-established and often science-based institutions, play a key and unique role in the capture, maintenance and use of ex situ conservation collections. Recognising this, the '*National Strategy and Action Plan for the role of Australia's Botanic Gardens in Adapting to Climate Change*' was adopted in 2008 (CHABG 2008).

Botanic gardens aim to achieve plant conservation by:

1. providing a safety net against loss of species and genetic diversity through living collections, seed banks and germplasm collections;
2. providing knowledge and expertise of horticulture, species distribution and taxonomy (often in association with herbaria); and,
3. increasing education and community awareness.

Botanic gardens are hubs for the taxonomic, horticultural and seed bank knowledge and expertise and associated research required for ecological restoration (Hardwick *et al*. 2011), although biogeographic gaps exist, and collections focus on vascular plant species (Mounce *et al*. 2017). An estimated 25 % of botanic gardens globally have ex situ conservation programs; 19 % have seed bank facilities, while 10 % have **micropropagation** facilities; and 12 % have restoration ecology programs (Hardwick *et al*. 2011). In Australia, there are 10 conservation seed banks (Figure 1.6) working within the Australian Seed Bank Partnership, often within botanic gardens (see Case Study 2.6). Unlocking the knowledge and expertise within botanic gardens that have such programs is critical to improving many steps within the restoration framework and requires policy and management systems that encourage collaboration and interdisciplinary partnerships. Knowledge transfer and education are strengths within many botanic gardens so recording, reflecting on and encouraging best practice and sharing this information has the potential to benefit many facets of the restoration framework.

1.5 Relationship to other guidelines and knowledge systems

The ANPC plays a key role in facilitating knowledge transfer among all stakeholders involved in conservation by coordinating the production of best-practice guidelines such as those for translocation (Commander *et al*. 2018), seed-based restoration (Commander 2021) and this edition, as well as professional development workshops for different audiences.

The *Guidelines for the Translocation of Threatened Plants in Australia* (also known as the 'Translocation Guidelines'; Commander *et al*. 2018) provide a how-to guide for the process of translocation, commencing with decision making, through assessment of biology and ecology, site selection, preparation, implementation, monitoring and evaluation. The guidelines also provide information on policy and legislative approvals as well as community participation. The Germplasm Guidelines complement the Translocation Guidelines by providing more detailed information than the brief sections in the Translocation Guidelines (chapter 3 'Identifying appropriate propagation techniques' and '**Mycorrhizal** and **rhizobial** associations' and in chapter 6 'Establishment and maintenance of an ex situ collection').

The *Florabank Guidelines* (Commander 2021) is a set of best practice guidelines for native seed collection and use in restoration. The Florabank Guidelines focus on ecological restoration of non-threatened flora (but can apply to threatened ecological communities) and complement the

International Principles and Standards for the Practice of Ecological Restoration (Gann *et al*. 2019). The Germplasm Guidelines complement the Florabank Guidelines as they provide more detailed information on long term seed storage, ex situ storage of non-orthodox species, and propagation and storage of vegetative material.

Informed by existing international plant germplasm conservation procedures for crop species, and an expanding literature base for ex situ wild species conservation globally, these guidelines on ex situ conservation methods aim to be applicable to Australia's diverse and specialised native flora including Australian crop wild relatives. Though written specifically for use with Australian vascular plants, the principles and procedures described here may be applicable to other floras.

The Germplasm Guidelines draw upon a diverse set of guidelines with similar goals and seek to place the relevant scientific principles and global practices into an Australian context for conservation of wild species using ex situ methods.

Other guidelines that can be explored by Australian practitioners include:

- *IUCN guidelines for reintroduction*[8] (IUCN/SSC 2013) and IUCN ex situ conservation guidelines[9] (IUCN/SSC 2014),
- *Millennium Seed Bank Partnership (MSBP) Seed Conservation Standards*[10] (2015, updated 2019) and associated resources,
- *CPC Best Conservation Practices to Support Species Survival in the Wild*[11] (Center for Plant Conservation 2019),
- *The Bioversity International Handbook of Seed Handling in Genebanks*[12] (Rao *et al*. 2006),
- *FAO Genebank Standards for Agriculture*[13] (FAO 2014),
- *ISTA Rules for Seed Testing*[14] (ISTA 2021) and
- *Nursery Industry Accreditation Scheme Australia Best Management Practice Guidelines*[15] (Greenlife Industry Australia 2019).

Implementation of practices within these Germplasm Guidelines should be navigated with due respect for the experience and intellectual property of knowledge holders, whether of scientific tradition, Indigenous knowledge and experience developed over thousands of years, other cultural, ethnic or social traditions, or legislatively prescribed property law. In Australia, attention to the Indigenous knowledge system and rights is particularly important as more than 44 % by area of the National Reserve System is managed or co-managed by Indigenous groups for biodiversity conservation (DAWE 2020c). The complex, contemporary problems of conservation require sharing from multiple knowledge systems, followed by action shared by interested communities, individuals, government agencies and organisations. Researchers are encouraged to open their minds to Indigenous knowledge systems and co-design mutually rewarding projects with Indigenous knowledge holders, starting with ample time for genuine engagement. Given that short-term grants often don't accommodate the necessary timeline for relationship building, practitioners are encouraged to make this exchange a 'lifelong project'. The Florabank Guidelines Module 2 (Van Leeuwen *et al*. 2021) and Translocation Guidelines (Commander *et al*. 2018 Chapter 5.2 and 9.2) provide background information and a list of resources on traditional knowledge including guidelines for engagement with Indigenous knowledge holders.

8 https://www.iucn.org/content/guidelines-reintroductions-and-other-conservation-translocations
9 https://portals.iucn.org/library/sites/library/files/documents/2014-064.pdf
10 http://brahmsonline.kew.org/msbp/Training/Resources
11 https://saveplants.org/wp-content/uploads/2020/12/CPC-Best-Practices-5.22.2019.pdf
12 https://www.bioversityinternational.org/e-library/publications/detail/manual-of-seed-handling-in-genebanks/
13 http://www.fao.org/3/a-i3704e.pdf
14 https://www.seedtest.org/en/international-rules-for-seed-testing-_content---1--1083.html
15 https://nurseryproductionfms.com.au/niasa-accreditation/

Guidelines such as the *AIATSIS Code of Ethics* (Australian Institute of Aboriginal and Torres Strait Islander Studies 2020) and *Our Knowledge Our Way in caring for Country* (Woodward *et al*. 2020) are a useful starting point. The onus is on conservation practitioners to seek guidance on respectful approach and engagement with all stakeholder groups.

Box 1.2: Guidelines for sampling for ex situ conservation vs ecological restoration

In a review of collecting guidelines, Broadhurst *et al*. (2008) compared the differences between collection of germplasm for conservation activities, such as translocation of a single species or restoration of a threatened ecological community, and the ecological restoration of communities on a landscape scale (from small, 1–10 ha, to large 10^2–10^6 ha). The aim to create a self-sustaining population through translocation, or to restore a self-organising ecosystem through ecological restoration, often requires germplasm sampling on a different scale and of a different nature (Merritt and Dixon 2011).

For example, The Center for Plant Conservation (2019) recommends that that a conservation collection has maternal lines differentiated, and diverse genetic representation of species' wild populations with seeds, tissues, or whole plants of at least 50 unrelated mother plants, and accessions from at least 5 populations across the range of the species. Both are important for viability at the smaller restoration scale and in all threatened species translocations, but maternal lineage tracking may be less of a factor where much larger scale restoration is the target.

It is therefore important to try to anticipate both the currently intended and the potential future end uses of germplasm collections *before these are made*, in order to apply collection (sampling) strategies that will optimise the captured germplasm for the intended end use, while still being consistent with available resources and sustainably sourced. It is equally important to consider the different guidelines that may be available for these intended uses, and to seek expert advice on recent, often unpublished, developments in current knowledge and local knowledge.

Collection of germplasm for ex situ conservation and translocation involves consideration of existing genetic data, if available, and a strategy to capture diversity within and between sites (Chapter 3). If genetic data are not available, consideration of factors such as reproductive system, breeding system, pollen and seed **dispersal**, and distribution of the species within the landscape and the distribution of genetic diversity (considering isolation by distance or by environment) can guide development of sampling strategies (Chapter 3, particularly Figure 3.1). Other issues to consider include **inbreeding**, potential taxonomic uncertainty, and **chromosomal** variability; some of these may be obvious as morphological variations but frequently may only be apparent following genetic study. Key points for acquiring collections of these types are summarised in Chapter 3.

Seed sourcing and provenancing strategies and collecting strategies for ecological restoration are not specifically dealt with in this current publication; instead, see Florabank Guidelines Module 5 – Seed Sourcing (Harrison *et al*. 2021); and Module 6 – Seed Collection (Crawford *et al*. 2021). However, the background information on genetics for acquiring and maintaining ex situ collections (Chapter 3 in the Germplasm Guidelines) is a useful and accessible introduction to the topic for restoration practitioners.

1.6 Purpose and content of these guidelines

These guidelines outline standards for the ex situ conservation of Australian plant germplasm where the intention is that the germplasm contributes to the conservation, recovery and management of Australian native plants and ecological communities. Close adherence to these guidelines is particularly important for the conservation of threatened plant species or threatened ecological communities (TECs), and in the case of entities that currently have non-threatened status but that may become threatened at some point in the future, or whose conservation may contribute to a wider conservation agenda, for example of critical habitat support for other specific organisms, or ecological restoration (see Box 1.2). While all species have intrinsic value, many species also have economic, ethnobotanical, horticultural, landscape-management or aesthetic values that may guide their inclusion in ex situ conservation projects where resources are limited. For example, the 1,000 Species Project of the Australian Seed Bank Partnership targeted collection of endangered, endemic and economically significant species (CHABG 2014).

The main objectives of these guidelines are to:

- address the needs of germplasm collection and storage of Australian flora;
- demonstrate that germplasm storage for effective conservation purposes is a complex activity requiring detailed planning, substantial resources and long-term commitment;
- emphasise the necessity of obtaining appropriate permissions and establishing who else is doing similar work;
- provide guidance on which form of germplasm is most appropriate in each situation: can seeds be collected, stored and germinated? What alternative techniques are appropriate?
- highlight the importance of collecting protocols to establish a valuable long-term germplasm collection for conservation;
- provide practical details on storing germplasm as seeds, tissue cultures and living plants;
- provide options for germplasm storage at low temperatures, including cryopreservation, as indicated by seed storage behaviour and expected **longevity**;
- provide an overview of plant regeneration strategies including seed germination issues, particularly **dormancy** mechanisms and ways to overcome them;
- highlight the need for continued research to establish storage and propagation protocols and understand the basic biology of many species;
- highlight the importance of collaboration, record keeping, data and **metadata** collection, management and sharing;
- identify probable end uses for germplasm collections and timeframes for storage under different conditions; and,
- show that stored germplasm can be used for multiple purposes complementing in situ conservation by providing material for restoration, translocation, bulk propagation or seed production areas, research, education, horticultural display and sustainable plant development; depending on the breadth and depth of the collection.

The first six chapters provide a step-by-step guide to germplasm conservation, starting with planning, followed by genetic guidelines, collecting strategy, seed storage and germination. As a guide to the planning process, Chapter 2 details the major considerations and provides guidance on plant germplasm conservation including benefits, risks and limitations, obtaining permissions, priority setting, storage options, and the importance of considering the intended uses of material. Chapter 3 provides genetic guidelines for acquiring and maintaining collections, including the

development of a collecting strategy to capture the genetic diversity of a species over its range, whether genetic data are available or not. Chapter 4 details the background information required when planning a collection and explains the practical steps involved in seed and vegetative material collecting. Chapter 5 provides an outline of seed storage techniques for **orthodox** seeds along with pre-storage operations, seed testing, seed longevity and seed bank management. Chapter 6 outlines methods for identifying and conserving non-orthodox seeds. Chapter 7 explains the critical process of seed germination, including how to identify and overcome the various types of seed dormancy.

The next four chapters discuss techniques that may complement seed storage or be the only mechanism for ex situ conservation of '**exceptional** species' (see Chapter 2, Case Study 2.5). Chapter 8 outlines the role of the nursery in supporting ex situ conservation, including the process of vegetative propagation of threatened species and handling of collections with biosecurity risks such as disease. Chapter 9 explains applications and types of tissue culture conservation options and Chapter 10 discusses the application of cryopreservation to germplasm storage, including reference to recent research in Australian species. Chapter 11 covers the method of conserving germplasm as living whole plants and the alternative types of collections that can be held ex situ. These chapters begin with the associated benefits and risks of each option.

The following two chapters discuss in detail the types of germplasm collection suitable for species with special requirements such as orchids, fungal symbionts, ferns, carnivorous and parasitic plants. Chapter 12 deals with collection and storage of orchids and symbionts, including mycorrhizae and rhizobia and Chapter 13 addresses the storage and maintenance of special collections such as spores of **pteridophytes** and **bryophytes**, pollen, parasitic and carnivorous plants.

The final two chapters provide practical advice on human-systems aspects of germplasm management. Chapter 14 details practical steps to managing risk within collections and strategies for responding to crisis situations, when germplasm collections may be required as part of regional or national crisis response actions. Chapter 15 covers the overarching principles of maintenance and utilisation of all types of germplasm collections, with reference to supporting technology and the variety of end uses for ex situ collections.

Included in the chapters as support for the main text are:

- case studies to illustrate the application of theory;
- information boxes to provide additional information on important concepts or topics; and,
- checklists and flow charts to summarise processes.

In line with the *Guidelines for the Translocation of Threatened Plants in Australia* (Commander *et al*. 2018) and *Florabank Guidelines* (Commander 2021), we have avoided the use of technical terminology where possible; however, whenever technical terms or abbreviations are used, definitions are provided in the glossary at the end of the publication. The citation of references throughout these guidelines provides a portal to the major sources of information available at the time of publication.

This best-practice handbook brings together the experience of many scientists and practitioners to showcase current approaches to curating a wide variety of ex situ collections. This new edition seeks to draw lessons from the body of knowledge and experience gained from working on Australian plant species, which has vastly increased over the last 12 years, and to place these in the context of current conservation plans and strategies. There is an emphasis on the importance of partnerships for implementing ex situ conservation actions that support in situ conservation. The increasing prominence of translocation and the importance of input

from a wide range of specialists in translocation plans, including custodians of ex situ collections, is key to enabling effective integrated conservation of threatened species. This edition highlights the need for a long-term perspective in conservation planning, actions and monitoring, as plant conservation initiatives are multi-generational, and action taken now will benefit future Australians (Broadhurst and Coates 2017).

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Chapter 2
Options, major considerations and preparation for plant germplasm conservation

Catherine A. Offord, R.O. Makinson, Lydia K. Guja and Tony D. Auld

2.1 Introduction

This chapter provides a guide for the essential first step of determining whether **ex situ germplasm conservation** is necessary for a particular taxon, an overview of the general options available, and a guide to determine the methods that can be used and how the collection will be maintained.

Ex situ conservation is a complementary action, and should not be considered an alternative, to in situ conservation (Guerrant *et al.* 2004). With that in mind (Section 1.2), five questions are important to consider early in the planning process:

1. Do you have all available information on the species, including unpublished information from other people who may be working in the same area?
2. Can you determine the short, medium and long-term conservation goals for the species, and the role of in situ and ex situ actions?
3. Has a thorough field survey been conducted?
4. Will the collection of material endanger the species or other species that may rely on it?
5. Do you have the necessary permission(s) to proceed?

A flowchart of the decision-making process incorporating these points is given in Box 2.1. Each of the points within the flowchart are described in detail from Section 2.3 onwards, following a discussion of the relative benefits and risks associated with ex situ conservation (Section 2.2).

2.2 Benefits and risks associated with ex situ conservation of plants

2.2.1 Benefits

Ex situ conservation of plants provides material for **translocation** or **restoration** of threatened species, communities or **habitats** (for threatened animals for example) that may not otherwise be available. In a few cases, plants may no longer exist in the wild and the only source of material for translocation is stored **seed** or cultivated plants held over a period of time until **threats** are removed or minimised in the wild **populations** (e.g., *Allocasuarina portuensis*, Case Study 2.1) or new wild populations can be established (e.g., Zimmer *et al*. 2016).

Ex situ collections enable research to be conducted on the biology of threatened species, where conditions can be controlled, and factors involved in plant growth and survival can be tested. This may contribute to in situ management by providing information on the species' response to disease (see Case Study 2.2), fire, grazing, competition or nutrients etc; as well as addressing information gaps critical to the restoration of the species, such as breeding biology (e.g., Chen *et al*. 2019; Miller *et al*. 2017). Ex situ collections can also be used to understand the response and adaptability of species to environmental changes including climate, thereby informing future species management. Understanding a species' biology is also critical for translocation planning to increase the likelihood of success (Commander *et al*. 2018).

Ex situ collections can supply plant material of horticultural, forestry, agricultural, biosecurity or pharmaceutical interest from sources that may otherwise be under pressure in the wild from over-collection and other threats (Case Study 2.3).

Raising public awareness is an important element of conservation. Ex situ collections may be available for educational and display purposes, for example, in botanic gardens, which in turn can assist in protection of the in situ population by raising public awareness.

2.2.2 Risks and limitations

In situ conservation should always be the long-term goal and is almost always the most efficient way to preserve **genetic diversity**, although future conservation needs for species in the wild may be uncertain, e.g., climate change impacts (Potter *et al*. 2017). Ex situ storage of material should only ever be considered a complementary conservation measure, and not an alternative, to in situ conservation measures. Ex situ conservation should not be considered as an acceptable substitute for the continued existence of a species in the wild and should not be used to justify destruction of habitat.

Germplasm storage poses a number of significant risks, one of the most serious being the loss of ability of the stored material to regenerate populations. This may occur, for example, if the viability of stored seed declines over time, or if genetic diversity in a living collection is progressively diminished through the loss of individual plants. In addition, the process of germplasm acquisition and storage can often lead to inadvertent selection of **genotypes** most suited to storage and cultivation, while not capturing other useful genotypes and this should be taken into account during planning (Chapter 3). Over-sampling of germplasm may damage wild populations, depending on circumstances, but this impact can be minimised by following the guidelines outlined in Chapter 4.

A longer-term risk associated with germplasm storage is the process of gradual **adaptation** and potential loss of **fitness** to survive and re-establish in the wild. Plants species can display a range of adaptability to environmental changes. Some populations in the wild, especially those species with short generation times, may evolve relatively quickly in response to environmental changes (Everingham *et al*. 2021). Stored material is adapted to the environment as it was at the time of collection and represents genetic variation through time to that point. If the original environment changes significantly, for example if the soil becomes saline or the average temperature rises, then the stored material may be maladapted, and hence may be adversely affected in terms of either reproduction and/or establishment when returned to the wild. It is important to capture as much environmental information as possible along with each **accession** (e.g., soil nutrient status, associated species), perhaps coupled with biological research on the collection's stress tolerance and level of plasticity to change.

Box 2.1: Decision flowchart for ex situ conservation options

Taxon or population is considered at risk or in need of ex situ conservation

Chapter 1

Have all major considerations been addressed?

- Is there a clear link to an in situ recovery process?
- Is it a precautionary collection?
- Is it for research or display?
- Is it an 'exceptional species'? (Chapter 6)

What is the time frame?

What information is available?

Is seed available (preferred in most cases)?

Will you need to conduct preliminary trials on collection, storage or propagation?

Do you have the necessary resources? Can you get the necessary permits or permissions?

Section 2.3

Do the benefits of ex situ conservation outweigh the risks?

Section 2.2

No

Monitor populations/pursue other options

Yes

Decide ex situ option/s
If seed is not suitable, consider other options

Section 2.4, Chapter 8

Collect material appropriate to the option/s chosen

Chapters 3 and 4

Seed banking

Chapters 5, 6 and 7

Tissue culture, orchid and symbiont culture

Chapters 9 and 12

Cryopreservation

Chapter 10 and 13

Living plant collections

Chapter 11

Utilisation (translocation, research etc.) and risk management

Chapters 14 and 15

Case Study 2.1: The role of ex situ conservation in recovery of *Allocasuarina portuensis*

Tony D. Auld

An example of a translocation made from an ex situ collection is the Nielsen Park Sheoak (*Allocasuarina portuensis*), which is described in the 2nd edition of the ANPC *Guidelines for the Translocation of Threatened Plants in Australia* (Vallee *et al.* 2004). At one point, this species was **extinct** in the wild and only existed as seeds and cutting grown plants conserved ex situ. Representative plants of the original population of this species have been reintroduced to the original site, and weeds and other threats minimised through intensive management. The species has now been established at several sites, but few plants have survived. Further translocation and habitat management are required, particularly weed management and understanding the role of fire in promoting recruitment and maintaining the species (DPIE 2019). Ex situ collections have been held as seed in the Australian PlantBank and as potted plants at the Australian Botanic Garden Mount Annan. This work has been factored into an Approved Recovery Plan for the species (NSW National Parks and Wildlife Service 2000).

Figure 2.1: *Allocasuarina portuensis,* ex situ collections were used to translocate this species into its original Sydney harbourside location after becoming extinct in the wild. (Image: J. Plaza, Royal Botanic Gardens and Domain Trust, Sydney)

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Case Study 2.2: Fighting Myrtle Rust

R.O. Makinson

Genetically and geographically representative ex situ collections of a species can have great value in allowing a rapid research response to new threats or declines in the wild. An example is the case of Lemon Myrtle (*Backhousia citriodora*). In the mid-1990s, a genepool planting (an array of multiple plants from every known **provenance** across the entire wild range) was established near Beerburrum, Qld, by CSIRO and the Queensland Forestry Research Institute (now part of Agri-Science Queensland), as part of a research project to assess optimal genotypes for essential oil and leaf production. Disease resistance was not a research consideration at the time, but the fortuitous survival of the array, and most of its documentation, allowed its re-use for controlled assessment of Myrtle Rust susceptibility and resistance in 2011–12, soon after the advent of that disease in Queensland (Doran *et al*. 2012; Lee *et al*. 2016). That whole-species assessment would simply not have been possible without the ex situ germplasm collection. When comprehensive genotype collections of this sort are assembled, very close consideration should be given before the end of the project to 'archiving' them in long-term custody, whether via whole-plant, seed, or other **preservation** options. Such collections will become increasingly difficult to recreate from scratch in the future.

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Figure 2.2a: Lemon Myrtle plantation at Beerburrum in May 2013; **Figure 2.2b**: Data collection at Lemon Myrtle plantation. (Images: John Doran)

Case Study 2.3: Conservation strategy for the Wollemi Pine

Catherine A. Offord and Heidi C. Zimmer

The Wollemi Pine (*Wollemia nobilis* W.G.Jones, K.D.Hill, J.M.Allen, family Araucariaceae) is a conifer with an ancient lineage, which was discovered in 1994 in a remote area of Wollemi National Park, NSW. The species is extremely rare and is the only **extant** representative of the *Wollemia* genus. Four stands have been discovered, all within a single catchment, and encompassing fewer than 100 mature individuals. The Wollemi Pine is threatened by *Phytophthora* and wildfire.

Because of their **phylogenetic** uniqueness and connections with other popular ornamental Araucariaceae, Wollemi Pines are highly sought after for cultivation. The ex situ actions employed to conserve this species were decided using a framework similar to that shown in Box 2.1, starting with major considerations and leading to development of a conservation plan. Research since its discovery has resulted in the development of a complementary conservation strategy (NSW Government Department of Environment and Conservation 2006).

After deciding that ex situ conservation was an appropriate action, the option of seed conservation was considered. Seed production is limited in this species; they produce only a few thousand **viable seeds** in any one year, and most of these are not harvestable because of inaccessibility of the cones and the role of seeds in helping to maintain a juvenile bank of plants in the wild. Additionally, although seeds appear to be **orthodox**, they are likely to be relatively short-lived in storage and therefore seed storage was not considered to be the single most appropriate ex situ conservation option for this species. However, a small number of seeds are stored at the Australian PlantBank and in the Millennium Seed Bank (UK) and **germination** requirements have been determined (Offord *et al*. 1999; Offord and Meagher 2001). Although it was found to be technically possible to establish this species in **tissue culture**, the material regenerates very slowly and requires special techniques, and it is thus a very expensive and risky conservation technique when compared with seed and live plant conservation. **Cryopreservation** was not considered as the facilities were not readily available at the time.

A complicating factor in this story is that **DNA** technology has so far revealed very low variation between trees (Greenfield *et al*. 2016). This is highly unusual and the Recovery Team that oversees the management of the species considered that the priority was to represent as many individuals as possible, so that any genotypic variation that may exist (but is not yet quantifiable) is likely to be captured and maintained. As there are low numbers of mature trees in the wild, and because preliminary experimentation showed that trees could be cloned using vegetative propagation, a greater effort has been expended in collecting and propagating cutting material of this species. This has resulted in an ex situ collection that represents the majority of trees discovered so far, with some inaccessible trees not yet included.

The **clonal** plants of this species are held as potted plants, in the nursery at the Australian Botanic Garden Mount Annan, and documented representatives have been planted out in this and other botanic gardens. The nursery collection comprises three replicates of around 60 individual plants originally collected in the wild. This is a high-maintenance collection but has been used extensively as a source of material for scientific studies: more than 100 scientific papers have been written on this species, the vast majority using ex situ material, either sourced directly from Australian PlantBank or plants cloned from the original collections. These studies have included research into Wollemi Pine tolerance of temperature extremes (Offord 2011), drought (Zimmer *et al*. 2016a) and fire (Zimmer *et al*. 2015); its microbial community (Rigg *et al*. 2016) and susceptibility to pathogens (Bullock *et al*. 2000; Puno *et al*. 2015); and establishment in translocation (Zimmer *et al*. 2016b; Rigg *et al*. 2017).

From this collection, **clones** were generated for a major world-wide horticultural release (Offord and Meagher 2006), with Wollemi Pines present today in gardens of more than 30 countries worldwide. In this horticultural setting, Wollemi Pines also have a role as an ambassador for threatened plant conservation, not only as a conversation piece but in actively involving gardeners in conservation. It is planned that, over time, these established trees will be the major ex situ collection representing the wild populations, thereby decreasing the reliance on labour-intensive nursery-held stock. In the meantime, the seed biology of this species is being studied to improve its long-term storage potential.

Figure 2.3: *Wollemia nobilis*, an emergent rainforest conifer (top left); ex situ conservation includes commercialisation based on vegetative propagation from stockplants (taking pressure off wild stands) (top right); seeds are orthodox but few are produced (middle left); novel seed collection techniques were developed including by helicopter and suspended seed nets (bottom left). (Image: J. Plaza, Royal Botanic Gardens and Domain Trust, Sydney)

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2.3 Priority setting

The aims of an ex situ conservation program should be to reinforce or re-establish self-sustaining populations in the wild, to minimise threats or the effects of threats and as an emergency backup of genetic material. It is important to consider the large number of species in Australia which potentially require ex situ conservation (the Federal EPBC Act 1999 lists over 1,300 Threatened Flora from across Australia, with many more listed or yet to be listed by States and Territories) but resources for ex situ conservation are limited, with few repositories and personnel to manage collections, so priority setting for target species and intended actions is a critical activity.

Priority setting for ex situ conservation of threatened species requires complex considerations, including legal access to plants, detailed biological information, skilled personnel, and infrastructure to hold, store and grow germplasm. The Decision Flow Chart (Box 2.1) and the following considerations in this chapter can assist with decision-making on ex situ conservation options. An example of an ex situ program, integrated with other conservation considerations, is given in Case Study 2.3. Further considerations of the relative ongoing effort and resources needed for ex situ conservation methods that support conservation in the wild are found in CPC Best Plant Conservation Practices to Support Species Survival in the Wild (Center for Plant Conservation 2019). A priority framework for management of ex situ plant collections in a botanic garden is outlined in Case Study 2.4.

Case study 2.4: A priority framework for management of ex situ plant collections at the Australian National Botanic Gardens: aligning state and national listings, informed by collection history and research.

Toby Golson

Development of the framework, background and rationale

Within its extensive remit to grow, study and display the country's indigenous flora, the Australian National Botanic Gardens (ANBG) in Canberra is currently home to over 9,800 lineages of c.4,800 native plant **taxa**, each collection accessioned and databased with information regarding origin, botanical determination, propagation and planting history. Each lineage is a unique accession maintained through clonal (vegetative) propagation, where possible. Previously, the ANBG gave highest priority to species listed under the federal *Environmental Protection and Biodiversity Conservation Act* (1999) (accessible through Species Profiles Rare and Threatened database: SPRAT[16]). This national approach reflects our role in Parks Australia, a Division of the Commonwealth Department of Agriculture, Water and the Environment.

Over time, it became clear that we also held collections with other conservation values, for example threatened species listed under state or territory legislation, or subject to current research with conservation implications. This recognition coincided with the implementation of the Common Assessment Method (CAM[17]) in 2016 which is a nationally consistent approach to aligning threatened species lists across Australian jurisdictions. We recognised multiple criteria needed to be prioritised in our curatorial methodology and, therefore, developed a new priority assessment framework.

16 http://www.environment.gov.au/cgi-bin/sprat/public/sprat.pl
17 http://www.environment.gov.au/system/files/resources/36ece4ab-82dc-4de9-aac6-9cc54bd7a820/files/mou-cam.pdf

The priority assessment framework

The framework is based on a numerical scale which ascribes a priority ranking (from 1–5, with 1 the highest) to each clonally distinct living collection according to the following criteria:

1. All wild origin collections constituting at least one of:
   - current/ongoing collaborative projects with tangible conservation outcomes including unlisted species undergoing conservation interventions by state authorities;
   - all Critically Endangered SPRAT or equivalent state and territory **endemic** (under CAM) listed species;
   - genetically representative holdings of a listed taxon (e.g., multiple wild individuals and/or populations sampled)
   - taxa in the National Environmental Science Program Threatened Species Hub's list of 100 species at greatest risk of extinction[18];
   - undescribed or recently described, unlisted, narrow endemics recommended for highest conservation listing by their collector or author;
   - listed taxa identified under the Commonwealth Threatened Species Scientific Committee 10-point Bushfire Response Plan 2020[19].

Many collections qualify as eligible for the highest ranking under more than one criterion.

2. All wild origin collections constituting: SPRAT Endangered or Vulnerable or equivalent state or territory endemic listed species not included in 1 due to a lack of genetic representativeness resulting from sampling from a single population only; and, all unlisted bushfire affected species.
3. All horticulturally or historically significant holdings as well as those with ongoing research or taxonomic value not included in 1 or 2 (these may be of cultivated origin).
4. All remaining wild origin collections.
5. All remaining holdings (generally of display and horticultural value only).

Implications for curation of the living collection

Underlying the framework is a set of operational functions that have been in place for most of the ANBG's existence and ensure the ongoing security of our collections. These include continuous stocktaking and repropagation at appropriate thresholds. Security of collections not been seedbanked is enhanced by planting widely across the garden, maintenance of permanent pot holdings and transfer of material to likeminded conservation organisations where appropriate. Provenance information linked to herbarium vouchers underpins the conservation integrity of these collections. Additionally, the vouchers and associated data are curated by the Honorary Living Collections botanist who ensures nomenclatural accuracy and taxonomic currency. While curation and collection management operations such as these are ongoing, the new framework provides a means of more accurately and easily assessing a large collection such as ours. Staff can now readily identify in our database the highest value collections relevant to their planning and prioritisation of tasks. Assessments and reassessments of taxa and the framework are continuous and aim to reflect as closely as possible the dynamic shifts in rarity in wild populations and the results of emerging research. As at July 2020 there are c.420 clonal lineages of 174 taxa assessed as Category 1.

18 https://www.nespthreatenedspecies.edu.au/media/1aobde4s/2-4-red-hot-plants-findings-factsheet_f2.pdf
19 http://www.environment.gov.au/system/files/pages/289205b6-83c5-480c-9a7d-3fdf3cde2f68/files/summary-list-plants-requiring-urgent-management-intervention.pdf

2.3.1 Which species to collect?

The following criteria may be used to determine which species should be prioritised for conservation collection:

- **Endangerment** – a high degree of threat; rapid decline in population numbers or size; species that are poorly represented in collections, or that could become rare as climate changes etc;
- **Endemism** – restricted to a certain region or locality;
- **Evolutionary and taxonomic uniqueness** – monotypic species vs closely related species;
- **Ecological potential** – for biological management and recovery; probability of success of establishment in cultivation;
- **Economic value** – genetic relationship to economically useful taxa;
- **Ethnobotanical value** – culturally significant species, traditional knowledge (Chapter 1);
- **Emblematic value** – taxa that can be used as flagships for promoting conservation.

(Center for Plant Conservation 1991; Maunder *et al*. 2004b)

2.3.2 What are the perceived end-uses for the conserved germplasm? Collecting material that is 'fit-for-purpose'

The collection of germplasm for conservation purposes, from any plant growing in the wild, should be an action informed by a purpose (Chapter 1). This is especially so in the case of rare and threatened species, or threatened populations, where the very act of collection may increase the threat level unless care is taken (e.g., to avoid overcollection, disease introduction).

Even with the best technology available, germplasm cannot be held indefinitely in seed banks or other repositories so thought must be given to its timely uses (Walters and Pence 2019). The purposes of the collection, e.g., translocation, research, horticulture or breeding, or precautionary capture against future decline, should be clearly defined including the extent of the collection and maintenance of material. The collection plan should ideally include a timeframe for maintenance and end use of the collection, although there is also a role for precautionary collections.

The most likely end-uses for ex situ conservation material are:

- In situ actions e.g., translocation, **reintroduction**, enhancement plantings (Commander *et al*. 2018; Commander 2021);
- Field genebanks and seed production areas to minimise the impact of collecting on small populations and remnants (Section 11.3.2);
- A source of genetic material for future conservation needs;
- Research e.g., seed biology, genetics;
- Education and display.

When considering the end use of a germplasm collection, it is essential to understand the importance of collecting and using material with appropriate provenance (Box 2.2) and sound sampling and handling techniques.

The scarce resources for making and maintaining conservation germplasm collections demand a well thought out concept for end-use (including multiple future options, not just immediate needs), and the various biological and human/social aspects involved. We strongly advise seeking advice from a range of knowledge holders and species specialists when designing a collection program to ensure the integrity of your collection.

Box 2.2: Deciding where to collect germplasm – why is it important to conservation outcomes?

'Provenance' of seed or other germplasm refers either:

- to the *area of origin of the germplasm*, with an implicit assumption that this may have significance for the **genes** or genetically determined adaptive traits that are being captured; or,
- explicitly to the *genetic provenance* within the total genomic variation of the species – genetic provenance in this sense may be determined by DNA techniques that identify geographic zones, populations or variants with maximum similarity and dissimilarity within the **genome**, or genotypes thought to be most suitable for purpose.

Assessing 'appropriate provenance' for a conservation action must take into account the intended end use, the biological characteristics of the species (e.g., breeding system), the characteristics and history of the parent population and environment, and the characteristics of the recipient environment or projected future environment under climate change. For these reasons, detailed discussion of provenance is beyond the scope of these Guidelines. The ANPC Translocation Guidelines (Commander *et al*. 2018) give provenance considerations for threatened species conservation, and a discussion of the provenance considerations for broad-scale restoration for common species is found in Broadhurst *et al*. (2008) and various modules of the Florabank Guidelines, particularly Harrison *et al*. (2021).

For a wide range of plant species, the following should be considered in selecting provenance for conservation end uses:

- Fragmentation threatens long-term persistence of remnant plant populations even for common species;
- Fragmentation may have an almost immediate effect in reducing the suitability of a population as a source of germplasm for use in conservation actions;
- Small populations (< 100–200 reproductive plants) are highly susceptible to declining seed set, loss of genetic diversity, or increased **inbreeding**, leading to poor seedling vigour and increased **hybridisation** risk;
- While it is often best to collect locally because plants are sometimes adapted to local conditions, it may not always be the best option. Good 'condition' of a remnant does not mean good genetic health.

For threatened species, or small populations of rare species, the consequences of inappropriate decisions and sampling strategies are more serious than for common species. Hybridisation and introgression (Potts *et al*. 2003) and genetic **bottlenecking** are two of the main risks posed by inappropriate choice of provenance and such risks are magnified for many threatened species that are naturally low in number.

Genetic techniques are now allowing the delineation of seed collection zones within species (Francisco Encinas-Viso, CSIRO, pers.comm.), and the identification of genotypes that may relate to end use. See Chapter 3 for detailed information on genetic considerations (particularly Case Study 3.2) as well as Harrison *et al*. (2021).

Long practice in **rehabilitation** plantings in some places has tended to show that 'local provenance' material being planted often 'does better' in terms of survivorship, and this is usually assumed to reflect genetic adaptation to local conditions, although this has rarely been validated by structured experiments. Given our poor knowledge of breeding and genetics for most species, there is an incentive to play it safe by using local provenance for conservation plantings. However, maximising genetic variation by mixing seed sources from multiple populations may be a suitable option if only small populations are available for seed collections (see Chapter 3 and Harrison *et al*. 2021).

The experience-based evidence should not be downplayed, but also should not be universalised – survivorship of planted individuals does not equate to success in establishing breeding populations over the long-term, and recent DNA studies show that over-emphasis on 'local' provenance may result in selecting less fit or less fertile germplasm.

Plants of 'non-local' provenance will not *necessarily* have any adverse effects on, or breeding incompatibilities with, local plants – they may still come from the same 'genetic provenance' within the species, which may be quite extensive. In other cases, however, there may be significant genetic differences, with effects that may range from an inability to interbreed, through partial to full interbreeding with either positive or negative effects on local **fecundity**, gene pools, and fitness of offspring to local conditions.

Key general recommendations for considering provenance for conservation purposes (adapted from Broadhurst 2007) include:

- Source germplasm from large reproductive populations (>100-200 individuals, noting that may be difficult for some threatened species);
- Improving and maintaining the genetic health of remnant vegetation will aid its long-term persistence and provide higher quality seed;
- Consider use of non-local germplasm to either maximise richness of large plantings or to avoid the adverse effects of sourcing from small remnants;
- Minimise assumptions about local adaptation if evidence is lacking;
- Accumulate, consider, and (if appropriate) publish as much species-specific and site-specific knowledge as possible, including breeding system and **chromosome** variation (i.e., ploidy level) if known.

Within those parameters, general rules include:

- Consult widely in the early planning phase – involve specialists;
- Ensure taxonomy of the species is understood (Chapter 3) and identification of source material is correct, including collection and lodging of an herbarium **voucher specimen** (Chapter 4);
- Source from geographically related and ecologically similar sites;
- If planning to use seed production areas, control inadvertent narrowing of the genetic base (see Havens *et al*. 2004; McKay *et al*. 2005).

2.3.3 Can you access and manage the material appropriately?

Many rare and threatened species have limited numbers of individuals from which to collect materials for ex situ conservation; they may also be **ephemeral**, sparsely distributed and/or difficult to access. Viable seeds are often in short supply and vegetative material sparse.

So, before embarking on collection, it is important to identify the following:

- Who else has an interest or knowledge in the species or the site? There is always someone who knows something, it is a matter of persisting to find out. It is vital to coordinate your proposed actions with others.
- Are there any genetic, reproductive or other studies existing or that could be carried out that may guide sampling? (Chapter 3).
- Is the species conserved ex situ elsewhere? This may remove the need for further collection, or to collect from other populations. Genetic studies may assist in targeting genotypes not already existing in ex situ collection(s) (see Case Study 8.3 on *Grevillea renwickiana*).
- Has a collection site been subject to previous germplasm collection? The cumulative impacts of collecting need to be considered if there is a need for further genotypes to be added.
- Do you have high confidence estimates of the level of sampling for your project (alongside any other impacts) that can occur without unduly damaging the source population?
- Can you collect at the appropriate time? When will seeds ripen or cutting material be ready? This minimises the risk of making poor collections and the need to recollect. It may be necessary to get local knowledge and/or reconnoitre sites prior to collection. See Chapter 4 for further guidance.
- How much relevant information is known about the species? If not well understood, look at a related species for some guidance.
- Do you have the required permits and permissions? (Box 2.3 and Chapter 1 Table 1.1).
- Consider the biosecurity implications for collection of the target species and access to the collection location e.g., timing, equipment and special procedures required, treatment of material and consultation with the recipient ex situ location.

It is recommended that organisations develop their own decision making and management frameworks to guide their conservation programs (see example in Case Study 2.4).

2.3.3.1 Have you identified other stakeholders?

Quite commonly, there are various stakeholders who have an interest in the conservation of a species or a particular population. Examples include government agencies, Landcare groups, traditional landowners or native title holders, and researchers. It is essential that these stakeholders are involved to discuss past, present and future actions and to coordinate conservation efforts. This is particularly important if it is someone else's area of interest geographically, legislatively or professionally. The opportunity to assist in work that is being done may well be lost if, because of poor communication, your interest is viewed as competitive (Touchell *et al*. 1997). Consider also lodging records of your work with other stakeholder organisations, and vice versa.

2.3.3.2 Are other agencies or individuals conserving this species?

Unnecessary collection from wild populations must be avoided and it is therefore important to determine whether useful ex situ collections of the target species already exist (ideally well-documented). Collections made for agriculture, horticulture or forestry may have some conservation value (Case Study 2.2). Even collections with low genetic representation may constitute a valuable resource. Conversely, earlier collectors of germplasm may be able to advise on difficulties with, or fitness of, material from your intended source. Information on storage and propagation may also be gleaned from the holders of such collections. Various sources may contribute to ex situ collections including special interest groups and individuals. Most large organisations have databases that reflect the depth and breadth of their collections, and many have information available on-line or by request.

2.3.3.3 What information is available on ex situ conservation of the target taxa?

Before embarking on an ex situ conservation action, get as much information as possible to ensure the best possible outcome. Check databases, published papers and networks (not limited to the science area). It is important to consider the existing frameworks that apply to conservation of target species. Check State and Federal listings for threatened species. Recovery Plans exist for many threatened species and ecological communities, sometimes as interim reports, and these outline the actions required, and in many cases, the agencies responsible. For many threatened species or populations, identified actions may include some relating to germplasm capture and deployment (e.g., seed banking, translocation), and these references may both help to mandate your project or to refine or broaden your options (see for example, the NSW situation in Zimmer *et al*. 2020).

2.3.3.4 Can you get the necessary collecting permits and permissions?

Before any collection from the wild, and particularly in the case of threatened species, various levels of permission for particular actions may be necessary (for examples see Box 2.3), and it is important to allow time for these. You may well be acting illegally without them and it is not appropriate to collect without a licence as this may adversely affect wild populations. The international Nagoya Protocol[20] guides access and benefit sharing of biological resources and its principles should be followed. You will need to very clearly think through the end use of the material you wish to collect. Commercial use may require a different permit from uses relating only to scientific research and/or conservation, and the latter permits often distinguish between threatened (listed) and non-threatened species. Indigenous land access and use permissions will be different again. When working with Indigenous knowledge holders, be aware that agreement may need to be reached for collection and use(s) of plant material as well as sharing and publication of intellectual property.

Permits and permissions can often take a long time (sometimes several months) to processed, for bureaucratic workload reasons or for cultural reasons. So, allow as much time as possible for the process.

20 https://www.environment.gov.au/science-and-research/australias-biological-resources/nagoya-protocol-convention-biological

Box 2.3: Obtaining permission to collect, hold and utilise wild Australian plants

See also Chapter 5 of the Translocation Guidelines (Commander *et al*. 2018)

The legal ownership and usage rights of wildlife, including most or all plant life, varies from one jurisdiction to another. Webpage addresses with information about the permit systems are not stable, so the following information relates to generic departments and agencies, which do tend to persist a bit longer (See Section 1.4). Direct contact with the permits sections of these agencies is always advisable prior to submitting the mandatory application forms.

If you wish to collect material of threatened species for experimental work (e.g., propagation), you should be able to demonstrate that you have both researched the literature and preferably have performed trials on non-threatened related or analogue species, if these are available, so as to minimise the risk of wasting scarce material of the threatened taxon.

Observing legal permit requirements and cultural protocols is essential not only for your own work, but also for subsequent collectors needing to access the same areas or knowledge. Failure to secure appropriate permissions may also compromise your insurance coverage.

On Commonwealth lands, one or more permits must be obtained from the Commonwealth Department of Agriculture, Water and the Environment[21] or its successor. Look on their website under 'EPBC Act' (*Environment Protection and Biodiversity Conservation Act* 1999) for 'Permits'. Any overseas export of biological material, even for scientific study, will require a separate permit under different legislation such as CITES for wildlife trade (but see same webpage for links), unless the export is from a scientific institution with a standing permit.

For the States and Territories, the starting point for permits is always the main conservation agency (Chapter 1 Table 1.1). General information on permits is usually on the agency website, but it is always wise to make direct contact with someone in the permits section, and for threatened species it is always imperative to make contact with relevant people with management responsibility for the species concerned (e.g., convenors of recovery teams where these exist, and/or regional threatened species officers). Note that there are variations from State to State in what lands are covered, for permit processes, by these agencies – in some jurisdictions they cover permits for conservation reserves only, in other cases they are the permit authority for all lands. Try to anticipate whether you will wish to transfer any of your material to repositories in other jurisdictions in the future and build this sort of need into your application.

Some other classes of public land may require permits from other agencies, notably State Forests and in some jurisdictions the departments that control aquatic biodiversity (not always the main conservation agency). Collecting from roadsides or other easements, or travelling stock reserves, may require permission from the relevant utility company or authority.

21 At 2021, <www.environment.gov.au>

For Aboriginal lands and Indigenous Protected Areas, you must contact the relevant Aboriginal Land Council or Aboriginal Corporation. Aboriginal community land is not public land. As with private freehold, issuance of permits is discretionary and the more liaison you have, the more likely you are to win support for your project. Be aware of the need to co-design projects for mutual benefit, agree on the process for sharing information with the local community, and familiarise yourself with protocols for scientific or other work on Country. The Aboriginal liaison officers with your local conservation agency will be able to guide you to these and advise on best process. Regardless of the written permissions you receive prior to a trip, you should always allow time to visit the relevant person with the council or corporation prior to collecting, as cultural use of sites may not be predictable in advance.

For private freehold, permission must always be sought from the landowner or manager (as also for leasehold land). In some jurisdictions the freehold owner has outright legal ownership of wildlife, including plants; in others there may be Crown ownership, but the landowner or lessee may have rights of use and benefit. The days of a chat over the gate and a handshake for permission to access are not yet gone but are rapidly going for major collecting exercises. In all cases you need to give landowners/managers clear information as to the nature of your project and the end use of the material. Developing your own networks of landowners prepared to give access or working through existing networks (e.g., Landcare groups) may take time but is always beneficial. It is an advantage if you, or someone in your organisation, takes responsibility for monitoring legal developments in the biological property area and the resulting legal obligations towards the various classes of landowners and managers. The conservation agency in your jurisdiction (or sometimes a unit in another department like Agriculture) should be able to help; your State Herbarium may also have someone with knowledge of current practices and legal developments.

The legal situation regarding 'biological property', including seed, is complex, varies from one jurisdiction to another, and between public and private tenures. It is also evolving rapidly, and unfortunately it is not possible to give absolute guidance. In all cases, the general principles are 'prior informed consent', and 'no commercial use of material collected for non-commercial purposes'. You must also be very careful to anticipate any likely wish on your part to transfer biological material to anyone else in the future – standard consent agreements are increasingly likely to specify no transmission of material or extracts to third parties without permission.

You must keep thorough and permanent records of what has been collected where, and what permissions were obtained. You and your organisation are also responsible for, and may need to demonstrate, a capacity to maintain indefinitely records of agreements that constrain, or place conditions on, the use of particular batches of seed or other material.

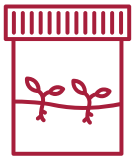
2.4 What are the possible ex situ conservation storage options?

Germplasm can be stored as seeds, plant material or plants but for most species, seed banking is the major ex situ storage option due to the relative ease of storing genetically representative collections (Table 2.1). Other options to consider are conservation of plant material through tissue culture, cryopreservation and living plant collections (i.e., nursery and garden collections, field genebanks). Such techniques may be the only option for species that fall into the '**exceptional**' category (Case Study 2.5). The storage potential of a species' seeds should be determined before seeds are banked to avoid unnecessary waste (Chapters 4 and 6). In order to establish any of these collections, technology is required for seed collection, storage and germination, vegetative propagation of plant parts, tissue and cell culture and plant cultivation (see Chapters 3–8). One of the most important factors in the conservation and utilisation of plants is the ability to effectively propagate target species for a variety of outcomes. Whatever ex situ option is used, specialist expertise and management are required to both establish and maintain the genebank collection and then produce living plants from this stored material.

There are distinctions among the ex situ collection/storage options, and each method should be assessed in terms of the appropriateness of the action. Maunder *et al.* (2004a) and Havens *et al.* (2004) set out a range of ex situ and in situ plant conservation methods to identify the relative ongoing effort and/or marginal resources required when considering collection storage options, e.g., seed banking (lower technology) versus cryopreservation (higher technology).

It is often appropriate to choose more than one ex situ conservation strategy for a species, especially for critically important species. The amount of effort and resources required varies greatly and should be carefully considered in planning collections. Remember to factor in the long-term commitment that is required for whichever options you choose.

Table 2.1 The major ex situ conservation options available in Australia and their relative advantages and disadvantages (see also individual chapters). Also see Case Study 2.6 for the location of current Australian germplasm repositories (Figure 2.4). Icons: CAM graphics.

Major ex situ conservation options available in Australia	Advantages	Disadvantages	Relative resource effort required
Seed banking **Chapters 5 and 6** 	Long-term storage of orthodox species; relatively space efficient; relatively low cost.	**Desiccation sensitive** species are not suitable; protocols for storage and germination for a lot of Australian plants are still to be developed; potential loss of viability/loss of genotypes as a disadvantage of aging seed collections in seed banking.	Usually the least resource intensive.
Tissue culture **Chapter 9** 	Suitable for a limited number of genotypes for short-term storage (up to five years); high volume production of plants; allows various manipulations; symbiotic culture.	**Genetic variability** of collections is usually low; success highly dependent on tissue/environment response; expensive to develop protocols for new taxa; high technical skills and facilities required; genetic changes possible over time.	Resource intensity is high – reliant on established protocols and skilled staff.
Cryopreservation **Chapters 10 and 12** 	Long-term storage of plant parts including seeds.	Success is highly dependent on the tissue/environment response; expensive to develop protocols for new taxa; high technical skills and facilities required; requires use of liquid nitrogen (LN) so an affordable and reliable source of LN is essential.	Resource intensity is high – reliant on established protocols and skilled staff; still highly experimental for native wild-source germplasm.
Living collections **Chapters 8 and 11** 	Living plants are available for a variety of conservation-related purposes e.g., species research and horticultural display and education; metacollections; seed and vegetative material can be propagated for translocation in facilities used for other types of cultivation; may be low relative cost.	The genetic variability held is usually low, except in the case of some intensive production systems, e.g., herbaceous species; cultivation may select against some genotypes; hybridisation or genetic bottlenecking may occur in seed produced.	Medium to high intensity resources required, especially in establishment phase. Aim to integrate into botanic gardens or equivalent for optimal care.

2.4.1 Seed banking

Most species are orthodox in terms of seed storage, that is, they tolerate the drying required for long-term conservation at freezing temperatures (Chapter 5). However, some species are **non-orthodox** or 'exceptional' species as they have seeds that lose viability when dried to low **moisture contents**, or they die at low temperatures (see Case Study 2.5). Such species, also sometimes termed '**recalcitrant**' or '**intermediate**' depending on their behaviour during drying and freezing, will require specialist storage procedures rather than conventional seed banking processes and this needs to be determined as early in the planning process as possible (Chapter 6).

Large seeded species from high rainfall areas and particular taxonomic groups should be suspected of having recalcitrant or intermediate seeds (Dickie *et al.* 2002; Ashmore *et al.*, 2007; Wyse and Dickie 2018). Where there is no information for a species, the status might be inferred from a very closely related species. Caution should be exercised when inferring at a higher taxonomic level, as families may contain species with a range of **desiccation tolerances** e.g. the family Myrtaceae contains fleshy-fruited recalcitrant *Syzygium* species, through to extremely desiccation tolerant *Eucalyptus* species. Species within a genus are likely to have shared storage behaviour (though exceptions are known, e.g., *Araucaria* species). Alternatives such as cryopreservation, tissue culture and living collections need to be considered for 'exceptional species' (Case Study 2.5, see Chapters 8–11 for techniques).

For most species, seed collection and storage are the most appropriate actions. Seeds are preferred because they are the plant's natural storage device for their genetic material and are often naturally 'packaged' to facilitate survival and **longevity**. Seed collection and storage may be an appropriate option for ex situ conservation if you can answer 'yes' to some or all of the following questions:

- Are seeds of this species known to survive storage in seed banking conditions?
- Does the species produce a sufficient quantity of seeds?
- Does the species produce viable seeds?
- Are seed processing and storage facilities available?

Often information on the first three points may not be known, but can be inferred from information on taxonomic allies, or from habitat or seed characteristics. Local knowledge often provides information on seed production times and should be sought whenever possible. Otherwise, small initial collections can be made and used to determine this information and inform the plans for ex situ conservation.

Case Study 2.5: A network and tools for conserving 'Exceptional Plants'

Valerie C. Pence, Joachim Gratzfeld, and Abby Meyer

The term *exceptional species* has been developed as a way of providing focus to 'plant species that cannot be efficiently and effectively conserved long-term ex situ under the conditions of conventional seed banking, requiring alternative conservation approaches' (Pence *et al*. 2020). This includes species for which seeds are not available, whether they are inadequately produced, inaccessible, or non-viable, as well as species with desiccation sensitive seeds, species with freeze-sensitive or short-lived seeds, and species with deeply **dormant** seeds. It is estimated that desiccation sensitive (recalcitrant) species will number over 30,000 globally, and species with short-lived seeds may be even more numerous (Colville and Pritchard 2019). Numbers of species without adequate or accessible seeds to bank or with deeply dormant seeds (which present several obstacles to banking) are more difficult to project and will need to come from expert comment on individual species.

A working, global List of Exceptional Species has been initiated to identify and classify the exceptional plants of the world (Pence *et al*. in press). The goal of the list is to focus attention on species that require alternative methods for long-term, ex situ conservation. It also classifies each species by the cause of exceptionality, information that can be used to target the most appropriate methods for conservation. Because this current list falls short of the large number of projected exceptional species, additional information is required from researchers and conservation practitioners worldwide. The Exceptional Plant Conservation Network[22] has been established to facilitate the gathering of such information, which can be entered online, as well as to facilitate practical conservation measures that can be applied to exceptional species. It also provides a platform for linking researchers and practitioners specialising in exceptional species conservation through the Directory of Expertise[23], developed by Botanic Gardens Conservation International (BGCI).

Ex situ conservation of exceptional species will require alternative conservation approaches, including coordinated consortia of living collections in botanic gardens or field gene banks. Several Global Conservation Consortia have been or are being established for taxonomic groups containing large numbers of exceptional species by BGCI, including species of oaks, magnolias, maples and cycads (Westwood *et al*. 2020). These consortia will coordinate institutions and experts working collaboratively to develop and implement strategies to prevent extinction of exceptional species, including genetically representative ex situ collections as well as in situ population recovery programmes.

An additional conservation approach for exceptional species is the use of **cryobiotechnologies** for long-term storage of seeds, pollen, or vegetative tissues in liquid nitrogen. Such methods are more resource intensive than conventional seed banking, but have been demonstrated for a wide variety of plant tissues and have proven safe and effective in maintaining viability for at least 20–30 years (the length of time such studies have been underway, thus far) (Pence *et al*. 2020; Walters and Pence 2020).

22 http://cincinnatizoo.org/conservation/crew/exceptional-plant-conservation-network/
23 https://www.bgci.org/resources/bgci-databases/directory-of-expertise/

Exceptional plant species pose a significant challenge to global plant conservation efforts. However, we now have information and tools as well as networks to facilitate access to resources and coordination of effort to begin to meet these challenges and ensure that exceptional plants are conserved into the future.

References

Colville L, Pritchard HW (2019) Seed life span and food security. *New Phytologist* **224** (2), 557-562. doi:10.1111/nph.16006.

Pence VC, Ballesteros D, Walters C, *et al* (2020) Cryobiotechnologies : Tools for expanding long-term ex situ conservation to all plant species. *Biological Conservation* **250**, 1-8. doi:10.1016/j.biocon.2020.108736.

Walters C, Pence VC (2020) The unique role of seed banking and cryobiotechnologies in plant conservation. *Plants People Planet* **3** (1), 83-91. doi:10.1002/ppp3.10121.

Westwood M, Cavender N, Meyer A, Smith P (2020) Botanic garden solutions to the plant extinction crisis. *Plants, People, Planet* **3** (1), 22-32. doi:10.1002/ppp3.10134.

2.4.2 Other ex situ conservation options

If it is known or suspected that a species does not produce seeds, or seed quality is low, or germplasm does not survive drying and/or freezing, various options may be available (Table 2.1).

When considering non-seed ex situ conservation methods, consider the following:

- Has the species or a close relative been conserved by this method before?
- What is the best tissue to sample?
- Does it have specific horticultural or other requirements?

2.4.2.1 Vegetative propagation

Plants may be propagated vegetatively by cuttings, bulbs, corms, **rhizomes**, **tubers** or any other plant part demonstrating **totipotency** (ability of a plant cell to form into an organ or whole plant) (Chapter 8). These techniques are often applied where unique or potentially useful genotypes need conservation, to ensure that clonal replicates are maintained.

Taking whole plants (transplants) is generally not recommended, rarely appropriate and often not successful. It can be considered in certain circumstances such as when a population would be destroyed (see Commander *et al*. (2018) Translocation Guidelines, section 1.3.2 and 2.2.3). It is also recommended that other forms of conservation be carried out, such as taking cuttings or divisions (Chapter 8).

2.4.2.2 Cultivation of living collections

The options for living plant conservation should be carefully considered in terms of benefits and risks because living collections are often highly labour intensive, and therefore costly in terms of time and resources (Chapter 11). It may also be difficult to adequately conserve the full extent of genetic variability for a species. It should be noted that the living collection method itself is not the issue, but the practicality of using it and doing it thoroughly at the scale needed is where it can fall short.

Botanic Gardens and other specialist gardens generally contain dedicated documented collections for display, reference, research, education and other uses (see Chapters 8 and 11). Many models for managing or indexing dispersed cultivated collections exist, especially in Europe and North America, albeit often with an emphasis on ornamental plants or crop varieties e.g., FAO Genebank Standards[24] including field genebanks. For native plant examples, see the [USA] National Collection of Endangered Plants[25]. Though not restricted to native species, the PlantSearch database[26] of Botanic Gardens Conservation International collates data from the organisational databases of 1,000 gardens.

Other cultivation options include agricultural field or pot collections (primarily for breeding), community or household gardens, metacollections, commercial holdings and incidental collections. Commercial production may be considered a conservation option if sufficient material is disseminated and pressure is taken off wild populations e.g., cultivation of plants for seed and cut-flower production to replace unsustainable or illegal harvesting[27].

Field genebanks include standing in-ground collections, seed production areas, seed orchards (see Chapter 11) and populations grown **inter situ** (plants cultivated in or near natural conditions). These may be established from seeds or vegetative material and used as sources of material for conservation purposes. Field genebanks have the potential to be an intermediate cost option between seed banking and intensive cultivation although they are not commonly used for wild species conservation (but see Case Study 3.3).

2.4.2.3 Tissue culture and cryopreservation

Plants, seeds, organs or cells cultured **in vitro** by tissue culture techniques or preserved cryogenically require specialist personnel and facilities; protocols for new species may take years to develop (see Chapters 9, 10 and 12). This option is particularly important for orchids, fungal spores and **hyphae** e.g., orchid **mycorrhizae**, and other **symbionts** such as **rhizobia** (Chapter 12). Cryopreservation is key to spore-banking of **pteridophytes** (ferns and fern allies) and **bryophytes** (mosses and liverworts), as well as pollen storage (Chapter 13). Storage of these special collections is in its infancy in Australia but has potential to become increasingly common.

2.5 Identifying resources required for ex situ conservation

Before committing to ex situ conservation of a collection, consider the following:

- What are the immediate and possible longer-term purposes of the collection?
- How long is conservation required?
- Are the appropriate resources available for the required time-frame?

It is vital that there are adequate resources available for storage of all forms of plant material needed for conservation purposes. Different conditions may be required for storage depending on the purpose of the collection, with the viability of the material being the governing factor (Chapter 5, Table 5.1). For example, seed being held for long-term conservation in a freezer is likely to require far more stable and permanent conditions than seed being accessed from a refrigerator

24 http://www.fao.org/3/a-i3704e.pdf
25 https://saveplants.org/national-collection/
26 www.bgci.org/plant_search.php
27 https://www.environment.nsw.gov.au/-/media/OEH/Corporate-Site/Documents/Licences-and-permits/cut-flower-sustainable-management-plan-170678.pdf

more frequently for restoration (see Commander *et al*. 2021), research, display, short-term use or education purposes. However, consider storing such collections under more strictly controlled conditions (i.e., low moisture content, low temperature) if possible (Merritt and Dixon 2011), thereby increasing their future usefulness and lifespan.

When framing the purpose of the germplasm collection, it is important to give at least some consideration to its potential for use by other stakeholders now or in the future. Such consideration may complicate the permissions process, and future use of a commercial nature may need to be excluded, as may secondary use of some species that are subject to cultural permissions, but where it is possible to leave some window for future non-commercial use (e.g., by incorporation into the general conservation holdings of an institution) this is desirable in principle. Similarly, institutions that are custodians of germplasm collections should adopt policy and practice that precludes overly casual deaccessioning of potentially significant germplasm collections – usually a range of stakeholders should be consulted before material is dispersed or destroyed.

One of the most important actions in establishing ex situ conservation collections is ensuring their curation and maintenance over time (Case Study 2.4), as collections often outlast the personnel who made them (Whitehouse *et al*. 2020). Good planning, documentation and recording systems should help to overcome this problem (see Chapter 15). Summaries of documentation should be lodged with appropriate conservation agency offices, especially for rare or threatened species or communities.

In some cases, it may be appropriate to replicate collections in one or more locations. This minimises the risk of complete loss of material due to equipment failure, natural disaster e.g., bushfire, disease, political instability etc (see Chapter 14). Re-testing of seed collections at appropriate intervals will guide the need to recollect species (see Chapters 5 and 15). This is especially important for species known to be short-lived in storage such as orchids (Chapters 5 and 6).

2.5.1 Sharing of resources

The resources required to adequately conserve the Australian flora ex situ are beyond the capacity of any one organisation or institution. The formation of partnerships by complementary organisations is a means of achieving successful plant conservation outcomes and goals for the whole of Australia (Case Study 2.6).

Partnerships can give considerable benefits to conservation programs, as can be seen in various case studies (e.g., 'Tropical Mountain Tops' project – Case Study 8.2, 'Care for the Rare' and conservation partnerships for Myrtle Rust susceptible species – Case Study 8.2 and 11.4). Through such networks, information, expertise, resources and germplasm can be shared, minimising risks to collections (Chapter 14).

Case Study 2.6: Partnerships for seed conservation: The Australian experience

Damian Wrigley and Tom North

In 2001, the Australian partners of the Millennium Seed Bank Project – the Royal Botanic Gardens, Kew's international ex situ seed conservation initiative – formed the Australian Conservation and Research Network (AUSCaR). The establishment of this network brought together the expertise and facilities of Australia's leading native flora seed banks to deliver ex situ conservation. In 2010 this network of seed banks embarked on a new initiative to strengthen collaboration across their facilities and deliver a more strategic, national approach to ex situ seed conservation of the Australian flora.

The Council of Heads of Australian Botanic Gardens, Australia's peak body for botanic gardens, formalised the creation of Australian Seed Bank Partnership (the Partnership) with the release of its first Business Plan (2011–2020). In the years since, the Partnership has worked collaboratively to achieve its vision of a future where Australia's native plant diversity is valued, understood and conserved for the benefit of all.

Over the past decade the Partners have participated in numerous collaborative projects, sharing knowledge and information to support improvements to shared practices and approaches to ex situ conservation. The Partnership represents an evolving network of facilities with many Partners experiencing upgrades to equipment and facilities over the past decade, ensuring that collection and research programs are delivered to the highest standard (Figure 2.4). Since 2011, each of the Partners have been assessed against international standards for best practice ex situ seed conservation, delivering high quality collections for use in research and future restoration activities.

Australia is sufficiently large enough with a well-documented and rich diversity of native taxa to be capable of sustaining a network of facilities across the country, rather than a single large facility. Despite the challenges of working across a continent the size of Australia, the Partners collaborate regularly on research and conservation projects, aligning these based on plant assemblages (rainforests, see Case Study 8.3), areas of expertise (orchids, see Chapter 12) or responses to specific opportunities or threats (*Austropuccinia psidii* or *Phytophthora cinnamomi*).

Since 2012 the Partnership has secured collections of almost 1,500 new taxa through collaborative Partnership projects. Many more species have been secured by the individual partners during this time with approximately 20,000 collections secured in seed banks throughout the country and nearly 10,000 taxa duplicated in the United Kingdom in recognition of almost 20 years of financial support and capacity building provided through the Millennium Seed Bank at the Royal Botanic Gardens, Kew.

The Partnership holds a unique space within the Australian conservation sector with seed banks hosted by capital city botanic gardens and environment agencies, an arrangement that provides ready access and close engagement with local, national and international threatened species policy development and implementation (Figure 2.5). The Partnership also brings together the only organisations in each state and territory that carry responsibility for the collection, storage and maintenance of germplasm collections of Australia's native flora, including legislatively threatened native flora. The seed banks work toward securing both locally and nationally legislated threatened species, delivering better representation and conservation for those taxa most at risk.

The Partnership has maintained collaborations with associate organisations at the regional, national and international levels, contributing significantly to the targets of the Global Strategy for Plant Conservation. The Partnership's engagement with the Australian Network for Plant Conservation and Greening Australia has helped to maintain strong linkages with Australia's leading plant researchers and restoration practitioners. In addition, collaborations with Botanic Gardens Australia and New Zealand (BGANZ) and Botanic Gardens Conservation International (BGCI) help to provide opportunities for collaborations and recognition of our work, including through initiatives such as the Global Seed Conservation Challenge Award. The Partnership also shares the knowledge it creates through conferences, online seed and image data bases and publications.

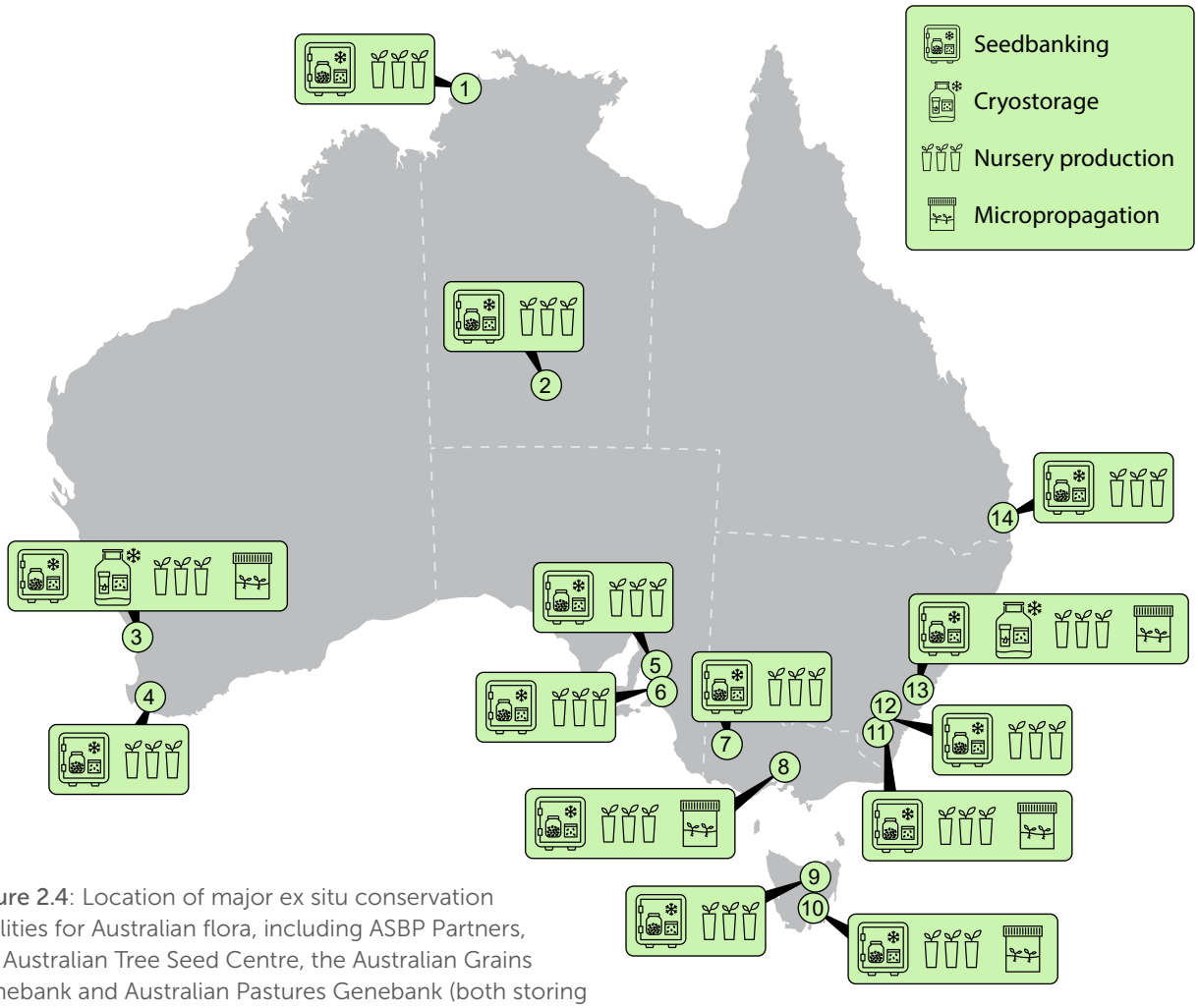


Figure 2.4: Location of major ex situ conservation facilities for Australian flora, including ASBP Partners, the Australian Tree Seed Centre, the Australian Grains Genebank and Australian Pastures Genebank (both storing crop wild relatives) and major forestry seed banks with conservation collections. (Image: CAM Graphics)

1. George Brown Darwin Botanic Gardens conservation seed bank
2. Alice Springs Desert Park
3. Western Australian Seed Centre, Department of Biodiversity, Conservation and Attractions, Kensington, and Kings Park and Botanic Garden
4. Forest Products Commission Seed Centre
5. Australian Pastures Genebank, South Australian Research and Development Institute
6. South Australian Seed Conservation Centre, Botanic Gardens and State Herbarium of South Australia (BGSH)
7. Australian Grains Genebank, Agriculture Victoria
8. Victorian Conservation Seedbank, Royal Botanic Gardens Victoria
9. Tasmanian Seed Centre, Sustainable Timber Tasmania
10. Tasmanian Seed Conservation Centre, Royal Tasmanian Botanical Gardens
11. National Seed Bank, Australian National Botanic Gardens
12. Australian Tree Seed Centre, CSIRO
13. Australian PlantBank, Australian Institute of Botanical Science, Royal Botanic Gardens and Domain Trust
14. Brisbane Botanic Gardens Conservation Seed Bank, Brisbane Botanic Gardens, Mt Coot-tha

Since 2011, Parks Australia's Australian National Botanic Gardens has hosted the Partnership's National Coordinator, a position that seeks to secure funding for collecting and research as well as facilitating engagement with a variety of conservation and flora-related initiatives, policies and programs. Each Partner seed bank carries responsibility for the collections in their facilities and deliver conservation outcomes based on the local, regional and national priorities for flora conservation. The role of the National Coordinator supports Partner efforts by providing a coordination and representation role predominantly for larger, multi-partner projects.

The post-2020 global **biodiversity** framework will be critical for plant conservation in Australia and indeed globally. The Partnership is therefore continuing into the next decade with a focus on collecting, storing and undertaking research into Australia's native flora, with the aim that the long-term ex situ conservation of plant germplasm in Partner seed bank facilities will provide the best chance for conserving Australia's native flora for future use.

Figure 2.5: In May 2018, Partners and Associates of the Australian Seed Bank Partnership came together at the South Australian Seed Conservation Centre at the Adelaide Botanic Gardens. (Image: ASBP)

2.6 Utilisation of material

Utilisation of plant material is the major objective of germplasm conservation. Keep this in mind while using the various options covered in these guidelines to ensure that the desired utilisation of material is possible. Each germplasm repository should establish clear protocols for the end use of their collections. Include in the protocols reference to conditions of the collection licences, as these are likely to change over time. Details for germplasm maintenance and utilisation are found in Chapter 15.

2.6.1 Base and active collections

Some seed banks split their collections into a 'base' collection that holds essential material for long-term conservation, and an 'active' collection that can be used for a variety of purposes. This adds extra complexity to collection management and should be carefully considered.

A **base collection** is a limited number of accessions (material with a unique identifying code) derived from an existing collection that has been chosen to represent the genetic spectrum of that collection. The base collection can be kept in a separate location or earmarked within the main collection and is only to be used under a specified set of circumstances.

An **active collection** will often be a larger collection of seeds or plants that can be accessed for end use in a variety of programs including research, further propagation and restoration. Information regarding these collections should be linked, and results of germination or other work on material from the active collection may inform storage or usage of the base collection (or vice versa).

One of the principles behind this approach is that utilisation of the material is an adaptive process. Trial and error, or replicated experimentation where resources allow, may over time improve the success rate of propagation and growth of the species, being mindful that for rare species, the collections may not be of sufficient size for large scale research. In such cases, 'analogue' or closely related non-threatened species may be suitable on which to conduct the necessary storage, propagation and establishment research. Base collections should therefore only be used where other suitable material does not exist and there is a high chance of successful regeneration.

Well before a collection is expended, particularly the active portion, consider recollecting or regenerating the material (Chapter 15). The data associated with expended collections must be maintained as it can guide future collection.

2.6.2 Access and benefit sharing

The Nagoya Protocol within the Convention on Biological Diversity (CBD), and other overarching conventions, encourage the beneficial sharing of genetic material, while recognising the rights of the owners of genetic material (whether political, private or cultural). When appropriate, and where material is reasonably available, germplasm repositories may share with other interested parties usually through a formal agreement process (Chapter 15). The obligation of the recipient is to respect intellectual property and comply with any genetic property rights of the supplier and original owner of the material, in the first instance by complying with requirements to use the material in the agreed manner only. In the case of some threatened species, a separate permit to hold or use material may be required.

2.6.3 Phytosanitary and biosecurity considerations and obligations

Phytosanitary considerations when making collections are aimed at minimising risks to the wild populations, and to susceptible biota. Phytosanitation should also extend to moving material between organisations, as it is reasonable to expect that material is disease-free and pest-free. The potential weediness of each species should also be considered when distributing plant germplasm. Phytosanitary conditions during collection and storage are discussed within the individual chapters. Quite often, phytosanitary and import certificates are required when moving material between countries, states and even adjacent areas (Chapter 11 section 11.8 and Chapter 15 section 15.4.2).

Acknowledgements

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Chapter 3
Genetic guidelines for acquiring and maintaining collections for ex situ conservation

Marlien van der Merwe, Linda Broadhurst, David Coates, Maurizio Rossetto

3.1 Introduction

Genetic diversity plays an important role in the survival and adaptability of species and **populations** and is recognised as a key component of **biodiversity** by the Convention on Biological Diversity, Aichi Biodiversity Targets, agreed in 2010[28]. To ensure long-term species persistence we need to conserve the raw genetic material that species need to evolve and not just individual members of a species. Populations that lack genetic diversity cannot evolve in response to change and have an increased risk of local extinction.

The purpose of a **germplasm** collection determines how the genetic diversity in the wild should be represented in that collection but in general, collections that are more representative of the natural diversity are more valuable for future proofing a species. Genetic data verifies the level of diversity present within populations and species and should be considered as a valuable tool to acquiring and maintaining germplasm collections.

Here, we provide a short overview of what genetic diversity is and the main reasons why it should be maintained. We provide a summary of the factors identified through theory and research that drive the distribution of genetic diversity. We outline how specific functional plant traits can guide collection strategies to help increase the genetic diversity in germplasm collections. Collections of species based on genetic data will be guided by those data, so we do not discuss specific collection strategies but rather highlight the value of such data.

28 https://www.cbd.int/sp/targets/

3.2 What is genetic diversity?

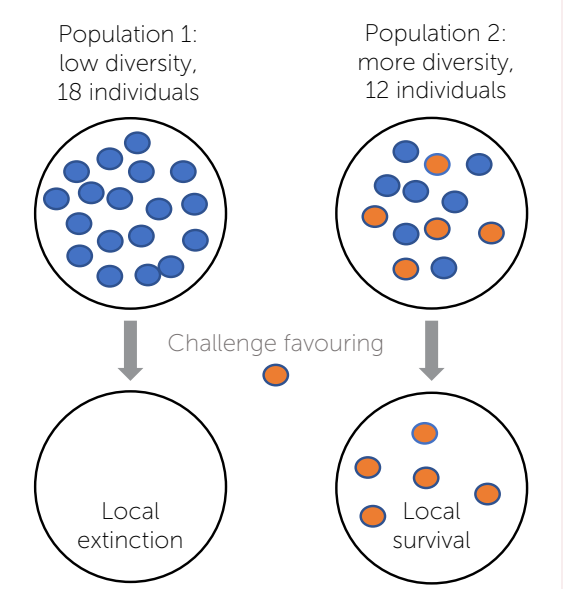
Genetic diversity is the term used to describe variation in **genes** or other **DNA** fragments that arise through **mutation** and **recombination**. The DNA sequence of a gene can differ among individuals and these are called gene variants (or **alleles**) or polymorphisms. How gene variants are distributed within individuals (number of genes with different variants), between individuals in a population, and among populations contributes to a species' genetic diversity. The different gene variants may contribute differently towards the **phenotype** (i.e., the traits we can measure such as flower colour, leaf thickness, salt tolerance, etc) and the **fitness** of an individual.

Genetic diversity is maintained through a broad range of dynamic factors and changes constantly in natural populations. Natural selection removes variants that reduce the fitness of an individual and contributes to the increase in variants that provide benefits. **Genetic drift**, on the other hand, randomly removes variants with small populations being particularly prone to loss of diversity through this process.

Box 3.1: Why is genetic diversity important?

Genetic diversity enables a species to respond and adapt to change or a challenge.

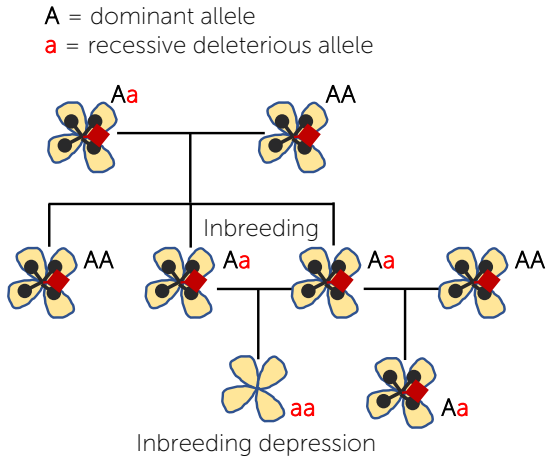
Here we illustrate how two populations with different levels of genetic diversity respond to a strong selective challenge such as drought, a heatwave, a new pest or disease. In our hypothetical example the challenge is a new strain of a disease. Individuals represented by the orange circle have a gene variant that enables recognition of the pathogen when it lands on the leaf, activating a response that prevents the pathogen from entering the plant cells. In population 1, this gene variant is absent, and all individuals become infected and die (**extinct**), whereas plants in population 2 with the pathogen resistant gene survived.



While this is a simple example, research has shown that genetic diversity influences many aspects of survival including the recovery of populations following disturbance, variation in fitness, species interactions, disease resistance and community structure.

Inbreeding and the risks associated with inbreeding depression

Inbreeding is the result of successful mating between two closely related individuals or through **self-fertilisation** (selfing). Inbreeding increases **homozygosity** where the two variants or copies of a gene are the same (in this example "aa" or AA" in comparison to the **heterozygote** "Aa"). Inbreeding is common in some plants, particularly those that can produce **seeds** through selfing.



When inbreeding leads to reduced fitness, such as lower seed production or reduced growth rates it is called **inbreeding depression**. In this hypothetical example a recessive allele (the red 'a') causes the loss of the reproductive parts of the flower. Plants that have both recessive alleles (aa) will be unable to produce seeds and contribute towards the next generation, whereas those without the allele (AA) or with only one copy (Aa) will be unaffected. In large populations, the effects of deleterious alleles such as this are often very small as there are many plants with other allele combinations to compensate. However, in small populations, such as the case for many threatened species this effect can be large and lead to extinction.

3.3 Germplasm collection strategies

We cannot precisely predict how future challenges will affect plant species and communities, but our aim should be to capture as much of the genetic diversity present in natural populations as is practically possible. This will ensure that these germplasm collections give species the best possible chance to respond to challenges such as changing environments, disease etc; in addition, this germplasm can be used to supplement existing populations that may be declining.

In recent years, increased attention has been given to genetic considerations in germplasm collection strategies, especially in relation to seed sourcing for **restoration** and **translocation** (Prober *et al.* 2015; Commander *et al.* 2018). Previously the focus was on using seeds from local plants or populations (local **provenance**) for **restoration** or **conservation** based on assumptions that local **genotypes** are best adapted to local environmental conditions (McKay *et al.* 2005; Leimu and Fischer 2008). More recent strategies, however, have recognised that environmental disturbances including climate change and **habitat** fragmentation are major challenges for plants and propose mixing source populations to maximise genetic diversity. These provenancing strategies are outlined in detail in the Florabank Guidelines (Commander *et al.* 2021), but broadly these recommend maximising genetic diversity and including genes that may enhance **adaptation** to changing climate. While these collection strategies have largely been centred around restoration, they can also be applied to other types of germplasm collections, particularly those that are conservation oriented. It is only very rarely possible to include the full extent of a species' genetic diversity in a germplasm collection, and we thus must try to maximise the extent of genetic diversity in a collection using carefully designed collecting strategies.

3.3.1 Key points for comprehensive sampling

While keeping in mind the information obtained with a background search for a species (see section 3.3.4), here are some general guidelines to maximise genetic diversity in germplasm collections:

- Collect from multiple sites to increase genetic diversity
  a. Genetic diversity is likely to increase with the addition of each new site, regardless of **mating system** i.e., whether the species is primarily **outcrossing**, selfing or a mixture of both (Hoban 2019).
  b. Sites should cover the geographic and ecological distribution (across known biogeographic barriers) of a species aiming to capture all potential **genetic variation**.
  c. Number of sites will vary according to the purpose of the collection, seed availability, mating system and other factors that influence genetic diversity (Box 3.3 isolation by distance and section 3.3.4 and Figure 3.4 below).
  d. Conservation collections should include as many sites as possible, but never over-collect at a site except where the site is approved for clearing (see also Chapter 4).
  e. Under no circumstance should seeds collected from different sites or different dates from the same site be pooled for a germplasm collection.
- Collect from multiple individuals at a site
  a. With the addition of each **maternal line** (mother plant), genetic diversity is likely to increase regardless of mating system.
  b. Avoid pooling of seeds from different maternal lines (Box 3.2, Why not pool maternal lines?).
  c. Focus on individuals that are wide-spread within a site (see Case Study 3.1; individuals that are close to each other are likely to be more closely related) and healthy (however, in some conditions, even unhealthy plants may have to be collected and included in conservation collections).
  d. Aim to collect approximately equal numbers of seeds from each maternal line. Some individuals produce more **fruit** than others and while it is tempting to collect more seeds from these individuals, this does not significantly increase the genetic diversity of the collections and can in fact lower the diversity.
  e. At sites where removal of vegetation for development has been approved, collections can be intensive if good records are kept, and **voucher specimens** are collected, databased and deposited in an appropriate institution. It may also be appropriate to collect more than one type of germplasm, such as cuttings and seed. Never pool the seeds with other collections when stripping a maternal plant.
- Sampling number: While the exact number of plants to be sampled will depend on different factors, collection from at least 50 individuals is recommended (however issues with strike rate, seed **germination** and survivability in a collection can rapidly reduce the size of a collection (Griffith *et al*. 2015) and there could be problems with clonality and inbreeding (see Box 3.1), i.e. selfing or crossing between related plants). For some threatened species with population sizes less than 50 plants it may be possible to sample all, or the majority, of individuals, and therefore a large proportion of the total genetic diversity available will be captured.

- If possible, collect leaf tissue material suitable for genetic work from all maternal lines (Steele, 2018). If these samples are processed and stored appropriately, these can be used for future research and will greatly improve the quality of the collection. Samples can also be freeze dried (**lyophilised**) and stored in airtight containers with silica, ideally in cold storage. In the absence of a freeze drier, samples can be dried with silica gel. These leaf samples will enable research such as parent identification and determination of mating system.
- All collections (seed, cutting, **tissue culture**, **cryopreservation**) made from ex situ collections (including living collections, seed orchards, and nursery grown plants) should be carefully labelled (see Chapter 11).
- Record keeping and **metadata** (see Chapter 15):
  a. Record keeping and maintenance of records with a collection is of utmost importance. All your efforts will be to no avail if the records are not maintained with the collection.
  b. Maintain location data for each collection and each maternal line, throughout the collection, storage and utilisation process. Field location data may be entered when herbarium vouchers are processed. See Chapter 4 section 4.5 for guidance on voucher specimen collection and field information recording.

3.3.2 Strategies for obtaining genetically representative collections

To acquire a genetically representative germplasm collection, it is important to know how this diversity is distributed in the natural populations. Ideally this would be through a targeted population genetic study and we highly recommend this an approach for all conservation management plans (Rossetto *et al.* 2021).

In practice, germplasm collections that aim to maximise genetic diversity can fall into distinct categories (Figure 3.1):

1. those informed by genetic data and
2. those where the collecting strategy was carried out in the absence of genetic data.

While genetic data can guide collection strategies, at present this data does not exist for most plant species in Australia. Consequently, we must rely on generalisations based on our understanding of the many factors that influence the distribution of the genetic diversity of a species (Figure 3.4). However, this approach can be problematic even between closely related and co-distributed species (Rossetto *et al.* 2020). In any case, following collection guidelines and maintaining high standards of record keeping (excellent location data and maintenance of maternal lines, Brown 1989), means that a collection can facilitate different provenancing strategies. Existing collections can then be augmented by new collections to increase genetic diversity. There are critical components relevant to genetic diversity in all collection strategies; to understand and monitor the level of genetic diversity in a collection it is best practice not to pool maternal lines (Box 3.2).

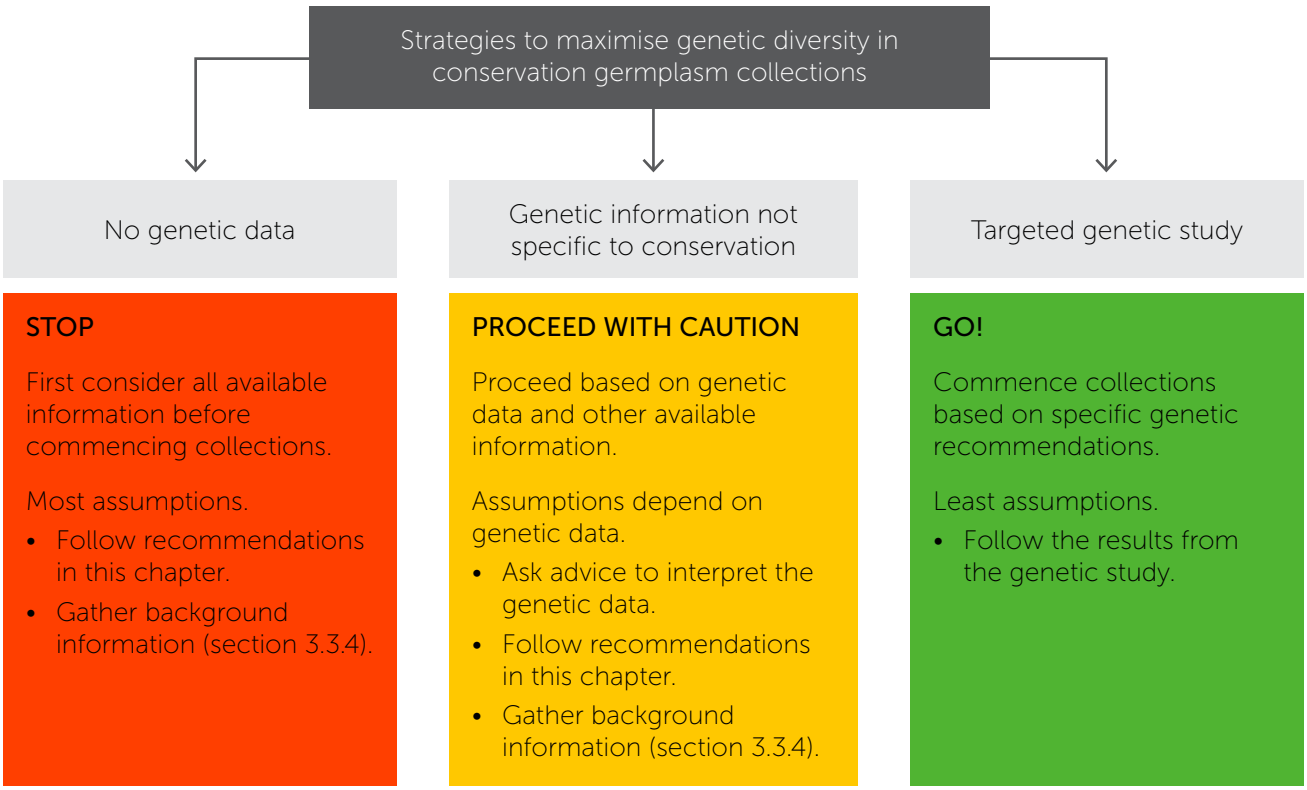


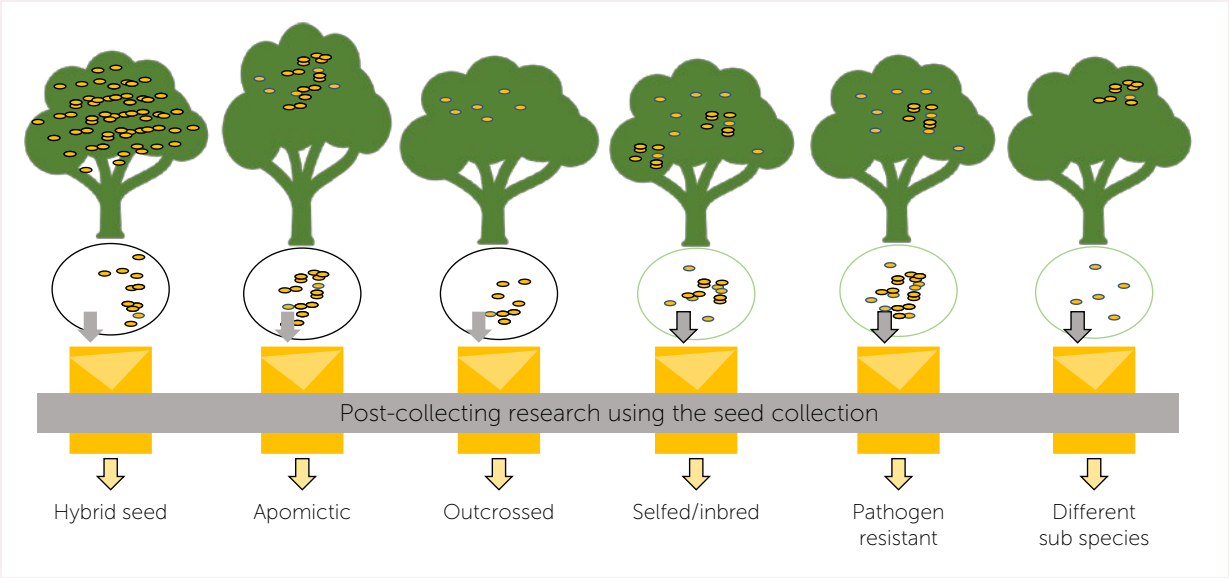
Figure 3.1: The information used to inform strategies to maximise genetic diversity in any germplasm collection will differ depending on the availability of genetic data. While all collections have some common features, the strategies to optimise genetic diversity will depend on the available information and how it is applied.

Box 3.2: Why not pool maternal lines?

Separation of maternal lines (seed from a mother plant) is one feature that distinguishes conservation collections from other seed collections (Center for Plant Conservation 2019). While pooling collections from multiple maternal lines saves time in the field, there are several good reasons why pooling seeds is not good practice.

Here are a few good reasons for keeping maternal lines separate:

- Taxonomic issues (species identification, **hybridisation** and polyploidisation) can be dealt with more easily.
- Contamination (pest, disease or other) and cross-contamination is limited to maternal lines.
- Seed viability and germination rate often vary across maternal lines (Roach and Wulff 1987) but cannot be detected in a pooled sample.
- Relative contribution of lineages can be impacted by kinship of maternal parents (Bragg *et al.* 2020).
- Long term storage can affect genotypes differently, leading to a loss of genetic diversity (genetic erosion) in the collection.
- Greater flexibility in use of the collection.
- Increases the value of the collection for future work.



Imagine if all these collections were pooled: How would the collected seeds be used? If these collections were pooled, it would be harder to select for specialised restoration outcomes such as replanting different subspecies or using pathogen resistant lines.

Remember that on average full siblings (same mother and father or pollen donor) will share about 50 % of their DNA, while half siblings (different pollen donors but same mother) will share about 25 % of their DNA. The actual amount may vary slightly, since recombination will shuffle the DNA differently for each seed.

3.3.3 Collections informed by genetic data

Existing genetic data will be useful in developing a collecting strategy, but a targeted genetic study is likely to provide the best options for the planned **ex situ conservation** collection and translocation and/or restoration activities. Both types of genetic data will inform the process of collecting but the first will still require some assumptions. A targeted genetic study is preferable for conservation and/or translocation because although predictions can be made, many factors play a role in the genetic diversity of a species and population (Figure 3.4, section 3.4). Targeted genetic studies can save time and resources and guarantee that collections are representative of the species (see Rossetto *et al.* 2021).

Various types of genetic data is available for many native Australian species. A literature search may provide valuable information (including Broadhurst *et al.* 2017 particularly the supplementary data and the Florabank Guidelines, Commander *et al*. 2021); while other information sources include Restore and Renew[29] for restoration species along the east coast of Australia. Although some published studies were not necessarily carried out with germplasm collections in mind, some of the results can still inform collecting strategies (see van der Merwe *et al*. 2021 for an example of the Queensland species *Acacia purpureopetala*). Many of these studies are published in scientific journals that may have open access to everyone, however if there is no access you can ask the authors for a copy. You can request help from the author, or a population geneticist or molecular ecologist to interpret the results.

Consult plant population geneticists or molecular ecologists, particularly when planning and designing a project and seek their advice to analyse and interpret data. They will also provide information on best practise in collection and storage of material for genetic research. There are several commercial services that extract DNA and do the **sequencing** or **genotyping** but analysis and interpretation will require specialist help.

The application of the genetic results to germplasm collections will depend on the sampling strategy and molecular technique used in the genetic study. For example, to describe boundaries for **genetic provenance**, Restore and Renew (Rossetto *et al.* 2019) uses a collecting strategy focussing on a large number of sites but fewer samples per site. Such data provide information on the **gene flow** and connectivity across the whole distribution of target species, enabling restoration practitioners to follow specific restoration strategies (Commander *et al.* 2021). On the other hand, when dealing with a threatened species and planning a translocation project (such as outlined in the case study for *Hibbertia puberula* subsp. *glabrescens*; Case Study 3.1; Bragg *et al.* 2020) most remaining individuals are genotyped. This genotyping provides details on site information for the collection strategy and subsequent downstream use of the collection such as translocation. Projects aiming to optimise a conservation collection according to a specific trait (for example, Myrtle Rust resistance, in *Rhodamnia rubescens*; Case Study 3.2) or for other purposes such as development of a seed orchard (see case study on *Eucalyptus benthamii*; Case Study 3.3) *and* maximise genetic diversity at the same time will use whole **genome** sequencing technologies and far more intense screening for specific genetic markers across a living collection. Genetic data can be highly informative for living collections where space and resources are limited (see Chapter 8 and 11 and the examples of *Pimelea spicata* and *Eucalyptus cattai* in Bragg *et al.* 2021).

Even with excellent genetic data, good record keeping and maintenance of these records for the life of the collection and beyond is crucial.

29 www.restore-and-renew.org.au

Case Study 3.1: Genetic principles for designing a translocation population: the case study of a critically endangered Hibbertia

Jason Bragg

Hibbertia puberula subsp. *glabrescens* is a perennial plant. It has a single small population, and occurs in a very small area, near an airstrip in western Sydney, NSW. To reduce the risk of extinction, translocation populations of *Hibbertia puberula* subsp. *glabrescens* have been established at several sites. The genetic composition of the translocation populations was determined carefully, with the goal of reducing the risk of genetic problems that can occur in small populations (Bragg *et al*. 2020). The approaches that were used to do this are potentially applicable to many species and are described below for one translocation as a case study.

Hibbertia puberula subsp. *glabrescens* is a prostrate shrub with small leaves and bright yellow flowers late in the spring. It has a small population (< 50 individuals), and narrow distribution, and is Critically Endangered under both state (NSW *Biodiversity Conservation Act* 2016), and federal (*Environment Protection and Biodiversity Conservation Act* 1999) legislation. The exemplar translocation consisted of taking cuttings from georeferenced individuals of the natural source population, propagating these cuttings in a nursery, and planting them at one or more different sites. Several sites were selected for the establishment of translocation populations, based on criteria including soil characteristics and other biotic and **abiotic** factors.

We set out to design the translocation population in a way that would minimise the risks of genetic problems. These problems include difficulty adapting to changing conditions due to low levels of genetic diversity (a lack of 'adaptive capacity'), and the possibility of reduced fitness of individuals due to inbreeding (Frankham 1995). These risks can be reduced by making the population as genetically diverse as possible and minimising the levels of relatedness between individuals in the population. The target size of the translocation population was set at 300 individuals, constrained by the space available at the translocation site. We therefore needed to determine the best number of clonal copies ('**ramets**') of each individual from the source population ('**genets**') to use in this translocation population. To do this, we started by generating a genetic dataset (Figure 3.2). This consisted of determining the genotypes of the individuals from the source population at thousands of locations in the genome. This helped establish that there was genetic variation among the individuals in the population, and that genetic variation among the individuals reflected their spatial distribution, in that very closely related plants tended to be close together.

We then considered different possible translocation populations, and used the genetic data to estimate their diversity, and the mean level of relatedness between individuals. We 'optimised' the design of the translocation population using an algorithm, resulting in a population design with high diversity and low mean relatedness between individuals. Cuttings were propagated according to this design, with adjustments that allowed for variation in propagation success among genets, while obtaining the highest diversity possible. Finally, we used genetic data to inform the spatial arrangement of the translocation plantings, with the aim of placing closely related individuals (especially **clones** of the same genet) apart in space.

In sum, a translocation of *Hibbertia puberula* subsp. *glabrescens* illustrates a set of methods for using genetic data to design a translocation population, with the aims of maximising diversity, minimising relatedness among plants, adjusting this design in light of propagation success, and placing closely related individuals apart at the planting site (Figure 3.2). These methods are potentially applicable to translocations of many endangered plant species.

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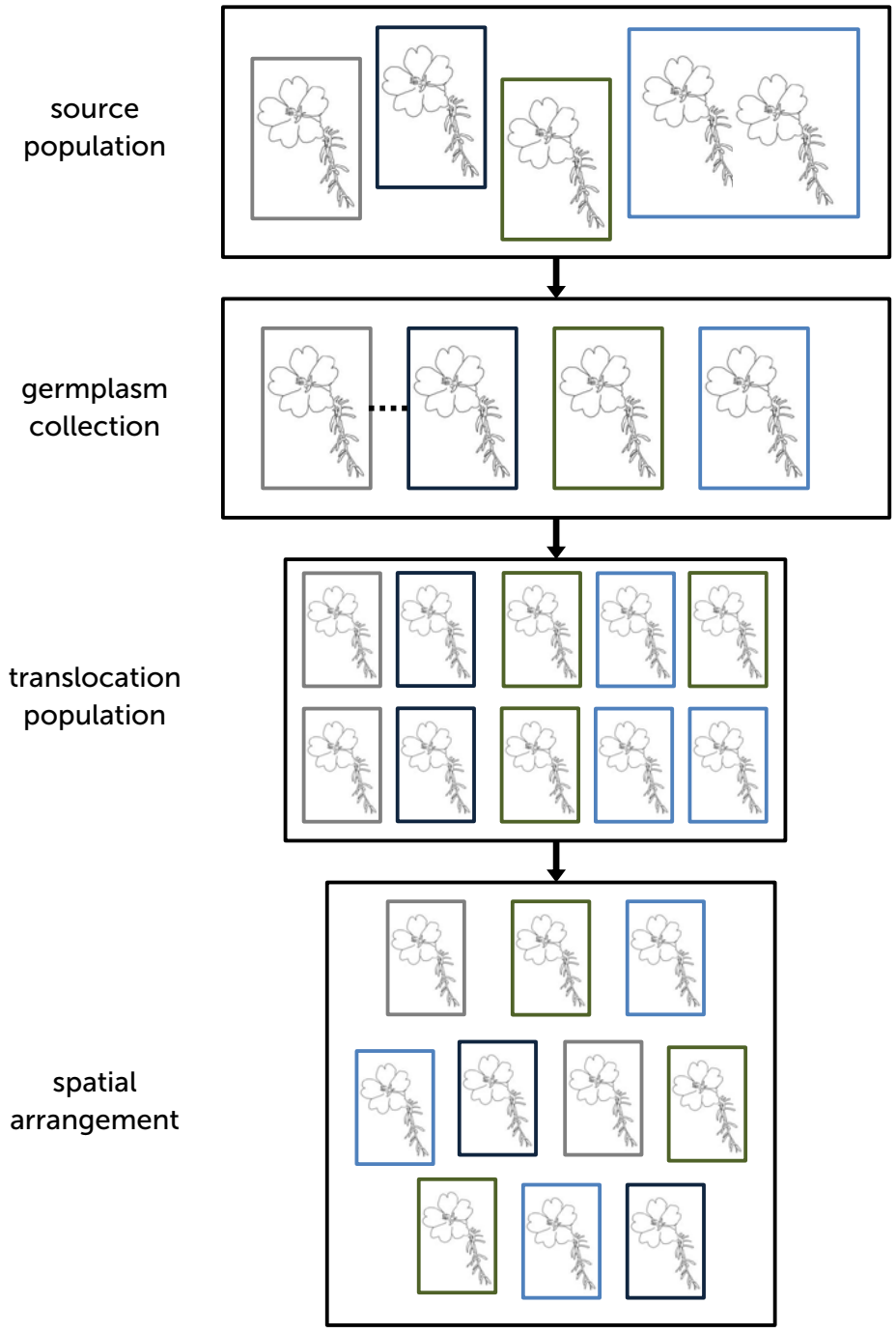

Figure 3.2: Idealised representation of approaches for designing a translocation population. A source population was sampled, revealing that some plants (light blue) are clonal ramets. The germplasm collection aims to include each genet from the source population. A translocation population is designed using cuttings from the germplasm collection, with reduced representation of ramets for two genets that are related to each other (connected by the dotted line). The translocation population was arranged in a way that aimed to avoid placing related individuals (especially clones) close together. (Image: Jason Bragg)

Case Study 3.2: Conservation genomics for Myrtle Rust impacted species

Stephanie H. Chen

Many Australian species of the family Myrtaceae are impacted by Myrtle Rust (*Austropuccinia psidii*) which was first detected in Australia in 2010 (Chapter 1 Box 1.1; Carnegie *et al*. 2010; Soewarto *et al*. 2019). For instance, the rainforest tree species *Rhodamnia rubescens* (Scrub Turpentine), which occurs in coastal districts north from Batemans Bay in New South Wales to areas inland of Bundaberg in Queensland, is declining rapidly due to Myrtle Rust infection. In 2019, it was listed as Critically Endangered under the NSW *Biodiversity Conservation Act* 2016 and Endangered under the QLD *Nature Conservation Act* 1992.

Genome scale data are becoming more widely adopted in conservation, facilitated by rapid technological advances in sequencing coupled with decreasing cost (Supple and Shapiro 2018). The increased feasibility of generating a reference genome for non-model organisms, including rare and threatened plant species, provides many benefits to common conservation genetics analyses. Notably, the reliability of genotype calls from genotyping-by-sequencing (GBS) datasets, also referred to as reduced-representation sequencing (RRS), is improved and this enhances downstream inferences (Wright *et al*. 2019). A key outcome of a genome scale research project on *R. rubescens* is to translate genomic information into a conservation program (Figure 3.3) through the identification of adaptive and disease resistance alleles. This will enable the spread of individuals with these alleles into populations and promote establishment of a genetically diverse and representative ex situ plant collections.

In the field, leaf tissue and cuttings were collected from approximately ten individuals per population across the species distribution for genotyping and propagation. Along with location data, phenotypic data on rust infection and general plant health were recorded for each sampled individual which contributed to the ongoing monitoring of Myrtle Rust and an enhanced understanding of how genotype interacts with the environment.

A high-quality reference genome of *R. rubescens* has been assembled. It is currently being used to characterise genetic variation and facilitate the identification of resistance alleles to optimise conservation populations to be genetically diverse and disease resistant. Consequently, these genomic data will allow us to understand population structure, inter- and intra-species variation in resistance and adaptation in Myrtle Rust affected species to develop recommendations for conservation management.

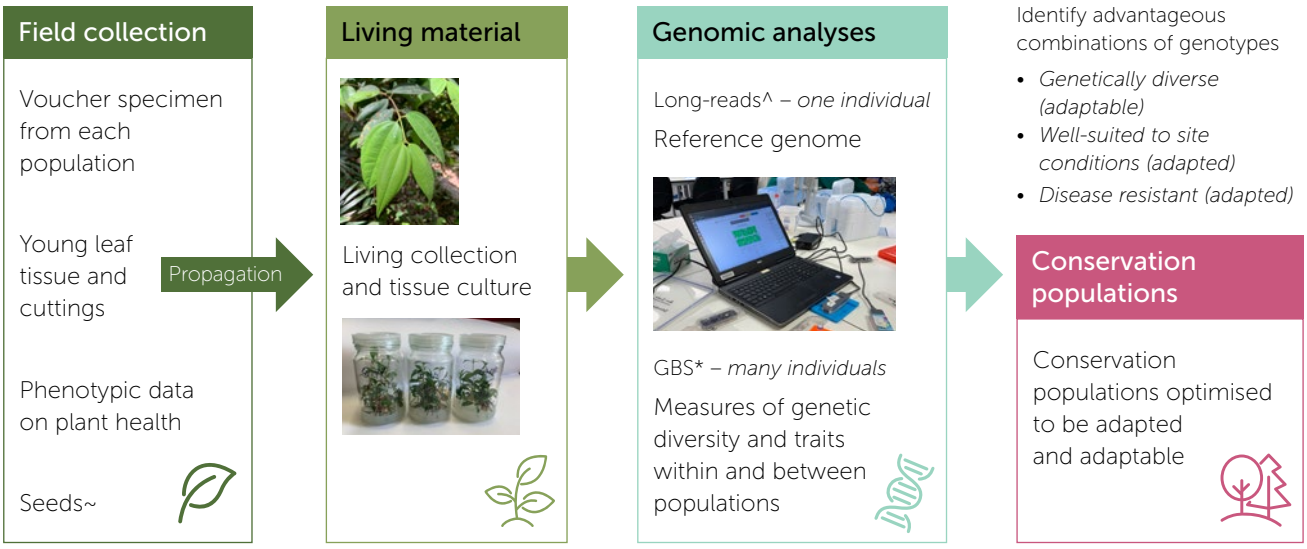

Figure 3.3: Flowchart outlining steps from field collection for genomic analyses to conservation outcomes.
~Not collected as **recalcitrant** and reproduction was affected by Myrtle Rust.
^In-house long-read sequencing using the Oxford Nanopore Technologies MinION.
*DArTseq was used as the genotyping-by-sequencing method (GBS). (Images: Stephanie Chen)

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3.3.4 Optimising genetic diversity without a targeted genetic study

In the absence of genetic data, there are two key steps to inform the development of a collection strategy – gather as much background information as possible and sample as comprehensively as possible. Record keeping is of particular importance during and after collections and will play an ongoing role in estimating and understanding the genetic value of a collection.

> The golden rule to optimise genetic diversity in collections is "*few seeds per plant but many unique maternal plants and visit most or all wild populations*" (Hoban *et al.* 2020).

3.3.4.1 Background information to inform genetically diverse collections

Numerous factors play a role in the distribution of genetic diversity within a species (Figure 3.4; Hamrick and Godt 1996; see Broadhurst *et al.* 2017 for an Australian perspective). Before you start collecting material from any species, we recommend that you do a thorough literature search to see what information you can find about the factors outlined below for the species of interest. These factors largely affect how genetic diversity is distributed at a site, how it changes between sites and across the distribution of the species (Box 3.3). Other sources of important information include government agencies, local Landcare groups, local seed collectors, citizen scientists, plant enthusiasts and NGOs. Literature searches can extend across **congeneric** species, but care should be taken when transferring information even between species as cryptic traits can influence genetic diversity (Rossetto *et al.* 2020). As no single trait should be considered during collection, the following information can be used to guide your collecting strategy.

A note on past practice: While life-form (tree, shrub, herb) is often used to infer levels of genetic diversity, it has not been found to be a consistent indicator given the interaction with other variables such as reproductive and life-history traits. However, an assessment of Australian species has indicated that long-lived trees generally have higher genetic diversity than shrubs and herbs (Broadhurst *et al.* 2017).

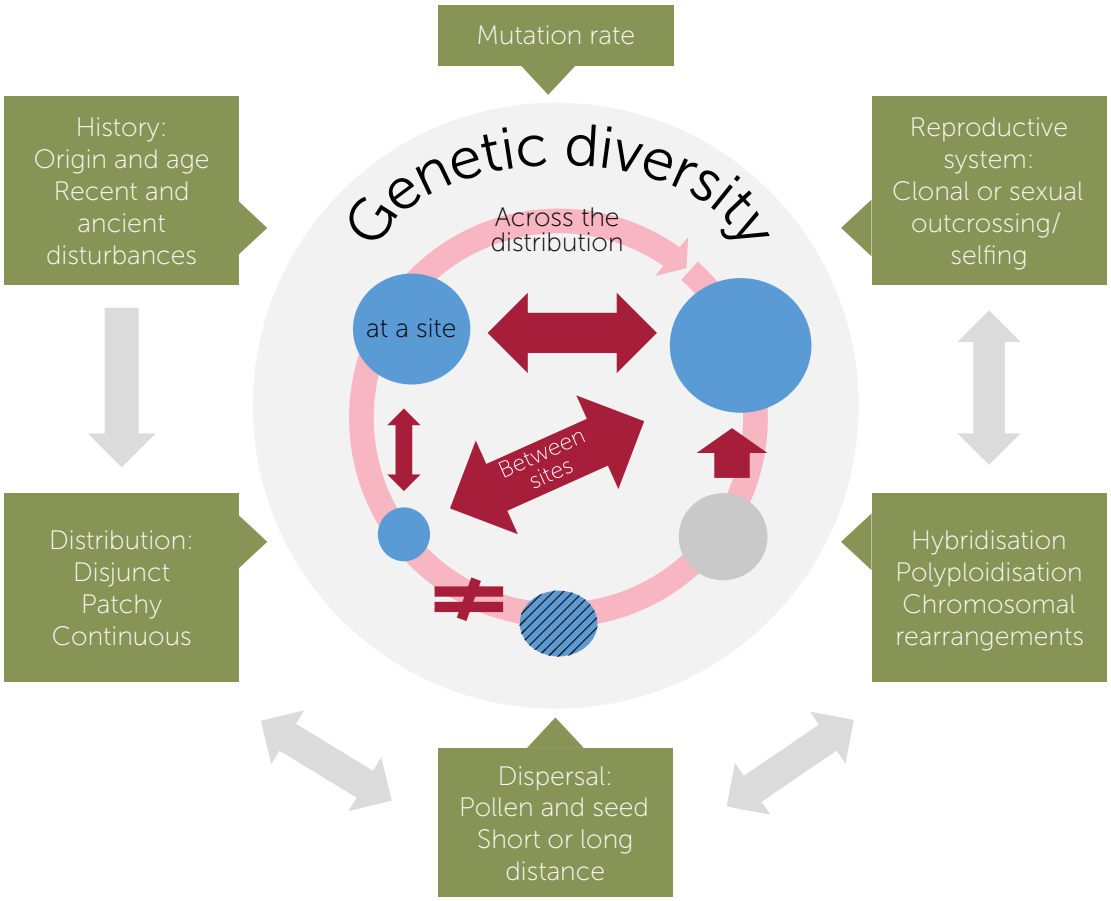


Figure 3.4: Many factors play a role in the distribution of genetic diversity in a species, at a site, between sites and across the distribution of the species. These factors (green boxes) should be considered when optimising the genetic diversity in a germplasm collection. Distribution of genetic diversity (Box 3.3) operates across the species distribution, between sites, and at a site. The amount of diversity can vary from site to site, as illustrated with the size of the blue circles and the level of genetic differentiation can vary across sites as illustrated by the width of the dark pink arrows. The grey circle represents genetic **introgression** from a congeneric species while the striped circle represents genome arrangements that can prevent successful sexual recombination.

3.3.4.2 Reproductive system and mating system

Plants can reproduce through sexual recombination and clonality (or both) and these systems affect how the genetic material of the offspring differs from the parents. The mating system determines how gametes (pollen and **ovules**) combine in natural plant populations and how genetic variation may vary from one generation to the next.

Mating system can be determined by genotyping maternal lines and offspring. Mating systems can vary across individuals, sites and time. Germplasm collections where seeds from maternal plants are not pooled can be used to determine the mating system. Hand **pollination** and/or intense "bagging" experiments where the flowers are enclosed in a bag before they open can be used to assess if the **taxon** is physiologically capable of self-fertilisation or can outcross with the organism used in the hand pollination experiment. This is often referred to as the breeding system and contrasts from what happens naturally in the population (the mating system).

Mating systems – Fertilisation between pollen and ovules on the same plant is known as self-fertilisation (selfing, selfed, **self-compatibility**) whereas if the pollen has come from another plant it is termed **cross-fertilisation** (outcrossing, outcrossed) (Figure 3.5). Plant species exhibit a wide array of mating systems from predominantly selfing to predominantly outcrossing and these have contrasting effects on patterns of genetic variation and rates of inbreeding (Box 3.1; Brown 1989; Hamrick *et al.* 1992; Whitehead *et al*. 2018). Plants have evolved many reproductive traits to promote these different mating systems. Mating system influences genetic diversity at a site and the level of **divergence** between sites.

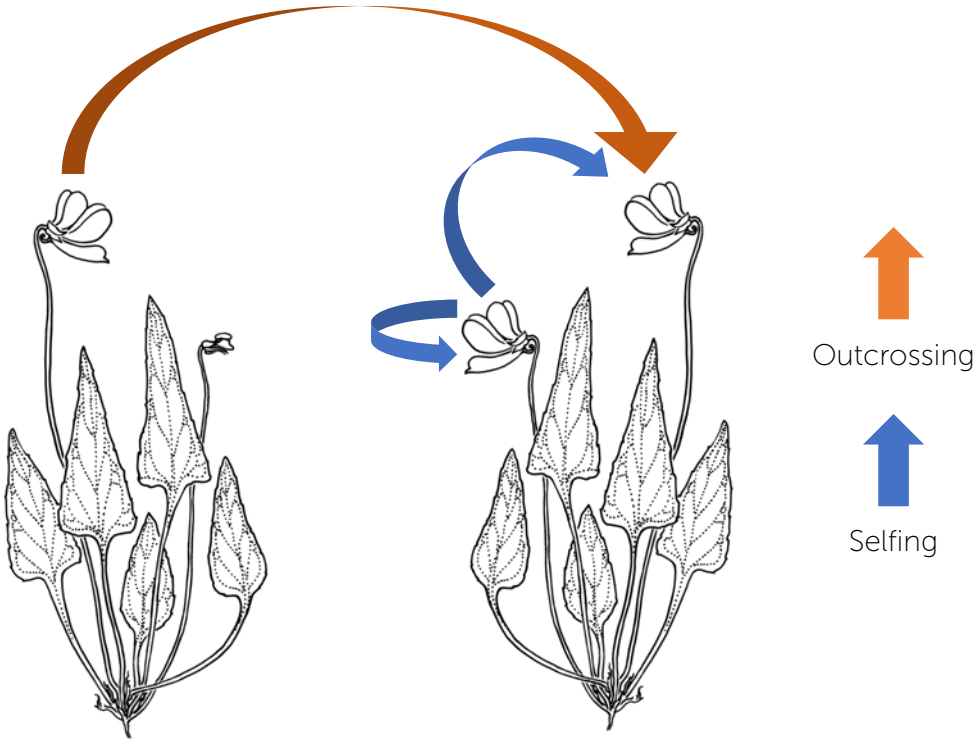


Figure 3.5: The mating systems of plants vary between selfing and outcrossing. Adapted from an original illustration from the Flora of NSW, reproduced with permission. ©Royal Botanic Gardens and Domain Trust.

Self-fertilisation – Seed from siblings of self-fertilised individuals are very similar to each other and to the mother plant and collections with many of these siblings will largely lack diversity. However, seeds from different mothers can be divergent and divergence increases across sites as the chance of maternal plants being highly related decreases.

For some species, studies have been conducted where the flowers are bagged prior to ripening of pollen and stigmas and successful **viable seed** production from these bagged individuals indicate successful self-fertilisation (see Adam and Williams 2001 for a study of 30 sub-tropical rainforest species).

Research found that self-fertilisation is sometimes associated with smaller flowers, lack of scent or nectar, low amounts of pollen and a short distance between the stigma and the style. In some plant species such as *Coleus australis* the presence of flowers that do not open (known as **cleistogamy**) is an easy way to recognise the potential of self-fertilisation. However, in other plant species, such as *Hakea* and *Acacia*, **morphology** provides us with little no or clues to the mating system and different mating systems and prevalence of selfing.

Collecting from species that are self-compatible:

- More sites, even relatively close in proximity, will increase genetic diversity.
- At a site, focus on collecting from the more distant plants and evenly from each plant.
- Avoid collecting from areas where there is only one old plant with several young similarly aged plants in a cluster around it (these could be selfed offspring from the one maternal line).

Cross-fertilisation – Outcrossing plants have evolved different systems to promote outcrossing, including differences in the timing of male and female flowering and separate male and female plants (**dioecious** plants). Some species have a genetic mechanism to prevent seeds being produced through self-fertilisation (known as self-incompatibility). Genetic mechanisms to avoid self-fertilisation are not obvious (e.g., self-incompatibility systems such as that found in *Rutidosis leptorrhynchoide*s, Brown and Young 2000). In healthy, fit and diverse populations of outcrossing species, the amount of genetic diversity captured from seeds collected from one individual will be much higher than from one individual where the seeds are produced through selfing. However, pre- and/or **post-zygotic** barriers may prevent fertilisation between siblings or individuals that are too closely related. In small and inbred populations, single individuals may not be genetically distinct enough to produce seed. Research found that habitat fragmentation can restrict gene flow and reduce the numbers of compatible individuals at a site.

Collecting from outcrossing species:

- For obligate outcrossers, collecting should capture individuals that are capable of breeding with each other, otherwise the individuals in a germplasm collection won't be able to mate.

In the absence of genetic information:

- Collect from widely spaced plants which are less likely to be related. Seeds should be evenly collected from as many plants as possible.
- For dioecious plants, cuttings from both sexes should be taken.
- For species where no or low seed set has been observed within populations, self-incompatibility or **pre-zygotic** inbreeding may be an issue and seeds should be collected from multiple populations.

Apomixis and clonality – Some plants produce seeds that are genetically identical to themselves through a process known as **apomixis** (**agamospermy**). Known Australian examples include *Acacia aneura* and *Senna artemisioides* but apomixis has been found in many daisies and grasses. Care should be taken when collecting seeds from these species, since the seeds are effectively clones of the parent plant and will lack genetic diversity.

Clonal reproduction through vegetative regeneration and root suckers are found among a broad range of plant groups in Australia. Some species are predominantly clonal (*Elaeocarpus williamsianus*, Rossetto *et al.* 2004), while in other species some populations may be largely clonal and others sexual. Without genetic data it is difficult to know which populations are clonal and which are sexual and how the clones are distributed at a site.

Collecting from plants that can produce clonally:

- Focus on collecting from different sites, with collections from a few individuals at each site. Avoid collecting from living collections.
- Genetic work is strongly recommended, this will help avoid over representation of genotypes in collections and maximise use of space in living conservation collections.

3.3.4.3 Pollen and seed dispersal

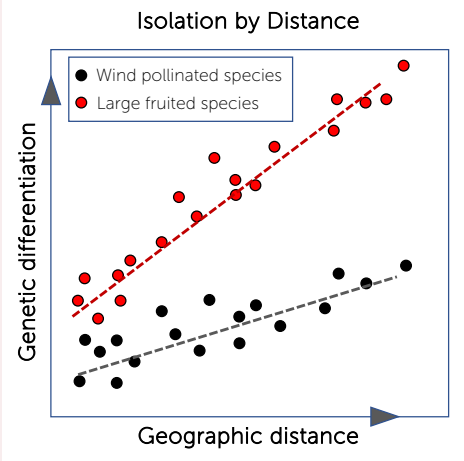
The behaviour of pollinators and seed dispersers influences the distribution of genetic variation at and between sites. Pollinators and seed dispersers that travel long distances will decrease genetic differentiation among populations (Box 3.3, little genetic change with geographic distance), whereas those that move small distances promote differentiation (Box 3.3, isolation by distance with a steep curve). Wind-dispersed species (pollen and/or seed) also show less differentiation among populations unless very strong barriers to **dispersal** occur. It is also important to note that various traits are involved in local dispersal: the effects on distance and dispersal can vary regionally as has been found for large fruited species in the rainforests of Australia where absence of dispersal agents have likely altered the distribution of genetic variation (Rossetto *et al.* 2015a).

Considering pollen and seed dispersal during collecting:

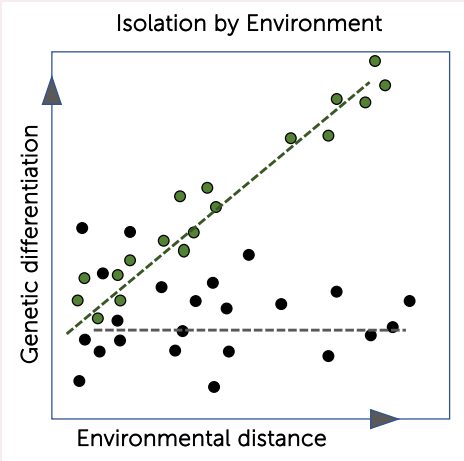
- For wind-dispersed species, distance between sites can be larger than for other syndromes.
- Bird dispersal is generally further than insect dispersal but much less than wind dispersal.
- For insect-pollinated species, increase the number of sites with a relatively short distance between sites.

Box 3.3: Isolation by distance (IBD) and isolation by environment (IBE) and provenance

It is common amongst plant species that populations that are geographically distant will be more genetically distant, as illustrated in the figure on the right. The scale of genetic differentiation changes over geographic space (the slope of the curve) and depends on how far pollen and seeds travel (gene flow). In this figure we illustrate two species with different levels of gene flow (genetic exchange). For the "red" species, gene flow is limited and therefore genetic differentiation increases rapidly across space compared to the "black" species. Traits such as seed dispersal mechanism and pollination mechanisms play a role in this relationship. The pollen or seeds of the "black" species may be wind-dispersed while the seeds of the "red" species are large and dispersed through gravity or an agent that does not travel long distance. Therefore, the genetic distance increases more rapidly for the red species than the black. Mating system also affects the steepness of the curve with higher levels of outcrossing likely to lead to less genetic differentiation as distance increases (black species).



When the genetic distance between populations changes with environment, it is referred to as isolation by environment (IBE) illustrated here by the "green" species contrasting with the "black" species where the genetic distance does not change. This can be a consequence of local genetic adaptation across heterogeneous environments. Examples of environmental gradients are altitude (where higher altitude is associated with lower temperatures), precipitation (rainfall) and salinity. The change in altitude between the coast and the upper Blue Mountains in New South Wales has likely contributed to genetic differentiation in many plant lineages found along this cline. For example, coastal and upland populations of the NSW Waratah *Telopea speciosissima* are genetically differentiated. Good collections will not just cover geographical distance but also environmental gradients to capture genes likely adapted to specific environments (known as **ecotypes**). Gene flow across environmental gradients can diffuse adaptation if the selective force is not strong.



IBD and IBE affect the size and shape of a genetic provenance.

Genetic provenance is a term used in restoration ecology and describes the size of a "local" provenance based on the amount of genetic connectivity across a geographic landscape. We can guess the steepness of the IBD curve based on life history traits and mating systems but genetic data across sites and populations provides information based on empirical data and can guide genetic provenance size based on the levels of gene flow detected amongst populations (Harrison *et al.* 2021). Isolation by environment is best studied with a combination of genetics, common garden, translocation and glasshouse experiments.

3.3.4.4 Hybridisation

Hybridisation occurs when two genetically distinct groups, usually species in the same genus, mate and produce offspring (**hybrids**). It is natural in many plant groups such as banksias, eucalypts, grevilleas, she-oaks, geebungs and orchids. Hybridisation is more common in species inclined to cross-fertilisation. While hybrids are often used in horticulture and agriculture because of their beauty or vigour, hybrids should be treated with caution in conservation and restoration collections.

Unfortunately, hybridisation has been found to occur between native and introduced species (such as the native Pigface, *Carpobrotus rossii* and the introduced C. *edulis*, Waycott 2016) as well as between threatened species and wide spread species (*Eucalyptus tetrapleura*, Rutherford *et al.* 2019; and *Eucalyptus aggregata* and *Eucalyptus rubida*, Field *et al.* 2009). One way (but not always indicative) to check if hybridisation is an issue is to walk the site and look for plants that have morphological features that are 'different' or 'intermediate' to the possible parental species. If this occurs, it is likely that seeds collected at these sites will include at least some hybrids even if the seeds are collected from "pure-looking" individuals. Options in this case are to move to another site or to collect seeds but make sure the collection is well labelled to indicate that hybrids may be present.

Collecting from species where hybridisation can be an issue:

- Collect from areas where the target species is found in abundance.
- Do not pool maternal lines (Box 3.2).
- Avoid single individuals (lone trees) that are geographically separated from other individuals of that species.
- Consider growing a subset of seedlings from the germination tests to check for intermediate traits, noting that not all genomic introgressions are expressed in the phenotype.
- Collect material suitable for genetic analysis.
- Collect vouchers to be lodged with a registered herbarium to allow taxonomic assessment.
- Remember the offspring (seed) can be hybrids while the parent is a "pure" individual.
- Avoid collecting seeds from living collections (botanic gardens) as the seeds could be products of hybridisation with other species present in the garden.

Options in this case are to move to another site or to collect seeds but make sure they are well labelled that hybrids may be present.

It is not always possible to distinguish between pure and hybrid individuals without genetic data (see Rutherford *et al*. 2019 on *Eucalyptus tetrapleura* for an example). Using the correct sampling techniques, genetic data will clearly distinguish pure forms of a species from those with mixed or introgressed genomes, and can even be used to identify F1 generation hybrids and backcrosses (the offspring of a hybrid individual and a pure individual of one of the parents).

3.3.4.5 Historical factors, species' distribution and genetic structure

Disruptions to pollen and seed movement (gene flow) can increase genetic differentiation among populations over time. This differentiation is known as population genetic structure and can sometimes bring about new species. Broad patterns in the distribution of genetic diversity across a species range may have been influenced by **temporal** changes in climatic, **edaphic** and **orographic** conditions in the past (see Broadhurst *et al.* 2017). Biogeographic barriers to gene flow are geographic features that disrupt or prevent the movement of genes and these may have helped the formation of genetically structured groups through drift and selection. Genetic drift means that in small populations, some alleles or genotypes may become reduced in frequency or disappear by random chance if they are not passed on to further generations. Several such barriers to gene flow have been identified in the Australian flora including (but not limited to) the Nullarbor Plain (southern Australia); central Australian arid zone; Hunter Valley and Shoalhaven River (New South Wales); Carpentaria Basin (Northern Territory and Queensland); Bonaparte Gap and Great Sandy Desert (Northwest Australia); and Black Mountain Corridor and Burdekin Basin (Far North Queensland).

Geographic disjunctions – Disjunctions in species distributions can be due to fragmentation of a larger population or it can be due to a long-distance dispersal event. Depending on the age of the fragmentation or dispersal event, the two **disjunct** populations may be genetically distinct. Disjunct populations that originated more recently may not be genetically distinct.

Genetic data can be used to infer genetic structure and such studies should include broad representatives across a wide distribution. Genetic data will identify how individuals from disjunct populations are related to the main populations, including whether they are recent founders or ancient **relictual** populations.

Considering species distribution in collections:

- Keep in mind that it is not possible to infer the degree of genetic structuring without genetic data.
- Collect material across the distribution and across known biogeographic barriers.
- Collect from geographically disjunct populations.
- Accurately record georeferencing of collection sites.

Species origin – Species age and origin affects how much diversity is present in the species. For example, species origin has been found to affect the level of population genetic structure amongst Australian Rainforest trees (Yap *et al.* 2020). In general, species that originated in Australia (**Gondwanan** or **Sahul** species) and have been in Australia for a long historical period have more structure and genetic diversity across their distribution, than species that originated in the Indo-Malaysian region (also referred to as **Sunda**) and migrated into Australia when conditions were favourable (Rossetto *et al.* 2015b; van der Merwe *et al*. 2019). Information on the biogeographic origin of many Australian plant species can be found in the literature.

- For Gondwanan species, make sure to collect across the distribution and in refugial areas (areas where the species survived during unfavourable environmental periods) while for the newer Indo-Malaysian arrivals sampling can be less intensive and large distances need to be covered to capture the little diversity available as the isolation by distance curve (Box 3.2) is likely to be much flatter.

3.3.4.6 Other issues to consider

Low diversity, inbreeding and recent bottlenecks – Many threatened species (but not all) have been found to have low levels of genetic diversity (see Case Study 3.3). While we can make assumptions, without genetic data it is not possible to assess the level of diversity in a population. Alternatively, collection strategies informed by genetic data will guarantee that collections capture the optimum amount of diversity at and among sites (see Case Study 3.1). Inbreeding (Box 3.1) can lead to low levels of genetic diversity within individuals and populations. While the mating system can provide some clues to inbreeding, levels of inbreeding can be best assessed with genetic data. Low diversity can be the product of a recent migration event (where only a small number of genotypes started a new population after dispersal) or a past **bottleneck** event (where due to some event only a small number of genotypes survived). Take note that the number of discernible individuals in a population is not always an indication of the amount of genetic diversity in a population

Taxonomic status – If there is any taxonomic uncertainty regarding the target species, then a multi-species, multi-sample genetic study can provide you with the necessary scientific evidence for making an informed decision on the taxonomic status of the group. This type of study involves sampling several individuals of the species under question and several individuals of each of the closely related species. While such a study may seem time consuming and expensive, the cost involved will be significantly less than the resources that go into putting together a conservation management plan, trying to collect and maintain a genetically representative collection, and building a population for conservation via living collections or translocations (see the *Banksia* example in Rossetto et al. 2021).

Collecting when there is doubt about the taxonomic certainty of a species or subspecies:

- Collect material for genetic analysis.
- Prioritise a genetic study of the target taxa.
- Do not pool maternal lines (Box 3.2).
- Collect multiple vouchers at a site.
- Consult a population geneticist or molecular ecologist to help design collections.

Polyploidy and chromosome change – Polyploids are individuals with more than two copies of their **chromosomes** and is common in plants. The basic chromosome complement in many organisms is two (diploid) – one inherited from each parent. **Polyploidy** and other chromosome changes include the addition of one or more complete sets of chromosomes or of individual chromosomes or chromosome reduction through processes such as chromosome fusion or translocation. Successful mating between different ploidy levels is generally not possible even though plants with different chromosome numbers may occur in the same population. While polyploidy is often associated with grasses (such as *Themeda triandra*) it is found in many other plant groups such as *Acacia*, *Allocasuarina*, *Callistemon*, *Eremophila*, *Lomandra*, *Syzygium*, *Polyscias, Ptilotus* and *Stylidium*. Presence of different polyploids is generally determined through genetic work (e.g., counting chromosomes, flow cytometry or molecular markers) and occasionally morphological identification is possible after cross reference with genetic data (e.g. *Allocasuarina littoralis*, Rose *et al.* 2015).

Specific molecular markers can be used to detect variation in ploidy (the number of sets of chromosomes), but it should ideally be verified by either chromosome counts or flow cytometry.

Collecting from species where a variation in ploidy has been recorded:

- Treat these as for taxonomic issues and collect a voucher with georeferencing from each plant.
- To capture an ample amount of diversity and enable successful mating in subsequent generations from the collection, label clearly, take good notes on morphology, collect multiple vouchers and do not pool seeds of maternal lines.
- Collect material suitable for genetic analysis from each maternal line.

3.4 Maintenance of genetic diversity and representativeness in different types of collections

The most important aspect of maintaining and understanding the genetic diversity of collections is meticulous record keeping.

3.4.1 Seed collections

- For all conservation collections, it is best practice to keep the maternal lines separate throughout the life of the collection (Section 3.3.2 and Center for Plant Conservation 2019). This will enable a careful assessment of the number of contributing maternal lines and the percentage of contributing seeds from each mother, following each post-collecting procedure (processing, drying, testing and storage). Seed viability and germination success is not equal amongst individuals, site of origin and time of collection. It is therefore possible that not all maternal lines from which seeds were collected are represented amongst the viable seeds.
- For ex situ conservation collections, avoid recollecting from the same individuals if the purpose is to increase genetic diversity. Overcollecting is a problem in long lived, easy to access individuals. Careful record keeping and location data for each collection can help to avoid this. Excellent mapping tools are available these days and these can be used in the field using mobile devices (see Chapter 4 and Figure 8.22).
- Metadata on the seed origin (georeferencing, collection site location and provenance) is crucial in all seed collections and should be maintained with the collection. This will enable the seeds to be used in accordance to the end-user's restoration or conservation strategy (see Chapter 15; and Commander *et al*. 2021).
- Focus on understanding the loss of genetic diversity through time (Guerrant and Fiedler 2004). Germination tests from a small subset of seeds can provide information on how seeds from the different maternal lines respond to storage and re-collecting can be done accordingly (Chapter 15).
- Increase genetic diversity in seed collections by collecting from new sites and new regions not present in the collection, and by recollecting from sites across different years.

3.4.2 Living collections

- Living collections are often used for research and good record keeping is of utmost importance. See Chapter 8 section 8.6 for details.
- We strongly recommend genotyping conservation-purpose living collections.
- Maintaining records of each individual, genotype or at least maternal line enables continuous understanding of the level of diversity within the collection (Chapter 11). As well as random events, environmental conditions in botanic gardens can select for or against certain genotypes and if these are not monitored the diversity present in a collection can rapidly deteriorate over time. Low genetic diversity in living collections can be further exacerbated if clonality is present in the natural populations of the species and unknowingly the living collection consists of only a few genotypes. See Case Study 8.3 on *Grevillea renwickiana* for an example of genetic studies informing collection and conservation of clonal collections.
- Since individual plants in botanic gardens are rarely seen as conservation collections, it is important that seeds collected from living collections be labelled clearly as such.

Seeds collected from botanic gardens could be the product of agamospermy, self-fertilisation or hybridisation. Seeds collected from these types of collections are rarely useful for increasing genetic diversity in germplasm collections, though they can be used for research in some circumstances, for example, determining seed storage behaviour (Chapter 6). Good record keeping (origin or provenance, age) may in the future enable these plants to be used for pollen harvesting and increasing genetic diversity across living collections similar to the model used in zoological gardens across the world (Volis 2017; Wood *et al*. 2020).

- The potential effects of hybridisation in conservation living collections can be minimised by separating congeneric species. Where this is not possible, always keep in mind that seed collections from such plants may contain hybrids.

3.4.3 Tissue culture

- Tissue culture is a valuable tool for species conservation, particularly where seeds are not produced, or seeds cannot be stored (Chapter 9). With meticulous documentation of the origin, **accession** number and labelling of each successive round of culturing, these collections can form an invaluable part of plant conservation.
- However, there is substantial evidence that genetic changes, referred to as **somaclonal variation**, can occur in tissue culture. The changes include changes in chromosome number and structure, biochemical changes, changes at DNA level (single nucleotide polymorphism (SNP) mutations, insertions or deletions). Some of these changes can influence phenotypic traits (such as disease resistance or plant height). To document and understand the level of changes that can accumulate in tissue culture collections, researchers are advised to maintain a leaf tissue collection (Steele 2018) suitable for DNA analysis to the wild plants from which the tissue originated. This can be used in a comparative study. Somaclonal variation seems to occur less frequently where organised plant structures, as pre-existing meristems, are maintained in culture.
- Protocols followed during tissue culture procedures are likely to favour certain genotypes above others, and this may lead to collections being skewed towards these genotypes. While tissue culture collections of non-model plants are rarely representative of the diversity of the species in the wild, an increased understanding of the value and importance of genetic diversity in wild accessions of crop species has led to improvements in methodology. These advances can inform tissue culture collections of non-model species. Where possible, seek protocols that maximise genotypes across tissue culture collections.

3.4.4 Cryopreservation

- Genetic diversity in most cryopreservation collections would largely depend on the same principles as for seed collections. Cryopreservation of **embryos** do not have the same problems with somaclonal variation as tissue culture.
- However, in crop species it has been found that different genotypes perform differently under different cryopreservation protocols. This can lead to over representation of successful genotypes and under representation or absence of other genotypes. Monitoring along with the appropriate record keeping system will enable collection managers to obtain an understanding of the amount of diversity in a collection and whether protocols should be adjusted to capture genotypes not responding to the inhouse methods. This is particularly important if the aim of the tissue culture collection is to redistribute the plants, or augment living collections or **in situ** populations.

3.4.5 Germplasm collections as donors for seed production areas

- Demands on seeds for restoration purposes are increasing rapidly and continuous collecting from the wild is not sustainable (Pedrini *et al.* 2020; Hancock *et al*. 2020) and not available at the required scale (Merritt and Dixon 2011). The quality of the germplasm collection will determine the suitability for such application. Here quality specifically refers to metadata accompanying the collection and the level of genotypic diversity present in the collection.
- For establishment of seed production areas or seed orchards, careful consideration should be given to each individual used in the establishment of the production area. Case Study 3.3 outlines establishment of a clonal seed orchard considering the available genetic data. Prior to establishing a production area, we advise seeking help from appropriate practitioners and read the Florabank Guidelines Module 7 (Gibson-Roy *et al*. 2021).

Case Study 3.3: Conserving Vulnerable *Eucalyptus benthamii* – ex situ and *circa situm* conservation in action

David Bush

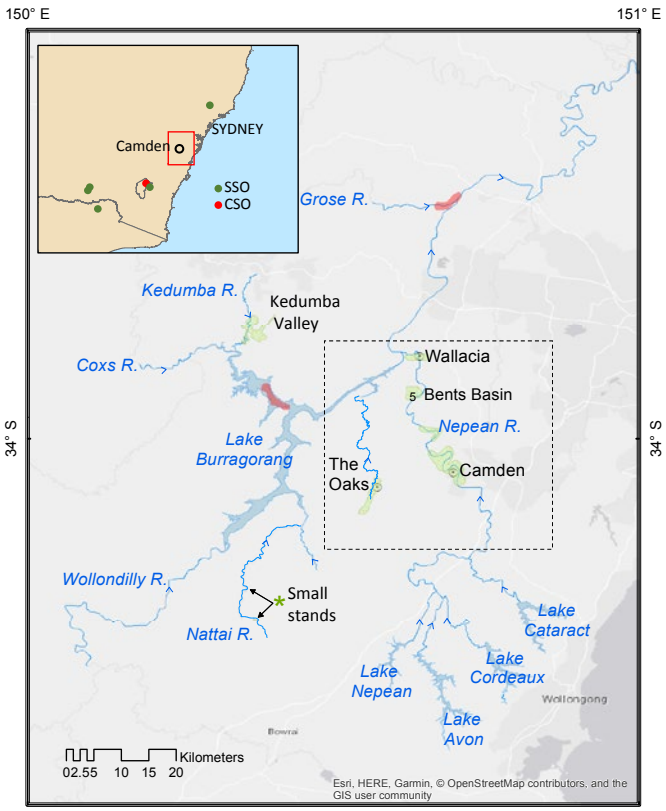
Eucalyptus benthamii (Camden White Gum) is a eucalypt (threatened: Vulnerable) with a restricted distribution to the south of Sydney (Figure 3.6). It grows on riverine floodplains along the Nepean River and its tributaries. Though a single population of several thousand trees exists in the Kedumba Valley at the top of Lake Burragorang, created by the Warragamba Dam, it is presumed that many more were lost when the dam was built. Agricultural clearing and urban development have caused further extensive losses and fragmentation of subpopulations growing along the Nepean River at Camden and other locations (inside dashed box, Figure 3.6). A subpopulation at the junction of the Grose and Nepean Rivers is now extinct (Benson 1985), though a recent survey has revealed that more trees than were previously thought to exist are still surviving along the Nepean, and a subpopulation previously thought to be extinct at The Oaks was found to still exist (Han *et al*. 2020).

CSIRO commenced action to conserve the species over 20 years ago. Ex situ seed orchards based on over 80 families (progeny of individual mother trees) from the Kedumba Valley were established in the ACT and locations in the NSW Riverina where the species is known to flower prolifically. Over 10 ha of stands have been established. These orchards have gone on to produce many millions of seeds, many of which have been used in industrial plantations overseas. These stands and the plantings derived from them constitute a significant ex situ conservation measure for the Kedumba Valley subpopulation.

Studies have shown that fragmented stands along the Nepean River are genetically distinct from those at Kedumba. These stands suffer from inbreeding and high levels of intraspecific hybridisation and often do not set seed crops (Butcher *et al*. 2005; Han *et al*. 2020). This is because individual trees are now physically isolated and stands suffer intense competition from woody weeds. As seed crops are often absent and/or of poor quality, ex situ conservation has been more difficult, necessitating grafted cutting propagation from individual trees, particularly those on the Nepean that do not produce good quality seed crops. The grafts have been used to form a clonal seed orchard (CSO). This orchard has been managed in very large pots and manipulated using plant growth regulating chemicals to promote flowering.

The seeds from the CSO are now being used for *circa situm* conservation. *Circa situm* conservation involves planting genetically diverse and appropriate trees, whose primary purpose is not conservation, adjacent to wild stands. The Camden Council is now using seedlings derived from the CSO seeds to replant public spaces and provide urban amenity. The circa situm plantings also provide pollen flow to closely situated wild remnants, giving the wild trees an opportunity to produce outcrossed seeds and prevent further loss of the entire subpopulation.

These conservation measures have greatly improved the security of this Vulnerable species. Future work will involve increasing the genetic diversity of both the seedling- and clonally-based ex situ conservations and encouraging further planting of the species in appropriate peri-urban locations along the Nepean and its tributaries.



Potted clonal seed orchard (Nepean)

Seedling seed orchard (Kedumba)

Figure 3.6: **Extant** (green shading) and extinct (red shading) populations of *E. benthamii* (after Han *et al*. 2020). The inset map shows the location of the populations (red bounding box) and locations of seedling seed orchards (Kedumba Valley subpopulation) and the CSO (Nepean River subpopulation). The dashed bounding box shows the extent of the Nepean subpopulation: many of these stands are in peri-urban areas and would benefit from *circa situm* conservation plantings. Photographs show the potted clonal seed orchard (CSO) and harvest of seeds from the seedling seed orchard (SSO). (Images: CSIRO)

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3.5 Molecular tools and genetic diversity estimates

Tools for population genetic studies are constantly advancing along with available genomic and **bioinformatic** technologies. In general, it has become much easier, quicker and resource efficient to obtain a genetic dataset than a few years ago. Some commercial services are available that develop molecular markers and genotype a set of individuals for any organism. These companies do all the work that was traditionally completed in the "wetlab" and it is thus not necessary to have access to a molecular laboratory to obtain genetic data for a project. However, it is still necessary to seek professional help from researchers with the appropriate background for data analysis and interpretation of results. More complex analyses, as outlined in the case study on the Myrtle Rust (Case Study 3.2) can be done in collaboration with a molecular biologist and bioinformatician.

While technology constantly changes, older tools and published data remain valuable resources. Published studies can be used to inform collections when the results are interpreted in a meaningful way (Figure 3.1). Population geneticists use a set of diversity measures to describe the distribution of the genetic diversity within a species. Most of these estimates are based on the distribution of genetic variants within populations and between populations. The commonly used measure, Fst, indicates the proportion of total genetic variance in a subpopulation relative to total genetic variance. It provides an estimate of the amount of connectivity or gene flow between populations (high Fst indicates restricted gene flow and low Fst indicates genetic connectivity with little divergence amongst populations). Other measures indicate the level of genetic diversity within populations (heterozygosity and allelic richness), the level of inbreeding (Fis) and relatedness (kinship). Note that estimates based on data acquired from different molecular marker systems (such as microsatellite markers and SNPs) are not directly comparable.

It is not within the scope of this document to discuss these measures in detail but there are some excellent references that can be consulted such as the textbooks "A Primer of Population Genetics and Genomics" by Daniel L. Hartl (Hartl 2020) and "An Introduction to Conservation Genetics" by Richard Frankham, M Jonathan D. Ballou, and David A. Briscoe (Frankham *et al.* 2019).

3.6 Conclusion

While we strongly recommend the use of genetic data to inform germplasm studies, collections that include multiple sites and multiple individuals across the distribution of a species are likely to capture a important level of genetic diversity. However, these collections are only of value if accompanied by a high standard of record keeping that is updated throughout the life of the collection.

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Chapter 4
Seed and vegetative material collection

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This chapter is a revision of the previous edition chapter by Anne Cochrane, Andrew D. Crawford and Catherine A. Offord, 2nd edition, pp. 35-62.

4.1 Introduction

This chapter provides an overview of internationally adopted protocols for best practice collection of plant material for **ex situ conservation**. These protocols aim to ensure ex situ collections provide a genetically representative sample of a **taxon**, while protecting the plants **in situ**, and will achieve the particular outcomes identified in Chapter 2. A checklist has been developed to guide this process (Box 4.1).

Box 4.1: Pre-collection checklist

Prior to collection

- Obtain necessary permits and permissions and consider biosecurity risks (see Chapter 2 for more detail).
- Inform relevant **seed** bank or nursery of potential incoming material.
- For cuttings, the number of individuals to sample should be determined by how many **genotypes** are required (Chapter 3) and can be maintained ex situ for the required duration (Chapters 2 and 11).
- During reconnaissance, correctly identify target taxon/taxa and suitable **population**/s for sampling including the presence of **hybrids** or closely related or visually similar taxa that may be confused during collection (see Chapter 3).
- Determine whether the population is natural e.g., not a previous **revegetation** effort.
- Determine whether collection has been made at the site before; consider cumulative impacts. See section 4.2.1 for potential sources of information.

During collection

- Collect seeds during dry weather conditions to reduce fungal contamination and spread of soil-borne diseases. Vegetative material collections can be made during wet or humid conditions.
- Maintain hygiene precautions such as cleaning of secateurs, boots and other equipment.
- Collect only ripe (mature) **fruit** or seeds (see Box 4.2).
- Sample seeds randomly from at least 50 widely spaced individuals to capture genetically unrelated plants and those exhibiting ecotypic variation. Where seeds from different plants will be bulked and not kept separate, aim to collect similar amounts of seeds from each plant sampled (see Chapter 3).
- Ideally, keep seed samples from individual plants separate (see Chapter 3, Box 3.2 for guidance on maternal collections). Seeds sampled from different populations should always be kept separate. This is particularly important for threatened species but may not be practical for species used in **ecological restoration**.
- Keep cuttings from individual plants in separate bags or wrapped bundles, each clearly labelled and identified to maintain clonal records.
- Collect no more material than the collection permit allows; if no limit is specified then collect no more than 20 % of available seeds so plants are not endangered by over-collecting (the only exception to this is if a population is to be destroyed).
- Reduce risks of insect **predation** and fungal contamination by collecting only fruits or seeds, where possible. Discard twigs, stems and leaves if possible.
- Use appropriate collecting containers – for dry ripe fruits and seeds use well secured *breathable* cloth or paper bags; for fleshy fruits use plastic bags that can be aerated to prevent decomposition or mould infestation – and keep seeds cool and dry to avoid overheating and mould.
- Use appropriate containers for cuttings – use moistened newspaper inside plastic bags; keep fleshy material and cuttings cool and moist in car fridge or esky, if possible, at moderately low temperatures – do not freeze or place any plant material on top of freezer blocks!
- Label bags and containers with relevant information for later identification.
- Record relevant collection information including date, precise location, number of individuals sampled, plant **morphology**, **habitat** and population characteristics.
- Make field observations of fire history, microclimate, and population structure (e.g., any obvious recruitment patterns).
- Examine plants for signs of disease or stress, especially when collecting cuttings, and for seeds, the quality (insect damage, empty seed and maturity) and quantity available. Record plant health information alongside collection information.
- Collect herbarium specimen(s) for lodging with State or other herbaria so taxonomic identity can be verified.
- Dispatch material for processing in a timely fashion.

Post collection

- Allocate and maintain **accession** codes for plant material linking to collection information.
- Pass all necessary information to relevant agencies as required.

4.2 Planning for collection

This section will guide the planning and development of a collection strategy. At this point bear in mind whether you are collecting seeds, vegetative material, or both. This section should be used in conjunction with Section 4.3 as the information provided here supplements that practical collecting information.

As the demand for seeds of native plants increases, it is important that collectors follow sensible and sustainable standards for collecting from the wild to avoid wastage, over collection or damage to plants and habitats. A recent seed survey by the Australian Network for Plant Conservation reported that future demand for seeds will be difficult to meet from wild harvest due to a general lack of seeds available from a broad range of species (Hancock *et al.* 2020). Problems caused by seed collectors over-collecting can alienate land managers and landowners, making future access to important collecting areas more difficult. It is worth discussing the collection strategy with seed bank and nursery staff, as well as other stakeholders (Chapter 2 section 2.3.3.1), to pre-determine nursery resource requirements based on volumes of plant material, propagation methods and any in-field pre-treatments (e.g., fungicide when collecting Myrtle Rust susceptible species) that may be required prior to collecting.

4.2.1 Planning information

To successfully plan a collecting trip, a range of information from a number of sources should be consulted including:

- Information sources:
  - Herbaria (e.g., The Australasian Virtual Herbarium, *Florabase*, or *PlantNET*)
  - Conservation databases and local land-use databases, e.g., Australian Seed Bank Online[30], NSW BioNet[31]
  - Land managers
  - Conservation agencies
  - Nursery propagators
  - Species profiles, listing statements/advice and recovery plans
  - Field guides
  - Local expertise
  - Bureau of Meteorology (rainfall maps)[32]
- Types of information:
  - Plant description and images (including any toxins and allergens)
  - Best material for collection e.g., seeds, cuttings
  - **Phenology** (timing of seed maturity, suitability of material for cuttings)
  - Plant number and health
  - Genetic data (see Chapter 3)
  - Locality description

30 https://asbp.ala.org.au/
31 http://www.bionet.nsw.gov.au/
32 http://www.bom.gov.au/jsp/awap/rain/index.jsp

- Maps
- Location coordinates (latitude/longitude, or eastings/northings – including datum used)
- Altitude
- Land tenure
- Site management history, including fire history (especially for disturbance dependent species) and previous collection or revegetation events
- Biosecurity issues such as presence of Myrtle Rust or *Phytophthora*
- Climatic data and immediate weather forecast for collecting
- Permits required (see Chapter 2)

4.2.2 Species identification

Identification of the target species is one of the most important tasks prior to collecting. Where species can only be identified using floral characteristics, identification may need to be made during flowering and plants marked for future collection using a GPS (or physically marking plants where it is unlikely to attract unwanted attention). The following assist in target species identification:

- Floras, guidebooks and botanical descriptions
- Taxonomic keys
- Images of target species
- Expert local knowledge
- Herbarium specimen/s to provide or confirm identification
- Botanical expertise

Be aware that plant taxonomy is constantly developing, meaning that printed reference material such as guidebooks can be outdated. Gathering an appropriate sample to save as an herbarium specimen is essential to verify the identity of the species collected, so despite any future name changes, the correct and current species name is known.

4.2.3 What material to collect

Plant material for ex situ conservation should be collected from known wild sources unless the species is extinct in the wild, collection will threaten wild populations, or plants have been grown with the purpose of seed collection (e.g., a seed production area). Plants of unknown origin found in gardens, revegetated areas or similar plantings might have limited **genetic diversity** that is usually inadequate as a genetically representative sample. In a garden situation this is often because plants collected for horticultural or display purposes are selected for a desirable trait, such as colour, shape or form, rather than for a representation of the genetic diversity of the species. Seeds collected from cultivated plants may have poor diversity due to the limited availability of suitable mates and pollinators. Additionally, **introgression** may occur following hybridisation with related taxa growing nearby posing a significant **threat** to the genetic integrity of the collection as a representative of the target taxon (Frankham *et al*. 2002). Although there are examples of cultivated collections that contain good genetic representation (see Chapters 8 and 11), in general, collecting from cultivated plants is not advisable so should only be used as a last resort or if the species is extinct in the wild and cultivated plants are all that remain. Material from such collections

may be suitable for research purposes, such as seed biology studies when wild-collected seeds are unavailable or for vegetative propagation trials.

When deciding which populations to collect, the end use of the collection needs to be considered. For threatened species **translocation** and habitat **restoration**, the **provenance** of the material used can be of paramount importance (see Chapter 3 and Commander *et al*. 2018; Commander 2021).

Once the target species and populations have been identified, the type of material to be collected must be decided (seeds or fruits, cuttings or divisions). Preferably this decision is made before collection, to allow the appropriate storage or propagation facilities and expertise to be prepared and available to receive the material (see Chapter 2). Often, information on the types of material that may be available is included in the sources mentioned in Section 4.2.2. However, for relatively unknown species, the decision may be based on the material available in the field or the circumstances of the collection expedition.

4.2.4 Equipment

A range of equipment is necessary for plant collection expeditions and includes apparatus required for:

- Safety e.g., first aid kit, vehicle recovery equipment, distress beacon i.e., Personal Locator Beacon (PLB)
- Personal protection e.g., appropriate clothing (weather dependent), eye protection, hard hat, steel capped boots, sunscreen, insect repellent
- Sustenance e.g., food, drinking water
- Communications e.g., radio system or phone
- Navigation e.g., GPS, maps, compass
- Plant identification e.g., sample specimens, photos, references
- Seed collection e.g., secateurs, pole pruners, paper or calico bags, cooler, labels, desiccant, data logger, **hygrometer** for eRH determination (see Chapter 5 section 5.3.2)
- Vegetative material collection e.g., secateurs, pole pruners, trowel, spade, plastic bags, newspaper for wrapping, spray bottle for wetting cuttings and newspaper, cooler/cool bricks, plastic labels, data logger, permanent marker/pencil, cooler bag/esky (see Chapter 8 for more details)
- Data collection e.g., field data book, plant press
- Photography e.g., camera
- Camping e.g., tent, swag, cooking apparatus etc
- Hygiene control e.g., disinfectant spray bottle, scrubbing brushes (see Box 4.4)

4.2.5 Authorisation – permissions and permits

Prior to collecting, consent from the land holder or manager is required. This includes access to private land, Indigenous Protected Areas and other lands under the control of indigenous owners, State Forests, National Parks or lands under other Federal, State or Local government control. Collectors must also be aware of conditions that apply to threatened species. For guidance on when a permit is likely to be required, see Chapter 2.

4.3 Seed collection

For most species, seed collection and storage are the most appropriate and common form of ex situ conservation for the following reasons:

- Seeds are the plant's natural storage device for their genetic material
- Seeds are 'packaged' for survival and longevity
- Seeds of most species can be dried and stored at low temperature which considerably extends storage life
- Seeds occupy very little space in storage
- Seed collections may represent a significant proportion of the diversity of population

Seeds are often contained within, or attached to, other structures which may also need to be collected and from which they may be separated **post-harvest**. Seeds of flowering plants are often contained within fruits which nourish, protect and disseminate seeds. Some fruits are obvious, such as the fleshy fruits of Lilly Pilly (*Syzygium* spp.), while other fruit types are easily mistaken for seeds, such as the caryopsis of grasses (family Poaceae) and achenes of Cyperaceae (e.g., *Isolepis*), Asteraceae (e.g., *Lagenophora* and *Brachyscome*) and Polygonaceae (e.g., *Rumex* and *Muehlenbeckia*). Seeds of **gymnosperms** are borne on **sporophylls** typically aggregated into a cone-like structure or reduced to one or two fleshy-coated seeds on a fleshy base which are openly exposed to the environment.

There are now several useful databases with images of Australian fruit and seeds, for example:

- Seeds of South Australia[33]
- Seed Information Database[34]

For more information on fruits and seeds, see books by Kesseler and Stuppy (2006), Kesseler and Stuppy (2013), Sweedman and Merritt (2006). For rainforest species see Cooper and Cooper (2013) and Dunphy *et al*. (2020).

A collection strategy for **germplasm** conservation should aim to capture a significant proportion of the genetic variation found within a species (see Chapter 3). Emphasis should always be placed on sourcing material that captures genetically diverse seeds of the highest possible quality, that will maximise the adaptive potential for recovery and restoration (Broadhurst *et al*. 2008). A genetically representative sample can be achieved by directing collection activities over a number of known populations of a species and by collecting within each population from a wide range of individual plants covering ecotypic and morphological variation. **Molecular data**, if available, can also be used as a guide to ensure that collections are representative of genetic variation within species (see Guerrant *et al*. 2004, for a detailed discussion and Chapter 3). It is vital that seed collections are limited to a size that avoids any impact on the long-term survival of the wild population (Section 4.3.6), or other species that rely on it for food, whilst maximising genetic diversity in the collection.

Before collecting, conduct a survey of the geographical and ecological range of the population to assess the number of plants and the potential seed crop that can be harvested. The size, density, age structure and extent of a population will inform the sampling strategy. This assessment may be done at any time during flowering or fruit production when plants are more easily located and can be correctly identified.

33 https://spapps.environment.sa.gov.au/seedsofsa/
34 https://data.kew.org/sid/

4.3.1 When to collect seeds

When designing a seed collection strategy, use data on reproductive biology (e.g., flowering and fruiting time, fruit characteristics and seed **dispersal mechanisms**) in conjunction with climatic conditions of target locations for formulating collecting procedures and establishing the timing for collections (see Box 4.2). Up-to-date local knowledge is very useful for planning the timing of collection. However, information on timing of fruiting and seed dispersal is often unavailable and is weather dependant, therefore, reconnaissance of populations during flowering is recommended to verify timing predictions. Knowledge of disturbance and weather patterns is often helpful for predicting seed production times for many species, especially **ephemerals** and fire- or disturbance-responsive species.

Seed collections should be timed to coincide with maturation of the seed and fruit, ideally at the point of natural dispersal (see examples in Box 4.2). At this point, seeds of **orthodox species** will have attained physiological quality traits, including **desiccation tolerance**, enabling them to be dried to the low **moisture content** required for survival in long-term storage (see Chapter 5).

Box 4.2: Timing of seed collection

- Flowering, fruit ripening and seed maturation can vary from species to species, year to year and often along altitudinal and longitudinal and/or latitudinal gradients.
- Prevailing environmental factors (temperature and moisture) have a major influence on time to maturity. For example, hot, windy conditions may speed the onset of fruit maturity and seed release; cold, wet conditions may lengthen fruit and seed development.
- Seed development may take weeks to over a year depending on the species.
- The timing of seed collection is crucial for species whose mature fruits and seeds are held for only short periods prior to dispersal (e.g., *Acacia* species). The method of natural dispersal may dictate the method of seed collection used (see Box 4.5).
- **Serotinous** plants (e.g., *Banksia*, *Hakea* and *Allocasuarina* and many small-seeded species in the Myrtaceae family such as *Eucalyptus, Callistemon*, *Calothamnus* and *Melaleuca*) may retain seeds on the plant within woody fruits for several years and the timing of collection after maturity can be less important than for **geospores** (see Seed *et al*. 2006).
- Seeds of orthodox species that are collected before they are fully mature will be of lower quality and, even if they do have **germination** potential, will not survive as long in storage as fully mature seed. This is because seeds acquire the ability to be desiccated during the maturation period, at the end of which they reach their greatest storage longevity potential (Hay and Smith 2003).

Indications that seeds have reached maturity include:

- Changes in fruit and seed coat colour
- Splitting of fruit
- Rattling of seed/s in fruit
- Hard and dry seed
- Some seeds have already dispersed
- Reduction in seed moisture content

4.3.1.1 Checking for seed maturity and quality

The first signs of dispersal in a population may be a sign that some, but not necessarily all, seeds are close to maturity and therefore ready for collection. It is possible however that early dispersal is a result of aborted or predated fruit. It is always advisable to check that a sample of fruit contains seed before making large collections.

Assess **seed fill** in order to avoid collecting empty, damaged or diseased seeds. The collection should always contain a high proportion of fully developed (filled, for example, see images in Case Study 8.4) and undamaged seeds. The maturity and quality of most seeds can be tested prior to collecting by conducting a cut-test (see Chapter 5.5.3.1, Figure 5.5) of a representative sample and assessing it. A hand lens is a useful tool for this task. Mature seeds should generally contain firm, white **endosperm** and/or an **embryo**. Seeds of some taxa, such as orchids, have dust-like seeds and are therefore difficult to assess in the field. In some species (e.g., in the family Apiaceae), embryos are small and may not be visible with the naked eye or even under a hand lens. Seed 'plumpness' is often an indicator of quality in such seeds. Fleshy fruits will generally become soft on maturity and are often coloured. There are some taxa (e.g., in the family Rutaceae), that have naturally low seed fill and this must be accounted for in the collection size.

Collecting fruit or seeds when immature can result in seeds with a poorer storage potential than if they were collected mature. For species where collection is difficult to achieve at maturity (e.g., many Rutaceae species with seeds that are explosively dehisced), or for species with sequential maturation within an inflorescence (e.g., *Senecio*) bagging or seed traps are recommended (see Box 4.5). These methods for seed collection should also be considered for high conservation value collections to ensure that seeds are collected at the point of natural **dehiscence**. As a last resort, immature seeds may be matured away from the parent plant if held under natural temperatures and high moisture levels (Probert *et al*. 2007). Care must be taken as these conditions can also lead to rapid aging of mature seeds especially if seed moisture content is also high. If a collection contains a mixture of mature and immature fruits/seeds then these should be sorted and treated separately (Schmidt and Thomsen 2003).

4.3.2 Seed collection methods

The method of collection will be determined by the type of fruits and seeds to be collected. Every plant type may require some modification to a general technique of collection, so assess each situation individually, be flexible and be resourceful. The safety of collectors is paramount, and it is important to make sure the collection can be made safely, especially in rough, steep or isolated terrain. It is worth considering a range of different techniques to assess advantages and disadvantages of each, for example see Frith *et al.* (2009).

The basic seed collection considerations are:

- Collect fruits/seeds when mature, ideally at the point of natural dispersal.
- Collect fruits/seeds off the plant rather than from the ground as those on the ground may be empty or may have been predated. There is also the risk of contamination from morphologically similar seeds of nearby related species or from other individuals.
- Collect fruits/seeds into buckets, bags or straight on to a collecting sheet (see Box 4.5).

- Use breathable bag materials, such as paper or calico, to allow moisture to escape and seeds to dry; minimising the potential of fungal attack and seed deterioration. Various grades of calico are available. Choose one that is strong and breathable, with over-locked inside seams, so fine seeds don't get caught in seams, otherwise bags can be turned inside out to avoid this. Be aware, not all paper bags are breathable.
- For fleshy, possibly **recalcitrant** seeds, collect the fruit in plastic bags as it may lose viability quickly if allowed to desiccate. Prevent the rotting of fruits and seeds by periodically opening bags to allow for gas exchange.
- For known orthodox fleshy species (e.g., *Solanum*), it may be necessary to do a preliminary clean of the collection in the field to prevent aging of seeds if left intact. Place in a calico bag and squash the pulp before drying and placing in a clean bag.
- During transport keep fruits/seeds under cool, dry conditions.

All seed batches must be labelled appropriately. Without labelling, it is often impossible to tell batches apart. Labels should include the species name (if known), the collector's code or the field number linking the collection to other data, and the date. Check that the materials used for labelling are sufficiently robust to survive the period between collection and processing e.g., use a pencil or indelible marker resistant to water and rubbing. Consider double labelling collections e.g., tag inside bag, information replicated on bag (if paper) or as a separate label attached to cloth bag. This will assist during processing prior to storage (see Chapter 5).

Allergy note: Be aware that certain plants may contain toxins or allergens e.g., some species of *Grevillea* (Proteaceae), *Ptilotus* (Amaranthaceae), *Anigozanthos* (Haemodoraceae) and *Pomaderris* (Rhamnaceae). Long sleeves, gloves and a mask may need to be worn during collection and cleaning of material. Sensitisation to these and other species may also occur with repeated exposure over time.

Box 4.3: Fruits and seeds of selected Australian taxa at maturity

Images: Andrew Orme, Kim Hamilton (*Syzygium*), Royal Botanic Gardens and Domain Trust

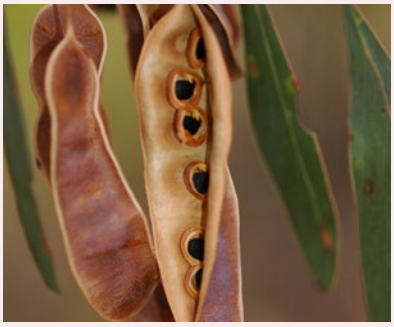
Asteraceae
Senecio diaschides

Casuarinaceae
Allocasuarina glareicola

Cyperaceae
Schoenus imberbis

Ericaceae
Epacris purpurascens

Ericaceae
Lissanthe sapida



Fabaceae
Acacia sp. New England
(J.B.Williams 97011) NSW Herbarium

Goodeniaceae
Goodenia glauca

Lamiaceae
Westringia rigida

Malvaceae
Hibiscus splendens

Myrtaceae
Corymbia eximia

Myrtaceae
Callistemon acuminatus

Myrtaceae
Syzygium australe –
desiccation sensitive

Orchidaceae
Cymbidium suave

Pittosporaceae
Pittosporum angustifolium

Poaceae
Austrostipa ramosissima

Proteaceae
Banksia ericifolia – serotinous species (note open and closed follicles)

Proteaceae
Grevillea robusta

Rhamnaceae
Pomaderris queenslandica

Rutaceae
Zieria smithii

Solanaceae
Solanum petrophilum

Sapindaceae
Cupaniopsis anacardioides

Thymelaeaceae
Pimelea ligustrina

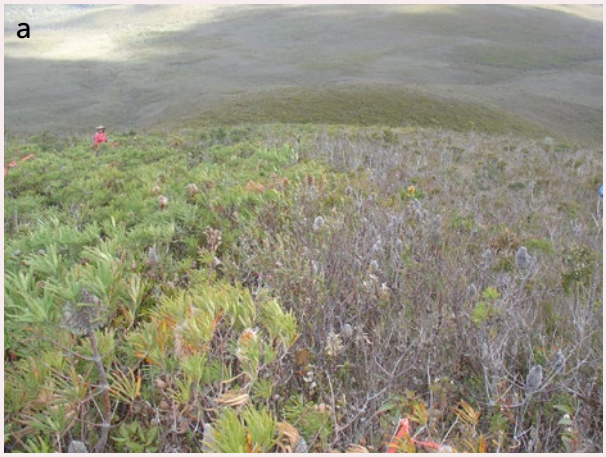
Xanthorrhoeaceae
Xanthorrhoea glauca subsp. *angustifolia*

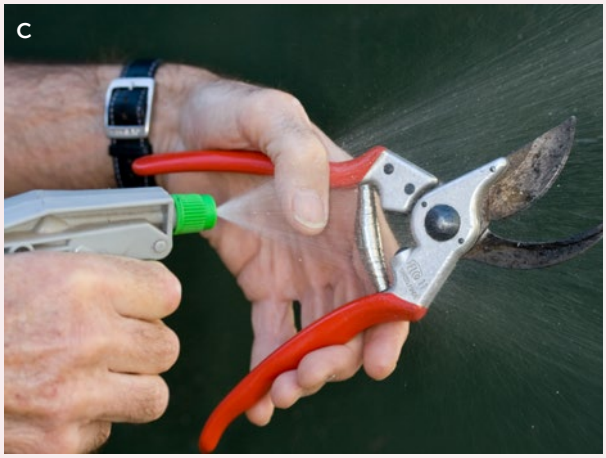
Zamiaceae
Macrozamia plurinervia

Box 4.4: Protecting plants from disease and weeds in the field

Regardless of the material being collected, it is vital that diseases such as the soil-borne Phytophthora Dieback (caused by *Phytophthora cinnamomi*) and air-borne Myrtle Rust (*Austropuccinia psidii*), and potential or known weed species, are not introduced or spread by collectors or their equipment, even on reconnaissance trips. Equipment must be cleaned between sites, and sometimes between plants, to ensure that pathogens or weed seeds are not transferred. Muddy shoes, vehicles and equipment can easily transfer potentially devastating organisms such as *Phytophthora cinnamomi*, and such items should be cleaned between collections and disinfected with disinfectant or alcohol prepared as a 70 % alcohol solution with water (State of NSW and Department of Planning, Industry and Environment 2020).

More information on weed and disease control can be found on State, Territory or Federal Government websites, but also see Makinson 2018; Makinson *et al.* 2020; Sommerville *et al.* 2019 for specific information on dealing with Myrtle Rust.





(a) Advancing front of *Phytophthora cinnamomi* infestation through susceptible vegetation in Stirling Range National Park (Image: D. Chemello). (b) Footwear and other items that come into contact with soil or plants should be cleaned between collection sites to stop the spread of diseases and weeds (Image: B. Summerell). (c) Disinfecting secateurs between plants. (Image: A. Crawford).

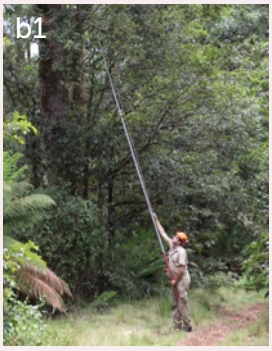
Box 4.5: Some techniques and equipment used to collect ripe fruit

(a) Hand picking allows an assessment of each individual fruit to be made on collection; heavily predated or damaged fruits can be avoided or discarded immediately. Gloves may be required if plants are prickly or spiny. Collect into paper bags, buckets, bags with open rigid mouths or into large calico bags. Collect individual hard woody fruits (e.g., *Banksia*) using secateurs.

(b) Pruning of ripe fruits and seed-bearing stems from plants that produce fruits on terminal branchlets can be done using secateurs or long handled pruners. A clean cut will prevent injury to branches and decreases the likelihood of infection of the wound by fungi or bacteria. Clean pruners between plants to prevent spread of disease. Fruits on some plants may ripen unevenly and stem cutting may lead to wastage of the resource. Some slightly immature fruits may continue to ripen after harvest.

Collecting fruits from tall trees requires specialised equipment. For heights of up to ca. 5 m pole pruners or saws may be used. In some cases, bows or sling shots are used to access very tall trees or rifles are used to shoot down branches. The canopy of taller trees may be accessible to arborists trained in tree climbing or in some situations may be accessible from elevated work platforms. Collecting from tall trees is hazardous and should only be done by experienced people who have the appropriate safety equipment and licenses. In some instances, it may be possible to collect immediately after timber-felling operations.

(c) Stripping fruits such as pods or grass seed heads, is an effective method of collecting fruit that is loosely held along stems. Gloves may be required.

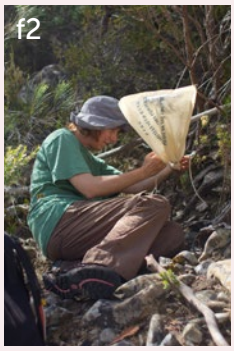


Images:
(a1) L. Perrins,
(a2) N. Tapson,
(b1) G. Phillips,
(b2) J. Wood,
(c) A. Crawford,
(d) A. Orme.

(d) Shaking branches or plants
Tarpaulins or drop sheets can be spread beneath plants to capture ripe seeds and pods that can be shaken from plants. Large quantities of seeds can often be collected in this manner (e.g., *Acacia*). Seeds should then be cleaned of small twigs and other debris using sieves.

(e) Seed traps

Given that a return visit will be possible, traps can be an effective way of collecting seeds for species that disperse their seeds over a long period of time or where fruit is too immature to collect. Traps can be placed under plants to catch seeds as they are shed. The traps should be made of a porous material such as shadecloth or flyscreen that can catch the seeds but allows water to pass through. This technique may not be suited to seeds with elaisomes as there is a high likelihood of the seeds being removed by ants. Seeds should be retrieved frequently otherwise they may become predated. Consider the amount of seeds to be trapped for each individual to avoid over collection.



Images: (e) A. Crawford, (f1) J. Wood, (f2) T. Rudman.

(f) Bagging

Bags can be placed over stems containing immature fruits to catch seeds when they are shed. A variety of materials can be used for these bags but all should have the following qualities: lightweight and breathable; moisture repellent or fast drying (to avoid rotting after rain); reasonably UV resistant if they are intended to be used for a number of seasons; and, have a low opacity to allow light to penetrate so the ripening process can continue. In some situations, bags can be obvious and draw unwanted attention; selecting colours that blend into the surrounds may help. Alternatively, stencil prints on the bags declaring institute ownership and conservation purpose may also help (see images f1 and f2). Organza mesh bags available from gift supply shops are inexpensive and suitable for most species. The durability of the material will also be important for prickly species. Care should be taken in selecting the stem on which to place bags so that flowers are not covered, potentially preventing **pollination** and therefore further seed production.

4.3.3 Collecting seed within a population

Genetic variation is the foundation which will allow a species to adapt to changing environments (see Chapter 3, Section 3.2, Box 1). This diversity will be spread both within, and between, populations (Brown and Briggs 1991). The aim of germplasm conservation is to capture as much diversity as practical. This means maximising the number of **alleles** represented within a seed collection. When samples are taken from a population, based on probability, alleles that are common in a population are more likely to be captured and collected early in the sampling process than less common alleles. As more individuals are sampled from a population the chance of more uncommon alleles being represented in the collection increases. There is however a diminishing return on collection effort with more effort required to include as yet uncollected alleles (Falk 1991). Sampling guidelines have been developed with the premise of capturing at least one copy of 95 % of the most common alleles within a population, that is alleles with a frequency of 0.05 or greater (Marshall and Brown 1975). How many plants needing to be sampled to achieve this goal will depend on the breeding system of the target species i.e., is the species capable of **self-fertilisation** or is it an **outbreeding** species (Brown and Marshall 1995). Information on the **mating system** is often not known, particularly for wild species. In the absence of specific genetic information to guide how many plants within a population should be sampled, it is recommended that a collection should aim to be made from at least 50 individuals if there are more than 50 plants in the population, or from all individuals if the population consists of fewer than 50 individuals (Guerrant *et al.* 2004).

Generally, plants growing closely together are related, as seeds generally fall below mother plants; a plant further away is more likely to be unrelated or less related. Seeds should be sampled from random plants throughout the extent of the population with the aim of collecting a similar number of **propagules** from each plant sampled if the seeds are to be combined to form a bulk collection. A number of sampling strategies exist (refer to Chapter 3 Genetic guidelines for acquiring and maintaining collections). Sampling should aim to capture as many unrelated individuals as possible in the collection. It is better to sample a number of fruits from different parts of the plant canopy from each individual plant rather than just one fruit as this is likely to increase diversity due to different pollination events (Brown and Marshall 1995).

Ideally, and where practicable, particularly for high conservation-value collections or where populations consist of fewer than 50 individuals, keep seeds from each individual plant (**maternal lines**) separate. Keeping maternal lines separate will allow for the balancing of these lines when used in translocation or can facilitate other research such as genetic studies (Chapter 3 Box 3.2 'Why not pool maternal collections?'). For a collection made from a large population, a composite collection can be made comprising a collection of individuals as well as a bulked collection, both sampled from across the population.

4.3.4 Collecting multiple populations

The aim of germplasm conservation is to capture as much diversity of a target species as practicable. This genetic diversity will be distributed both within and between populations. Multiple population collections are particularly important for selfing or clonal species (as they tend to exhibit higher diversity between populations) as well as for taxa that form part of a continuum (connected population across a large distance and/or a range of habitat types e.g., soil and water).

If time and resources permit, and in the absence of genetic information to guide collection, different and diverse populations should be sampled across a species' range with the aim of collecting up to five populations (Falk 1991; Center for Plant Conservation 2019). Where a species is known only from relatively few populations, then an effort should be made to collect from all populations. If this is impractical, the best option may be to identify and conserve key populations, although how these are defined will depend on features of the species (e.g., mating system, spatial range and demographics) or of the populations (e.g., size, genetic integrity and seed **fitness**), and the intended end use of the material.

In the first instance, the largest population of a species, or for common species a population of at least 100 individuals should be targeted. It may be sensible to initially collect from the largest population in order to obtain a broad sample of genotypes and hopefully, a good number of seeds with subsequent populations increasing the genetic representation of the species. Populations of a species that are identified as being at high risk of extinction due to a threatening process should also be prioritised for collection.

The following are the major considerations for choosing which population/s to sample:

- population size (being mindful that very small populations may be genetically **depauperate**);
- proximal threat of extinction;
- geographic isolation;
- ecotypic or morphological variation;
- frequency of disturbance; or,
- populations growing in unprotected areas.

Ensure that material collected from different populations is kept separate (e.g., given a different accession number), as taxonomic changes may occur in the future. Separation of material from different populations is vitally important for maintaining genetic integrity in a recovery program as well as for investigating genetic structure, assessing possible differences in tolerance to threats such as disease, and for understanding germination responses under a variety of environmental conditions.

Case Study 4.1: Patterns of genetic variation, ex situ seed collections and translocations of *Banksia brownii* (Feather Leaved Banksia), Proteaceae

David Coates, Rebecca Dillon and Sarah Barrett

Banksia brownii is a long-lived shrub or small tree to 4 m found over a range of approximately 90 km in south-west Western Australia. It is ranked as Critically Endangered under IUCN Red List criteria and is currently listed as Critically Endangered by both Western Australian and Commonwealth governments. The species is fire-killed and regenerates from seeds stored in woody fruits in the plant canopy, and is **self-compatible**, producing large conspicuous inflorescences pollinated by a range of nectar feeding birds, small mammals and insects. Plants are known from three bio-geographically **disjunct** areas occupying contrasting habitats. Population genetic studies have demonstrated significant genetic structure corresponding to the three population groups (Figure 4.1). High levels of differentiation among groups indicate long term historical isolation and significantly higher levels of genetic diversity in the Stirling Range populations, indicate larger and more stable population sizes over extended timeframes. The three population groups are considered to be discrete conservation units important for the management and recovery of *B. brownii*.

Banksia brownii is highly susceptible to the introduced soil-borne pathogen *Phytophthora cinnamomi* with 10 of the 30 known populations now extinct and most in significant decline due to the disease. Genetic diversity studies based on ex situ seed collections from **extinct** and **extant** populations indicate that some 38 % of total genetic diversity has been lost from *Banksia brownii* due to *P. cinnamomi* (Coates *et al.* 2015). The species is also vulnerable to short fire intervals given its requirement for a fire free period of at least 15 years to reach maturity and accumulate an adequate aerial seed bank. These threats highlight the importance of ex situ seed collections that were commenced in the late 1980s with a number of early collections made from now extinct populations. Many of these collections have been stored for nearly 30 years without significant loss of viability (Crawford *et al*. 2007). Genetic diversity estimates suggest that these ex situ collections harbor genetic diversity levels comparable or higher than current extant populations with samples from the extinct eastern Stirling Range populations showing higher levels of genetic diversity than the five extant Stirling Range populations (Coates *et al.* 2015).

While managing populations of *B. brownii*, primarily through the control of Phytophthora Dieback, is an important focus for conservation efforts, ex situ conservation through seed storage and the establishment of new populations in *Phytophthora* free areas through translocation have also been critical (see Barrett and Yates 2015). Given the recognition of three genetically and biogeographically distinct conservation units within *B. brownii* (Figure 4.1) three separate translocations representing each conservation unit have been implemented to date. Two of these translocations (T1 and T2, Figure 4.1) were based on seed sources from a mix of extinct and extant populations with genetic diversity higher than the combined estimates of current extant populations. Our studies show that all three translocated populations have been established with a suitably broad genetic base to maximise population persistence and that ex situ seed collections harbour sufficient genetic diversity for the future establishment of likely successful translocations.

References and further reading

Barrett S, Dillon R, Monks L (2008) Translocation proposal Feather-leaved banksia, *Banksia brownii* R. Br (Proteaceae). Department of Environment and Conservation, Perth, Western Australia.

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Crawford AD, Steadman KJ, Plummer JA, Cochrane A, Probert RJ (2007) Analysis of seed-bank data confirms suitability of international seed-storage standards for the Australian flora. *Australian Journal of Botany* **55**, 18-29.

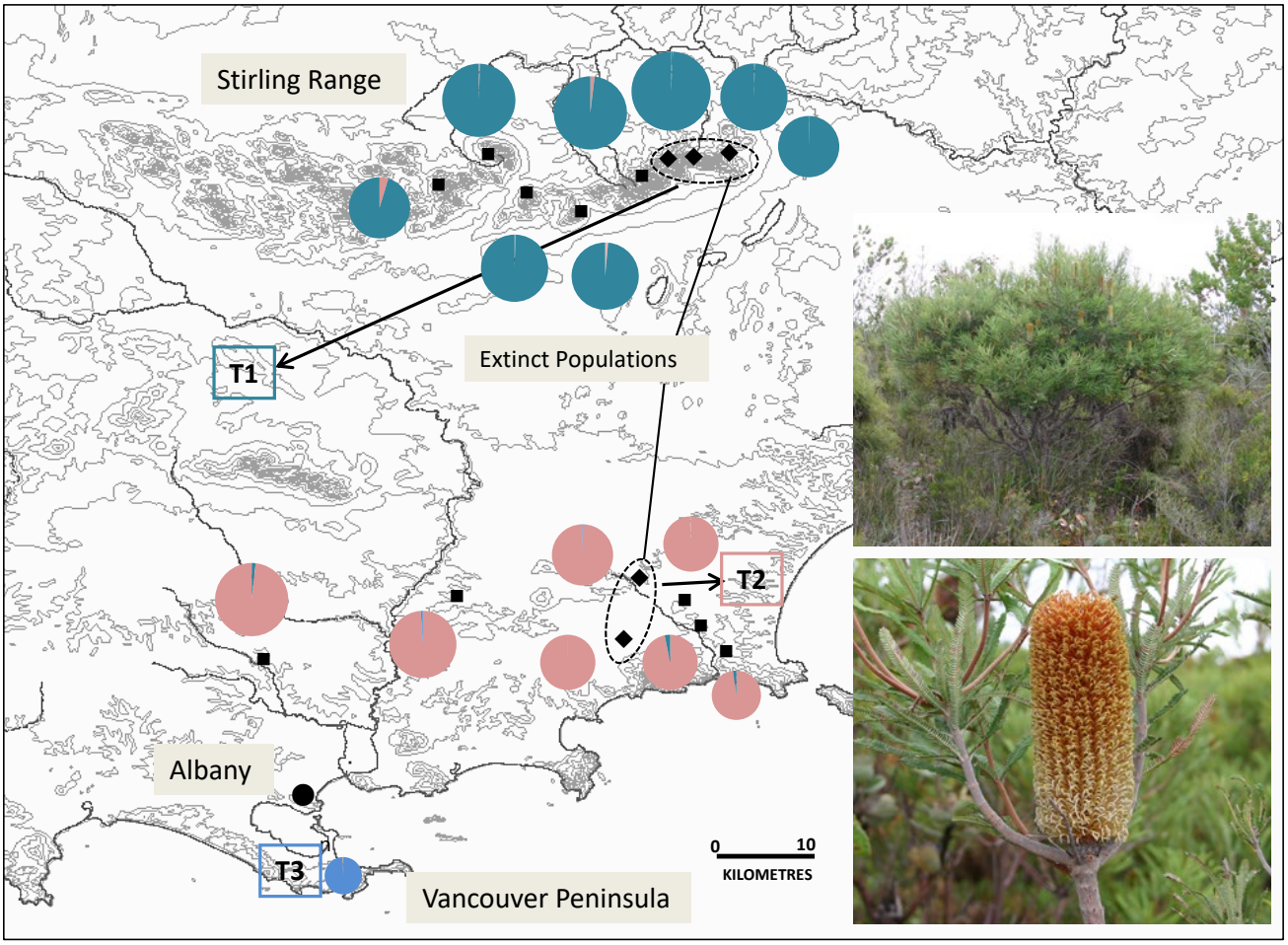

Figure 4.1: Mean q-matrixmembership proportions of *Banksia brownii* populations (pie charts)when K=3 from a STRUCTURE analysis (see Coates *et al* 2015). The size of pie charts is relative to the level of genetic diversity. Extant populations ■. Seeds from extinct populations ◆ was initially used to establish two separate translocated populations (T1 and T2) in *Phytophthora* Dieback free areas. Translocated population T3 was established with seeds from the single Vancouver Peninsula population. Reprinted from Coates *et al*. (2015), with permission from Elsevier.

4.3.5 Maternal collections

There are specific situations which may require the collection (including relevant documentation and mapping) of seeds from individual parent plants and keeping them separate (see Chapter 3, Box 3.2 'Why not pool maternal lines'). The need for this can include investigation of genetic integrity and/or hybrid influence in small populations of threatened species, and the selection of progeny from specific parents for translocation genetic planning.

4.3.6 Collection size

Ideally, a conservation seed collection should be of a suitable size to provide enough seeds to:

- Conduct initial viability and/or germination tests to assess quality. For threatened species, consult with appropriate institutions to ascertain if the germinants can be grown on for planting following testing;
- Monitor collection viability over time (see Guerrant and Fiedler 2004);
- Duplicate the collection with another seed bank for safe keeping;
- Provide sufficient material for the intended purpose of the collection e.g., translocation.

A collection size of between 3,000 seeds (Center for Plant Conservation 2019) and 10,000 to 20,000 seeds (Way 2003) is the recommended target number to meet these goals, providing that it can be obtained without threatening the survival of natural populations. These targets set minimum ideal levels of seeds for a collection. Often, particularly for small populations of rare species, even the smaller quantities might not be achievable in a single collection. In these instances, additional collections, or bulking up of seed material through the use of seed production areas will be required in the future (see Chapter 11).

It is often far easier to collect greater quantities of seeds from species with small seeds because they generally produce higher numbers of seeds in a season, than larger seeded species (Moles *et al*. 2004). So, as seed size increases, more time is likely to be required to collect a similar quantity of seeds. Collection size will also be limited by population size and available seeds, often making these ideal targets unachievable in any one collection. In these instances, repeat collections to achieve the required size should be considered (Section 4.3.7). Further consideration is required before collecting numbers of large seeded species, as considerably more space is required to store them.

Case Study 4.2: Seed collection sizes required for translocation success – allow for losses!

Andrew D. Crawford and Leonie Monks

The Department of Biodiversity, Conservation and Attractions (DBCA) has been undertaking translocations of threatened Western Australian plant species for over 20 years. Data on the demographic costs of each step of the translocation process have been collated for a wide range of species, some of which has been published previously in Cochrane *et al.* (2007). Modelling data for four of these species has been expanded and presented in Figure 4.2.

For the four species presented, average germination ranged from 74 to 94 % across all accessions of the species assessed over several years. Survival in the nursery was more variable, ranging from 43 % for *Acacia aprica* to 95 % for *Grevillea humifusa*. Survival of plants after planting into

the translocation sites to an age of five years was less variable but was low, ranging from 8 % for *Daviesia bursarioides* to 32 % for *G. humifusa.* Averaged over the four species of every 100 seed set up for germination, only 14 are likely to become a five-year-old reproductive adult plant in translocation.

This demographic data guides predictions of how many seeds of a species may need to be stored to establish a certain number of reproductively mature plants in a population. It is necessary to consider that seed germination and seedling establishment success will vary by species, from year to year and between seed batches. Currently DBCA's target for mature plants in a translocated population is 250 plants. This means that to achieve this goal in a single translocated population, the estimated seed requirements averaged across all four species was just over 1,850 seeds, ranging from 900 for *G. humifusa* to 7,900 for *D. bursarioides*. As a precaution, no more than 50 % of seeds stored in the seed bank would be used at any one time, so double these amounts would be required to achieve the goal of 250 reproductively mature plants at a translocation site and to have seeds in reserve. It should be remembered that there may be a need for more than one translocated population, in which case these estimates give an indication of the minimum seed requirements for a species in ex situ storage.

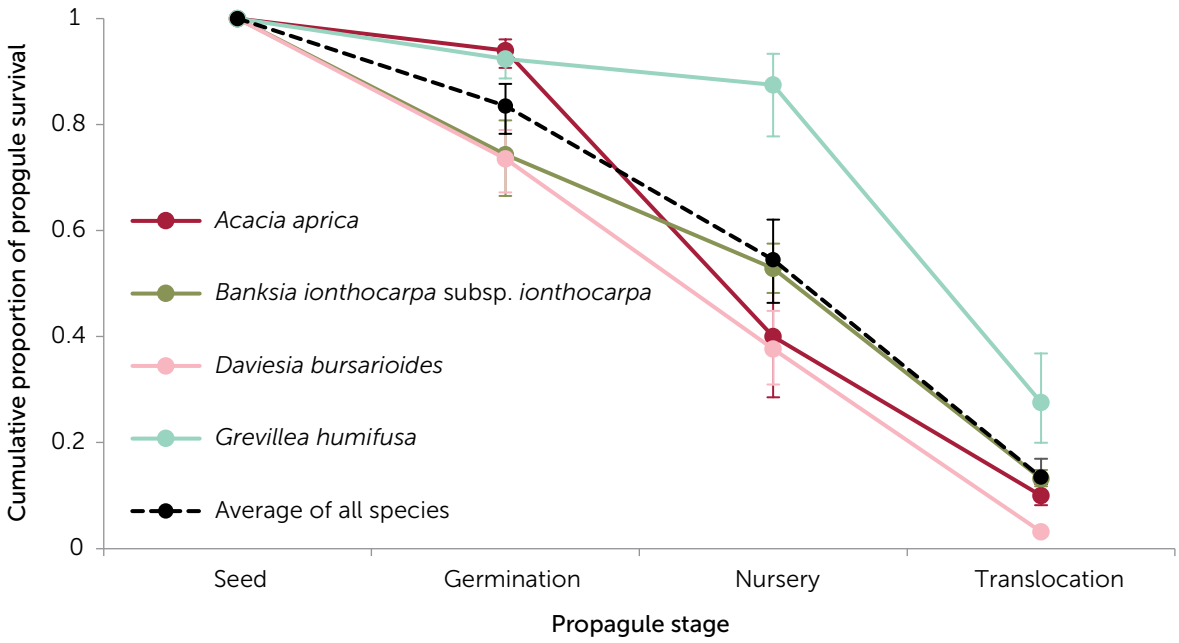

Figure 4.2: Proportion of propagules surviving through the translocation process from seeds to reproductive plants (≥ 5 years old) for four threatened Western Australian plant species: *Acacia aprica; Banksia ionthocarpa* subsp. *ionthocarpa*; *Daviesia bursarioides* and *Grevillea humifusa*. Average survival across all four species is also presented.

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Case Study 4.3: Tasmanian montane conifers – revising sampling strategies

James A. Wood

Tasmania is home to ten species of native conifer, seven of which are unique to the state. Of these seven **endemic** species, five are restricted to the Tasmanian highlands, **relicts** of past cooler climates (Hill and Brodribb 1999), with their stronghold in the Tasmanian Wilderness World Heritage Area. Of these five, two are trees (*Athrotaxis selaginoides* and *A. cupressoides*) that live to extreme age (up to 1,300 years) (Gibson *et al*. 1995). The remaining three (*Diselma archeri*, *Microcachrys tetragona* and *Pherosphaera hookeriana)* are shrubs, probably also achieving considerable age. With rising temperatures due to global climate change, Tasmania's montane conifers are particularly vulnerable to increasing drought and fire risk (Worth *et al.* 2016a). In fact, some populations have already been lost to fire and may not be able to re-establish naturally due to fragmented distributions and limited dispersal capacity.

The collection of genetically diverse, adequately sized seed collections for these species can be challenging. Three species – *A. selaginoides*, *A. cupressoides* and *D. archeri* – all produce seeds sporadically with several years of little activity, followed by a mass seeding event lasting about 2–3 weeks. Additionally, populations are now restricted to areas that have not experienced fire (Worth *et al.* 2016b) and so are widely scattered across Tasmania's upland areas. However, in addition to location and timing, the conifers also present one more challenge. Research conducted within the last ten years has revealed that stands of *A. cupressoides* and *D. archeri* are highly clonal (Worth *et al.* 2016a, b). This means that to ensure we capture as much genetic variation as possible, we will need to collect over much larger areas than is typically required.

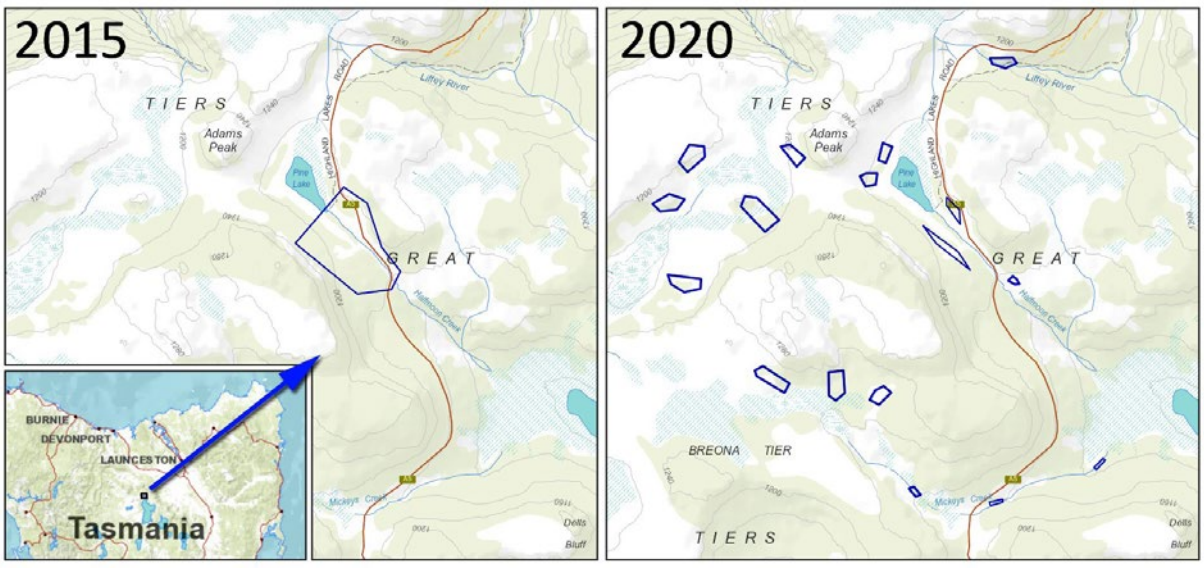

Figure 4.3: Pine Lake *Athrotaxis cupressoides* population sampling areas for the 2015 and 2020 masting events.

In recent years, two major collecting programs have been undertaken by the Tasmanian Seed Conservation Centre (TSCC) when **masting** events have occurred, in 2015 (Wood and Rudman 2015) and 2020. In 2015, the clonal nature of *A. cupressoides* and *D. archeri* was not appreciated and although large seed collections were made from good numbers of plants from several populations, with hindsight it is now likely that genetic diversity within collections is poor (Wood and Rudman 2015). For 2020, much broader sampling was planned. As an example, in 2015 the iconic population of *A. cupressoides* at Pine Lake (Figure 4.3), was sampled and >87,000 **viable seeds** from 60 trees over 52 hectares were collected. For 2020 the follow-up plan was to sample from 2 trees each from the 17 distinct stands across the local area of 1,150 hectares with the participation of 6–8 people. Unfortunately, the Coronavirus pandemic stopped collection so the TSCC now must wait for the next masting event which may be several years away.

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4.3.7 Setting a safe limit to seed collecting

Ecologically sustainable collecting practices should always be followed, regardless of the conservation status of a species. The activity of seed collection should never adversely deplete levels of seeds remaining in the natural population. Sufficient material should remain to allow for soil or **canopy seed bank** accumulation to allow for natural regeneration. Further, it should also allow for natural predation of reproductive material as seeds are often a major food source of native animals.

Modelling to assess the impact of seed harvest on populations has found that low intensity, frequent seed collections are expected to have a lesser impact on a population than more intense but infrequent collections (Menges *et al.* 2004). To reduce any adverse effects of over-collection, it is recommended that no more than 20 % of the annual seed production of a plant population should be taken for a once-off collection (Way 2003). If repeat visits to a population are likely to be required, then no more than 10 % of the annual seed production of a plant population should be taken (Menges *et al.* 2004). For some species, the nature of the fruit may require all the seeds be collected from one plant. In these instances, the amount of seeds collected should be based on the number of plants i.e., no more than 20 % (or 10 % as above) of seed-bearing plants should be harvested. In some States or Territories, the amount of seeds that may be collected may be legislated and may be less than these recommendations. A collection size of 3,000–20,000 seeds is recommended as a good size to cover a multitude of uses (Center for Plant Conservation

2019; Way 2003). However, in many cases (e.g., for rare or threatened species) collections of this size may be too great for the natural population to withstand. Rare or threatened species may not occur in many large, healthy, reproductive populations. For these and other geographically restricted species, it is vital that seed collections are kept within limits which will reasonably avoid any impact on the long-term survival of the wild population i.e., <20 % of available seeds. In certain cases, seed collection may be reliant on reduction of browsing pressure by caging of plants or the use of supplemental pollination to maximise seed set in the wild. In such cases, pollen should be taken from different plants to the plant that is to be pollinated (cross pollination) as this maximises **genetic variability** of the seed crop. Expert advice should always be sought before cross-pollinating plants in wild populations to avoid deleterious effects such as inadvertently creating inbred seeds. Following collection, particularly from small populations, measures to protect populations from threats such as fire and grazing should be considered to allow replenishment of the seed bank. More specific safe limits for collecting can be set if data are available on **longevity**, **fecundity**, masting, and establishment for the target species.

The only exception to these collection guidelines would be if a population is be destroyed. In this instance all seeds can be salvaged and if resources are available, vegetative propagation material should also be collected.

Collectors should be aware of the possibility that other collectors may be working in the same area and may target the identical population in the same year. Good communication and recognition of harvesting signs in the field are important to avoid over-collecting.

4.3.8 Repeat collecting

Multi-year or multi-season sampling is a good strategy to build up diversity and quantity of seeds, particularly when one collection may result in low seed numbers. Multiple collections within a season will allow for the collection of early- and late-flowering plants from within a population. Known or suspected short-lived species (Chapter 5, Table 5.3) might need to be flagged for regeneration or repeat collections sometime in the future (see Chapter 15 for a decision tree).

Collecting over a number of years or seasons may increase the likelihood of capturing a greater proportion of the genetic variation of the species, particularly in seasons where only a small proportion of the population flowers, or if the species is reliant on disturbance or rainfall events for emergence or flowering. Repeat collecting can reduce over-exploitation of the resource in any one year and allow collection of sufficient material for storage and recovery. Multiplying or bulking of collections through seed production areas may also be warranted (Chapter 11 and Gibson-Roy *et al*. 2021). Collections from different years should be kept separate.

Repeat collecting is recommended when:

- Species display low reproductive output;
- Populations are small;
- A species has a long flowering and fruiting period;
- Seeds ripen gradually over a season;
- Species display year to year variation in population size and structure;
- Climatic or **stochastic** events reduce seed set e.g., drought or fire;
- Existing collections have declined in viability (a decline of >15 % of original).

4.3.9 Post-harvest seed handling and cleaning in the field

Post-harvest handling begins immediately following collection and good practice is essential to maximise the quality and longevity of the collection (See Section 4.3.2). Transport seeds or fruits as quickly as possible to the processing and storage facility. During transport, protect the material from physical, moisture and temperature damage. Ensure that seeds are not physically damaged by heavy objects or careless actions. For the most part, seed collections should be kept cool and dry. This is usually achieved by keeping the seeds in the shade, but in very hot weather they may need to be artificially cooled e.g., kept in a cooler (at no less than 15 °C if fleshy-fruited, and no less than 5 °C otherwise). Do not leave seeds in a vehicle in full sun as they may overheat; exposure to sustained high temperatures can reduce viability. The collecting vehicle should be parked in the shade, or at the very least, the windscreen shaded. Try to maintain ventilation around the collections at all times. Vehicle air conditioning is useful to maintain a dry and cool environment and circulate air. Damp collections should be spread out as soon as possible to dry naturally, either outside in the shade or inside in a well-ventilated room. A temperature data logger can be used to monitor the conditions during the post-harvest period; this is often useful during shipping collections to determine whether seeds have experienced high temperature (which may explain poor viability).

Fleshy fruits may require careful handling, partial cleaning and rapid dispatch to a seed bank. Keep these collections aerated and cool to prevent fermentation, development of fungal growth and even germination.

In some cases, it is possible to do some preliminary cleaning of seeds in the field by removing dry, bulky fruit parts or extraneous material, such as leaves and twigs, or pulp from known orthodox fleshy seeds (e.g., *Solanum* species). In most cases, it is best to leave the task of cleaning collections to seed bank processing staff with the necessary equipment and facilities. Section 5.3 deals with this issue in detail, but it cannot be stressed too highly that fruits and seeds should be handled with great care at all stages.

4.4 Vegetative material collecting

When viable seeds are not available, or a **clonal collection** is being established for any reason, plants can be vegetatively propagated (for greater detail see Chapter 8). Cuttings are the most commonly used material for vegetative propagation. The best cutting material is clean, with healthy shoots that are neither too woody nor too immature; however, the optimal season and material for propagation is highly dependent on the taxon and what material is available (see Mathews 1999; Hartmann *et al*. 2002; Stewart 1999). See Chapter 8 on further guidance to determine whether or not material is suitable for propagation.

It is not typically recommended to take cuttings when plants are in full flower or seeding, however for identification purposes this may be the only time to reliably collect. Some species can be propagated by **rhizomes**, **tubers** or other structures that can be divided (see Chapter 8 for details). Great care should be taken if using division as a propagation method, as it is imperative that all soil material is washed clean and treated to reduce the possibility of importing soil-borne diseases into the propagation facility. Plant propagation using these methods is highly dependent on expert knowledge and is therefore best discussed with an experienced propagator, otherwise material may be wasted. Chapter 8 includes issues to consider when establishing collections such as cuttings in a nursery setting.

Collectors should aim to collect vegetative material when plants are in an active or semi-active growth stage. For many woody species, this may be in late spring or early summer, after the peak of active growth, and before the material is fully hardened (semi-hardwood cuttings). Only material that is healthy and disease-free should be collected.

4.4.1 Practical considerations for collecting vegetative material

When collecting vegetative material, the most important consideration is that the plants and their habitat are not adversely affected. Before taking any material for propagation assess the plant's health and vigour. Decide on what to take by pre-determining how much material to collect including the length of each cutting and where you will make the cuts. Cuts should be made, where possible, just above a healthy bud or node.

Care must be exercised to remove only the plant parts necessary for propagation, and to ensure that the regrowth and seed producing ability of the plant are not significantly diminished by taking material in bud or in the wrong season.

Equipment should be clean to avoid introducing diseases to the plant, either directly through unclean pruners (e.g., Myrtle Rust), or indirectly by transferring contaminated soil on shoes (e.g., Phytophthora Dieback) (for example, see Box 4.4). Secateurs should be sprayed with 70 % methylated spirits solution between plant collections to avoid cross contamination (State of NSW and Department of Planning, Industry and Environment 2020).

Always keep material from different plants separate. Collect enough material to establish at least three **ramets** (replicate plants) of each genet (original plant from the wild) in case of plant loss through disease or misadventure. Individual plant species and clonal collections must be kept separate but if a composite collection is being made from a number of **genets** within a population a single record can be made.

In order to successfully propagate cuttings, only healthy material should be taken. The conversion rate of cuttings taken to plants established may be low, and is typically 30–75 % but can be much lower. Therefore, sufficient material should be taken from each individual plant to take this into account. If possible, conduct preliminary propagation trials using a small amount of material to minimise the amount needed in the main collection. Horticultural information and expertise should always be sought when dealing with a new taxon.

Vegetative material should be kept moist, cool (in a refrigerator at 3–5 °C, never lower) and should be propagated as soon as possible after collection. If not returning from a field trip for some time, it is advisable to wrap cuttings in moistened newspaper, place in a plastic bag within a cool-box and airfreight the material to a forewarned nursery for immediate attention. If using a cool box with ice block, care should be taken to ensure material is never in direct contact with the blocks.

Transplanting of whole plants should be avoided if possible as the conservation of plants in situ is preferable. However, there may be circumstances where it is appropriate e.g., salvage operations where the population or plant is to be destroyed (see Monks *et al.* 2018). To determine the chances of success, it is best to discuss this technique with a horticulturist/propagator prior to attempting transplantation. The risks associated with transporting soil-borne diseases using this method are high and should not be under-estimated.

4.4.2 Collecting for conservation projects

Collection of vegetative material for ex situ conservation collections is usually done as a last resort (e.g., due to a lack of seeds, or the inability to store or germinate seeds), particularly when translocation is likely to be the eventual outcome. Where viable seeds exist, and long-term storage is possible, seed storage will always be the preferred ex situ conservation method (Guerrant *et al*. 2004). Ex situ collections established and maintained vegetatively are often either low in plant number, represent small populations or capture limited genetic diversity, or are made as an adjunct conservation measure. Examples of the latter include stock plants for species research, including horticultural development, or, most often, for display and educational purposes in botanic or other gardens (refer to Chapters 8 and 11).

Large clonal collections of a size that adequately represent the diversity of a taxon for translocation purposes may be prohibitively expensive to maintain, and other options should always be explored before taking this path. If the required diversity cannot be held as seeds, options involving vegetative regeneration of plants (e.g., cuttings, division, **tissue culture** and **cryopreservation**, see Chapters 8–11) need to be very carefully considered in terms of costs, the diversity able to be represented and the required or perceived outcomes. See Chapter 2 for resource costs for maintaining different ex situ collections.

4.4.3 Sampling vegetative material

Depending on the diversity within the taxon, the number of populations and individuals to collect from should be guided by the intended purpose and will be dependent on the diversity within the taxon. If the collection is made for conservation reasons, then it is necessary to collect from a large number of individuals, similar to or perhaps greater than the number required for a seed collection. This strategy differs from that for the collection of material for display or educational purposes when it is generally sufficient to collect from only a few representative individuals.

Given that some genotypes perform better than others during propagation and cultivation, more genotypes should be collected than the minimum eventually required. However, care should be taken to ensure that, if the purpose of the collection is for translocation, the individuals that do not respond well to cultivation are still represented if possible.

Read 'Planning for Collection' (Section 4.2) in conjunction with this section, as the information is not repeated here. The collection checklist is in Box 4.1.

As with seed collections, the number of populations to sample depends on the end-use and resources available to maintain the collections. Key populations should be represented if possible. See Section 4.3.4.

Vegetatively propagated conservation collections should aim to sample the available diversity of a population (greater than 95 % if possible). More extensive sampling of individuals is required to capture the desired level of diversity compared to seed-based collections, making vegetative material-based collections potentially very large.

Ideally, to capture maximum genetic diversity in an ex situ collection, it is suggested that vegetative material is sampled as follows:

- If a population has fewer than 100 individuals, sample from all plants;
- If a population consists of more than 100 individuals, sample from at least 100 individuals (unrelated if possible, i.e., not near-neighbours).

For many rare species, nearly every individual can be sampled. However, the establishment and maintenance of large clonal collections may not be appropriate or feasible, especially if multiple populations need to be maintained (Chapter 8; Hawkes *et al*. 2000; Guerrant *et al*. 2004). In reality, genetic diversity held in ex situ living plant collections is lower than can be held by seed collections and is ultimately controlled by the practical constraints of growing and maintaining the plants for the required time.

4.5 Botanical voucher specimens and field information

4.5.1 Botanical voucher specimens

Botanical specimens are taken to vouch for the identity of a seed/cutting collection and enable future taxonomic developments to be aligned with the collection. An herbarium specimen should always be lodged with the relevant State or Territory herbarium with duplicate material lodged if a local reference specimen is to be kept or if duplicate **voucher specimens** are required for another institution. A voucher specimen will ideally include a flower, fruiting structure, vegetative material, and other distinguishing features, as available, that represent a 'typical' individual of the target species (see Box 4.6). To conserve the structure and appearance of the herbarium material, specimens should be dried between papers and placed in a plant press as soon as possible after collection[35]. Where a population is being revisited and a specimen has previously been taken and lodged at an herbarium, and where the target species is readily identifiable at that location, it may be acceptable to use the existing specimen as a reference voucher. This can be particularly useful for rare species where there are few individuals at a location and the taking of a specimen may involve the taking of a whole plant e.g., an orchid.

35 https://www.dpaw.wa.gov.au/images/documents/plants-animals/herbarium/How_to_collect_herbarium_specimens.pdf

Box 4.6: Examples of a voucher specimens suitable for botanical identification and herbarium lodgement

a: Herbarium specimen of *Melaleuca glaberrima* F.Muell. (PERTH 09325379) from Western Australian Herbarium, Department of Biodiversity, Conservation and Attractions.

b: Herbarium specimen of *Spyridium scortechinii* (F.Muell.) K.R.Thiele (NSW 496913) from the National Herbarium of NSW.

4.5.2 Field information recording

Detailed information regarding location and field conditions of the collection site provides data for various purposes. This information assists with mapping species and vegetation communities and helps to ensure that collecting areas are not over harvested (see field notebook example in Box 4.7). Information collected with voucher specimens follows data standards, which include fields that should be recorded in set, consistent formats. Most major herbaria follow biological standards like Darwin Core[36] to enable easier transfer of data and more consistent recording of traits (see Chapter 15 section 15.2.2 for more details). Comprehensive data on the collection can provide an insight into the ecology and recruitment strategy of a species and its niche within the plant community. The data will be particularly useful for the reassessment of conservation status and aid recovery planning.

The following information should be recorded in the field:

- Species name.
- Common or indigenous name.
- Date of collection.
- Collector's name/s and collection number.
- Location description.
- Location coordinates. Include method used and the datum of the coordinates.
- Altitude.
- Number of plants sampled.
- Number of plants in the population and area occupied. Indicate whether these are actual numbers or estimates.
- Plant description (form, habit, flower colour etc.).
- Site description (associated species, threats, disturbance, landform, aspect, slope, soils etc.).
- Phenology of population (i.e., vegetative, bud, flowering, fruiting).
- Population health.
- Herbarium specimen details.

Any morphological variation, evidence of hybridisation or nearby plantings that may increase the risk of pollen contamination through hybridisation are also useful to note.

Photographic records are an important means of recording information about a species, population condition, associated species and landform. However, photos do not substitute for a voucher specimen.

36 https://dwc.tdwg.org/

Box 4.7: Example of the basic field information to be collected

TAXON: _____ ACCESSION No: _____

COLLECTOR(S): _____

POPULATION No: _____ CONSERVATION CODE:_____ DATE: ___ / ___ / 20__

LOCATION:_____

_____

_____ DATUM: _____

LATITUDE: _____°_____'_____"S LONGITUDE: _____°_____'_____"E

HABIT: Climbing | Prostrate | Decumbent | Erect | Compact | Open | Succulent | Rhizomatous | Caespitose | Bulbous | Tuberous | Floating | Submerged | Annual | Perennial

FORM: Tree | Mallee | Shrub | Dwarf Shrub | Herb | Grass | Sedge | Lily | Halophyte | Epiphyte

PHENOLOGY (Low/Med/High*)**:** Vegetative | Bud | Flower (colour) | (_____)

Immature fruit | Fruit | Dehisced

LANDFORM: Hilltop | Cliff | Slope | Valley | Swamp | Ridge | Flat | Outcrop | Breakaway Low Plain | Gully | Riverbank | Sand Dune | Drainage line | Lake Edge | Firebreak | Other _____ ASPECT: _____

GEOLOGY: Laterite | Granite | Dolerite | Limestone | Other:_____

SOIL: _____

VEGETATION TYPE:_____

ASSOCIATED SPECIES:_____

_____

_____

No. of PLANTS:_____ AREA OCCUPIED: _____ No. PLANTS COLLECTED: ____ Bulk/Ind.

COLLECTION NUMBER (seed): _____ VOUCHER SPECIMEN: _____ Duplicate for ____

PHOTO No's: _____

COMMENTS: _____

_____

_____

** Low = low number fruits, Med = moderate fruit crop, and High = heavy fruit crops.*

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Chapter 5
Seed banking: orthodox seeds

David J. Merritt, Katherine J. Whitehouse, Gemma L. Hoyle, Andrew Crawford, James A. Wood, Annisa Satyanti, Sally L. Norton, Graeme Errington, Amelia J. Martyn Yenson

5.1 Introduction

The storage of **seeds** in seed banks is the primary method for **ex situ conservation** of plant **genetic diversity**. Conservation seed banks may serve a variety of functions, but all are established with the objective of capturing and conserving a broad diversity of plant genetic resources. The collection and storage of seeds in secure facilities under controlled environments underpins global food security, serves as a safeguard against the extinction of traditional **landrace** varieties and wild species, and provides a source of **germplasm** for the propagation of plants for a range of activities. Seeds are stored for crop breeding and improvement, plant breeding and amenity horticulture, research and education, **translocation** of rare and threatened species, and **revegetation** and **ecological restoration**. The size and diversity of seed collections, the scale of the seed banking facilities, the stringency of their environmental controls, and the precision of the **post-harvest** handling and storage procedures all vary depending upon the purpose for which the seeds are stored. But the end goal remains the same – to maintain high quality, **viable seeds** until they are required for use.

There are more than 1,750 genebanks worldwide, and numerous examples of collaborative global initiatives and partnerships centred on seed banking. Prominent examples include the UK-based Millennium Seed Bank Partnership focused on conserving seeds of wild plants, and the Svalbard Global Seed Vault in Norway which serves as a back-up facility for seed collections of the world's food crops. In Australia, seeds of wild species are conserved principally through a network of seed banks in botanic gardens and other conservation agencies working together under the Australian Seed Bank Partnership (ASBP, see Case Study 2.6), as well as by other conservation and research organisations including CSIRO's Australian Tree Seed Centre, and some regional botanic gardens and universities storing seed collections. Since the year 2000, this network has banked seeds from over 6,500 species (CHABG 2021) and 67.7 % of Australia's EPBC-listed threatened flora are represented in ASBP seed banks (Commonwealth of Australia 2020). The Australian Grains Genebank (AGG), a partner of the ASBP, is the national conservation seed bank for crops, their **progenitors**, and wild relative species. The AGG conserves over 165,000 **accessions**, or types, of seed representing cereal, oilseed, pulse, and legume species, and is part of the global network of agricultural genebanks that operate for a food secure future.

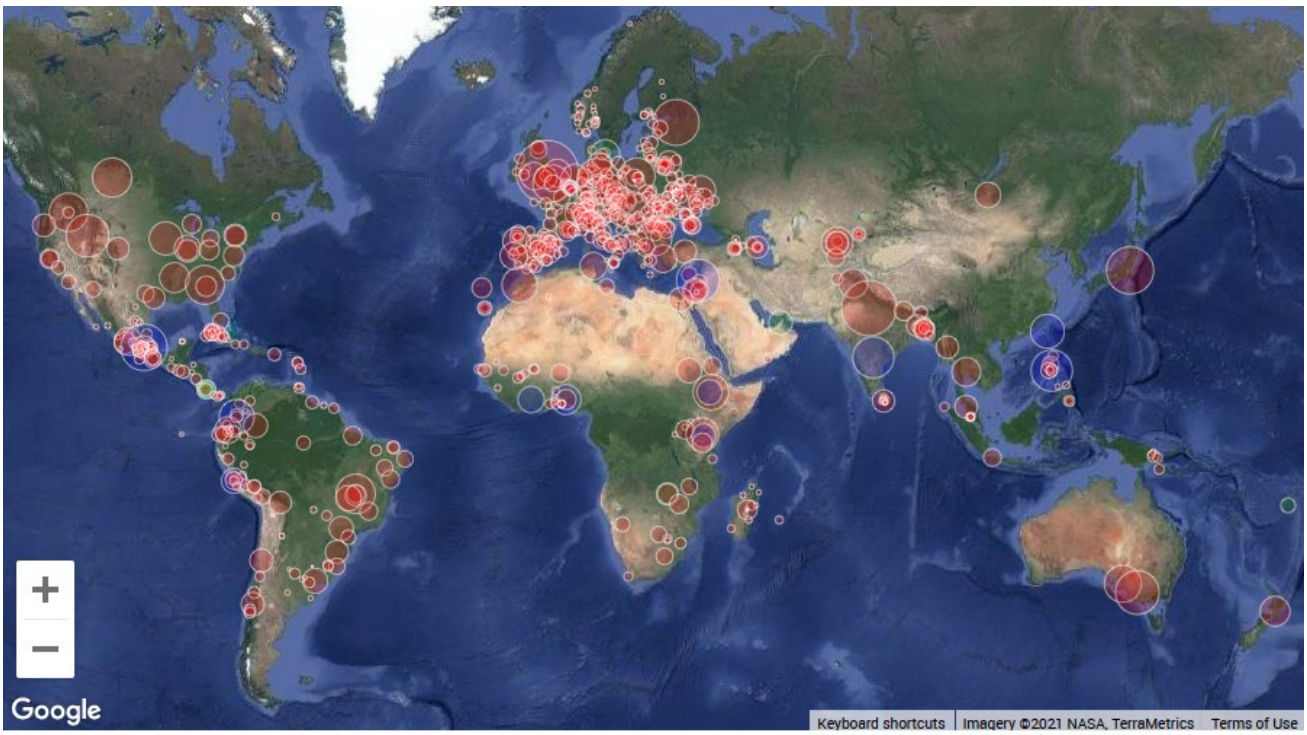


Figure 5.1: Global distribution of ex situ genebanks for food and agriculture with holdings >10 000 accessions. The map shows geo-referenced genebanks from countries that reported on Indicator 2.5.1 of the Sustainable Development Goals in 2020. Note that the Svalbard Global Seed Vault is not included as a genebank in this data set and some countries have not yet reported on their holdings. Source: FAO (Food and Agriculture Organization of the United Nations) 2020, 'WIEWS – World Information and Early Warning System on Plant Genetic Resources for Food and Agriculture'[37]. Reproduced with permission.

Worldwide, agricultural genebanks (Figure 5.1) conserve over 7.4 million accessions of plant **taxa** representing actual or potential value as food crops, and are essential resources that underpin the development of more resilient (e.g., against pests and diseases, or for drought tolerance) and productive varieties that are critically required to feed the world's growing population (Yu *et al.* 2016; Fu 2017). These seed banks contain the elite varieties grown today using mechanical agricultural systems, the traditional landrace or village varieties that are hundreds, potentially thousands, of years old, and progenitors and wild relatives. Many of the agricultural genebanks operate under the International Treaty for Plant Genetic Resources for Food and Agriculture to ensure there is a fair and equitable sharing of germplasm and potential benefits to countries of origin of the material. The modern crop varieties grown today have limited ability to tolerate the changing conditions regularly experienced season to season, let alone those expected into the future. As such, there is increasing focus on wild crop relatives as sources of diversity to withstand changing environmental, pest and disease conditions. Without the conservation of crop diversity in genebanks, the development of the more resilient and productive food crops required into the future would be difficult to achieve.

Conservation seed banks, including agricultural genebanks, differ from seed banks storing seeds destined for ecological restoration, in that seed samples are generally cleaned or processed to a higher purity, kept in smaller quantities, and stored under more stringent conditions suitable for long term storage. Conservation collections are intended to be kept for decades or longer and are usually duplicated at other locations for risk management. **Restoration** seed banks have a more rapid turnover of seed collections (generally from months to a few years, see Merritt *et al*. 2021). Conservation seed banks often store both threatened and non-threatened species, while

37 http://www.fao.org/wiews/data/ex-situ-sdg-251/maps/en/

restoration seed banks tend to store non-threatened species (though may also include species that are components of threatened ecological communities). Therefore, guidelines for the collection and storage of seed collections vary according to the end-use. Conservation collections, for example, usually employ lower storage temperatures to ensure seeds remain viable for longer (Figure 5.8, Table 5.1).

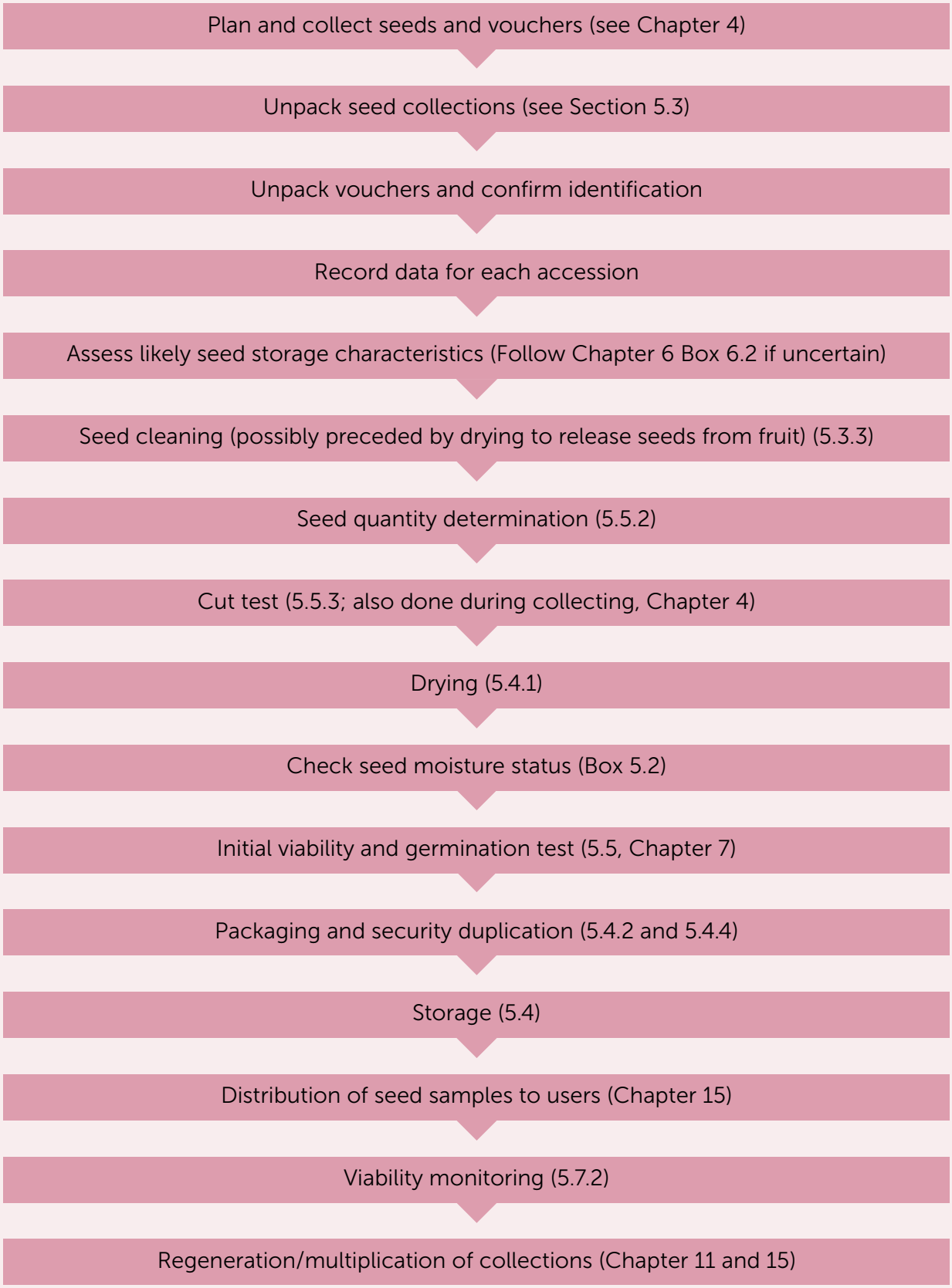
5.1.1 Seed storage behaviour

Research through the 1970's to early 1990's determined three categories of seed storage behaviour with respect to the response of seeds to desiccation and sub-zero storage temperatures – **orthodox**, **recalcitrant**, and **intermediate** (Hong and Ellis 1996). Orthodox seeds are most common – most crop species produce orthodox seeds, as do most plants from seasonally dry environments. Orthodox seeds naturally dry during the latter phases of their maturation with their **longevity** increasing in a predictable manner with a decrease in seed **moisture content** and temperature. They are tolerant of desiccation to low moisture contents and can therefore survive sub-zero temperature storage. Recalcitrant seeds are those that do not survive desiccation and are most commonly found amongst rainforest trees and mangroves. Recalcitrant seeds have high water contents at maturity, and subsequent drying to a degree that would allow storage at sub-zero temperatures is lethal. Because they are sensitive to desiccation, recalcitrant seeds are not well-suited to conventional seed banking and require more specialised procedures for conservation (refer Chapter 6). The intermediate seed category captures seeds that have properties somewhere between orthodox and recalcitrant seeds – they are generally **desiccation tolerant**, but often not to the same degree as orthodox seeds. The benefits of decreasing moisture content and temperature to the longevity of intermediate seeds varies between species, and some are sensitive to sub-zero storage temperatures (Walters 2015; Chau *et al*. 2019).

The best current estimate of the presence of desiccation tolerance vs desiccation sensitivity amongst species is c. 90 % and c. 8 %, respectively (Wyse and Dickie 2017). Whilst desiccation tolerance and orthodox storage behaviour are clearly predominant seed traits allowing for the wide applicability of seed banking, desiccation tolerance alone does not guarantee orthodox storage behaviour, or that seeds will be long-lived in storage. Many desiccation tolerant seeds have relatively short lifespans in storage (e.g., a significant viability decline in dry seeds under cool (5 °C) or cold (-18 °C) conditions between c. 10–25 years of storage; Probert *et al*. (2009); Chau *et al*. (2019); Colville and Pritchard (2019); and identifying these types of seeds, as well as those with **non-orthodox** storage behaviour, is one of the current areas of priority research for seed banking of wild species (Walters and Pence 2020).

It is important to recognise the diversity of seed storage behaviours amongst species and that not all seeds are capable of surviving desiccation or sub-zero storage temperatures (refer Chapter 6 for identification of these species). Variability in seed storage behaviour between species, rather than three discrete categories, is more appropriately viewed as a continuum with respect to the degree of seed desiccation tolerance and the impacts and interactions of seed water content and storage temperature on longevity (Walters 2015). The storage behaviour and longevity of seeds of most wild species is yet to be determined and it is this fact that makes the storage and curation of seeds of diverse species a continual learning process.

Box 5.1: Flow chart of seed banking procedures, from Smith *et al.* (2003) and Rao *et al.* (2006)



5.2 Benefits and risks of seed banking

Seed banking is widely accepted as the most effective method of ex situ conservation. The ability to store and maintain the viability of genetically representative germplasm of a species using relatively low-maintenance conditions is the key advantage of seed banking compared to other forms of ex situ conservation such as **tissue culture** (Chapter 9) or nursery-based plant collections (Chapter 8 and 11). Ex situ conservation also allows representative genetic- and species-level diversity to be collected from the wild and utilised for various purposes without putting the natural **populations** at risk through repetitive harvesting of tissue or seeds. Seed banks require relatively little space compared to nurseries, and the facilities can be scaled in size, technical sophistication and resourcing, depending upon the purpose of the collections – from desktops (e.g., seeds stored in airtight containers over silica gel) to the large walk-in vaults of global-scale genebanks. These factors make seed banking efficient and cost-effective.

For orthodox seeds, the maintenance of their viability over long periods of time is key to the efficiency of seed banking. All seeds have a finite lifespan, with the longevity of a given seed collection being determined by a range of factors including species characteristics and genetics, seed quality, and post-harvest and conservation storage conditions (see Section 5.7). Viability loss during storage and consequently the genetic erosion within a seed collection, or the potential complete loss of genetic diversity, is a risk of seed banking (FAO 2014). The monitoring of the viability of seed collections is therefore critical to maintaining the genetic integrity of a collection. Should significant viability decline be detected within a banked seed collection, decisions need to be made regarding regeneration, or re-collection of new seeds from the wild, if possible. Re-collection may not always be feasible in the case of population decline or species extinction (e.g., for collections of threatened species) and strategies for regeneration and multiplication that minimise genetic erosion are important components of genebank management (FAO 2014; CPC 2019). Maintenance, monitoring and regeneration of ex situ collections is addressed in Section 5.7.2 and Chapter 15.

The work of seed banks spans decades, even centuries. This presents not only technical and scientific challenges, but also funding challenges. A sustained source of funding is required for collections and facilities maintenance, and resourcing with appropriately trained staff. Seed banks require funding not only for specific infrastructure, but also to employ staff skilled in the sourcing, curation and use of collections, taxonomy, research and management (see Chapter 15; Engels and Visser 2003; Fu 2017). Further, the effective curation of banked seed collections that are ever-increasing in size and diversity requires a corresponding increase in resources for operating budgets and staff. This requirement can present limitations in seed bank operations, including lags in collecting, cleaning, drying, packaging and storing seeds. The viability monitoring and re-collection of accessions (or regeneration of irreplaceable accessions) in particular, is a major challenge to seed banks as the size and diversity of their holdings increases.

Duplication of seed collections is a sound risk-management strategy, with best practices duplicating important collections in-country, and even in seed banks in other countries (CPC 2019). International programs including the Millennium Seed Bank Partnership, the US National Laboratory for Genetic Resources Preservation, and the Svalbard Global Seed Vault all contain duplicate collections of wild and/or crop species from around the world. Duplication of collections aims to prevent the loss of valuable germplasm from localised events that may impact on seed bank infrastructure or operations, such as fires or floods, through to more major incidences of political unrest or outbreaks of war (Chapter 14).

5.3 Pre-storage operations

Careful observation, handling and cleaning of seeds prior to sorting is essential to ensure that high-quality seed collections are conserved, and valuable time and resources are not wasted. This section highlights important quality assurance steps required during post-harvest handling of seeds, including the importance of record keeping during curation, and key steps to cleaning and processing seeds prior to storage.

5.3.1 Record keeping and labelling

It is essential to keep track of each seed collection and ensure that corresponding data are stored appropriately for future access. Information collected during curation complements **provenance** and seed collection data, and all this information should be kept together in a centrally located electronic database system. The use of barcodes and/or Quick Response (QR) codes can facilitate sample handling and transfer of data through the process of conservation in seed banks.

Retaining the identities of individual seed collections during processing is essential if collections are to maintain their genetic integrity and their value. It is imperative that individual seed collections are not mixed or confused with other seed batches. Depending on the intended use of collections, seeds from each individual plant from a population can be stored separately (CPC 2019; see also Chapter 3 Box 3.2 'Why not pool maternal lines?'), or seeds can be stored as bulked samples from many individual plants from a population. The protocols for sample handling must be specific to the intended use of the collection and must retain a clear link to the provenance data and other information recorded at the time of collection. Clear labelling of collections is vital, and care should be taken whenever multiple collections are being handled to ensure that loss or confusion of identity is avoided. One way to achieve this during processing is to give seed lots two labels – one inside the bag or container with the seeds and the other on the outside of the bag or container or on the processing equipment (e.g., jar, tray or cleaning apparatus). The labels should have a clear, unique identifier for each collection (e.g., accession number and/or collector's number with species name). Labels must be legible and written in pencil or water-repellent ink and resistant to moisture. Jeweller's tags written in pencil are ideal. After any partial or preliminary cleaning, seeds should be returned to the same labelled container. Empty collecting bags, containers and processing equipment should be thoroughly cleaned before re-use to avoid cross contamination.

Information on seed cleaning, seed quantity, **cut test** results, **germination** testing and storage details (number of containers and location) should be recorded in a central database or folder, along with the provenance data and other field information on the collection. Notes on the methods used for cleaning, quantity determination and germination, as well as the outcome, can be useful in ensuring consistent handling between different members of staff and over time. These curation records can also provide insights into where potential quality issues with a collection may have arisen. As well as adding to the value of the individual collection, these data can assist with decision making for future viability and germination testing and help identify future collection needs such as the recollection of a particular species over several years to maximise seed numbers. Refer to Chapter 4 for more information on seed collection and record-keeping procedures.

5.3.2 Post-harvest handling

After collection, pay particular attention to the moisture content of the seed, as they will age rapidly at high moisture levels, particularly when also at high temperatures. For orthodox species, seed moisture content needs to be reduced to allow for storage at low (sub-zero) temperatures. It is always wise to check whether a species is known to produce seeds that can tolerate desiccation to the low moisture contents required for long-term storage, before the seeds are placed under conditions suitable for drying (Chapters 5 and 6).

Seed moisture content is simply the amount of water present in a seed. Seed moisture content is influenced by the seed maturation status at collection and the environmental conditions in which the seeds are held during post-harvest handling, as well as seed size and composition. Seeds gain or lose moisture depending on the temperature and the **relative humidity** of the surrounding air, eventually reaching equilibrium (Gold 2014). The relative humidity of the air, at a given constant temperature, at this equilibrium point can be measured and is termed the **equilibrium relative humidity** (eRH). A 'safe' moisture level to prevent rapid aging is around 50 % eRH or below (approximately 12 % seed moisture content on a fresh weight basis, depending on seed oil content), when the temperature is below 25 °C. Orthodox seeds tolerate desiccation to at least 10–15 % eRH (approximately 5–8 % seed moisture content) (Figure 5.2) and should be dried to this level before long-term storage. Seed eRH can be measured quickly and non-destructively using a **hygrometer** (see Box 5.2). Seed moisture content is measured destructively, by oven drying seeds at 103 °C for 17 hours (ISTA 2021), and expressed gravimetrically on a fresh or dry weight basis (see Box 5.2). Note that ISTA (2021) provides further guidance on moisture content measurement for crop, tree and shrub species, including the need to grind or cut large and water-impermeable seeds prior to measurement, as well as use of a high temperature oven drying method (130 °C) for some species.

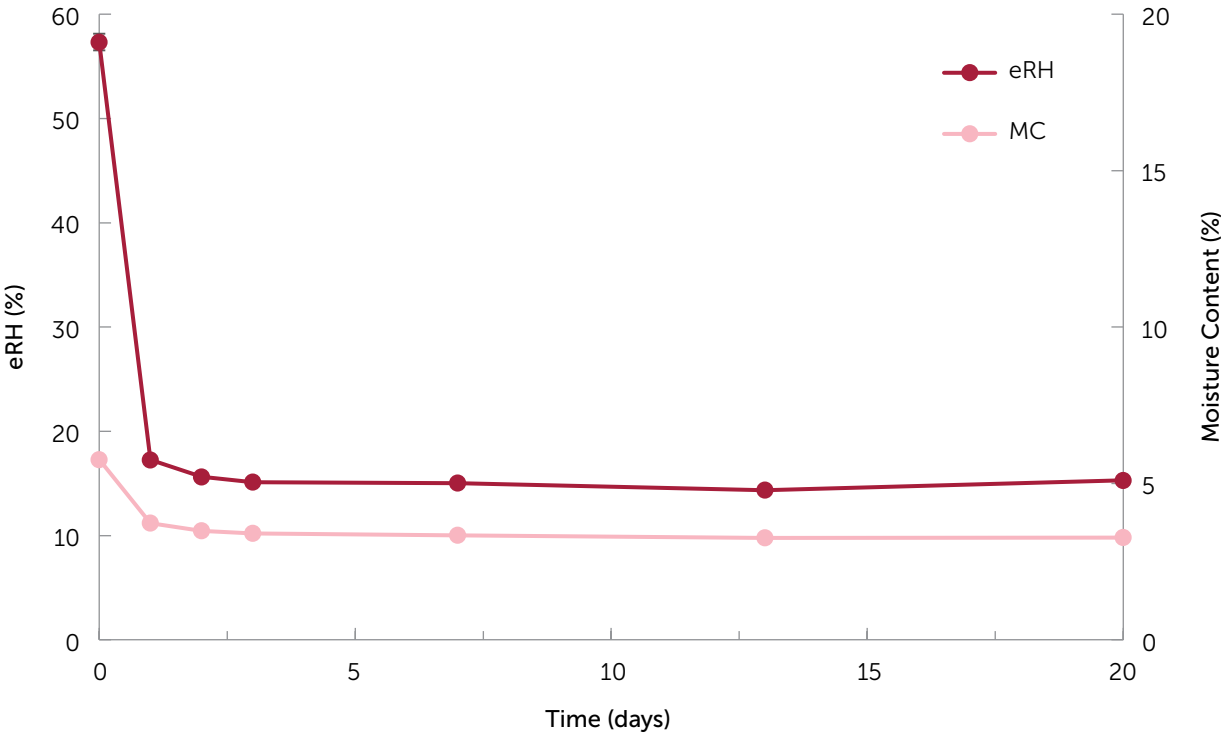


Figure 5.2: Seed drying over time. This graph demonstrates the change in moisture status of seeds of *Grevillea wickhamii* measured via two methods: equilibrium relative humidity (eRH) and seed moisture content (MC). Note that it took approximately 7 days for these seeds to equilibrate in a controlled environment drying room at 15 % RH and 15 °C (D. Merritt, unpubl.).

Box 5.2: Determination of seed moisture content

Seed moisture status can be measured as equilibrium relative humidity (eRH) using a hygrometer (Gold and Manger 2014a). This technique measures the relative humidity (RH) of air at equilibrium with seeds held in a sealed chamber. This method is rapid and non-destructive, so seeds can be returned to the collection following measurement.

Procedure:

- Place a sample of seeds into the chamber of the hygrometer. Ensure that the chamber is fully filled with seeds.
- To avoid inaccurate readings, do not directly handle seeds, do not touch the inside surfaces of the sample chamber, and do not breathe on an exposed sensor.
- Allow the seeds to equilibrate and record the end point RH and temperature displayed on the hygrometer. As the eRH will be dependent on temperature the measurements should be conducted under controlled conditions, ideally the same temperature at which the seeds have been dried.
- When seeds are dried sufficiently, the reading for RH should be between 15–20 % when measured at 15 °C.

Seed moisture content can alternatively be determined through low-temperature oven drying using the following procedure adapted from ISTA (2021). Note that this is a destructive method and seeds should be discarded after oven drying.

- Remove at least three replicate samples of seeds – ideally at least 0.1 g of seeds, depending on seed size and availability.
- Weigh the seeds in a suitable container, such as an aluminium weigh dish with cover, on a balance measuring to three or four decimal places. This is the "wet weight" of the seeds.
- Place the containers with seeds, with covers removed, into an oven at 103 °C for 17 hours.
- Remove the containers from the oven, replace the covers, and place the containers in a desiccator containing a suitable desiccant and allow to cool at room temperature for 1 hour.
- Re-weigh the seed samples. This is the "dry weight" of the seeds.
- Calculate seed moisture content on a dry weight basis using the following formula

$$\text{Seed Moisture Content (\%)} = \left(\frac{Weight\ Wet\ Seeds - Weight\ Dry\ Seeds}{Weight\ Dry\ Seeds} \right) \times 100$$

Note: These guidelines recommend calculating seed moisture content on a dry weight basis. However, moisture content is also often calculated and reported on a fresh weight basis. In any case, the method of calculation should be clearly stated.

Mature seeds collected as close to the point of natural **dispersal** as possible should be dried to a moisture content appropriate for storage as quickly as possible (Box 5.3). Initial drying of seeds for post-harvest processing may be possible under ambient conditions if the relative humidity is low (<50 %), but in humid conditions (including after rain), drying will require a desiccant such as silica gel, an air conditioned room or, preferably, specialised facilities such as a drying room (see 5.4.1). Collectors and curators need to make sure that 'dry' seed collections (<50 % eRH) do not take up any more moisture by avoiding exposure to humid conditions.

If seeds are immature at collection (indicated by a very high eRH of 85–100 %), they may benefit from being held for a short period in conditions that promote post-harvest maturation, rather than being placed immediately under rapid drying conditions. Seed maturation is encouraged by maintaining seeds within **fruits** or keeping the fruits on branches or stems under natural conditions for 1–2 weeks, rather than beginning to dry and clean the collection. Post-harvest maturation can also be encouraged by storing seeds at high humidity (e.g., 30 days at c. 80 % RH for *Rhododendron* spp.), but it is important to note that seeds handled in this manner do not appear to achieve their maximum potential longevity (Hay *et al*. 2006) and it is always preferable to collect fully mature seeds. Fleshy fruits can be kept in plastic bags until they can be cleaned, but the bags must be opened daily to aerate and remove condensation (Chapter 4).

Box 5.3: Post-harvest handling of seeds (adapted from Gold 2014)

<table>
<tr><th rowspan="2">Seed maturity</th><th rowspan="2">Seed moisture status</th><th colspan="2">Ambient conditions</th></tr>
<tr><th>'Dry'
(daytime RH < 50 %)</th><th>'Humid'
(daytime RH > 50 %)</th></tr>
<tr><td>Immature</td><td>85–100 % eRH</td><td colspan="2">Hold intact fruits under shaded and covered, or indoor, ambient conditions for 1–2 weeks. Leave fruits on branches or stems if possible*</td></tr>
<tr><td rowspan="2">Natural dispersal</td><td>'Wet'
50–85 % eRH</td><td>Dry seeds in a thin layer, in a well ventilated outdoor or indoor location. Minimise moisture absorption at night.</td><td rowspan="2">Transfer to seed bank as soon as possible, or dry with desiccant, or place in air-conditioned room</td></tr>
<tr><td>'Dry'
<50 % eRH</td><td>Hold seeds loosely packed in permeable bags in a well-ventilated shady or indoor location. Minimise moisture absorption at night.</td></tr>
<tr><td colspan="4">*Remove seeds from fleshy fruits as soon as **morphological** signs (e.g., fruit colour) indicate that they are fully ripe. Allow to dry slowly under ambient conditions before transferring to a cool dry-room.</td></tr>
</table>

Case Study 5.1 (below) indicates that there is potential for further optimisation of the post-harvest maturation and drying phase to improve seed quality.

Case Study 5.1: High temperature drying to improve seed quality in orthodox seeds: an example from crop species

Katherine J. Whitehouse

Post-harvest drying, following late seed maturation, prolongs seed survival in air dry storage. A relatively low drying temperature is usually adopted to reduce the risk of seed deterioration during the drying process, particularly during the later stages of drying when evaporative cooling will no longer suppress the temperature within the seeds (Nellist 1980; Cromarty *et al*. 1982; McDonald and Copeland 1997). However, research has shown that an initial exposure to a higher temperature (45 °C and sometimes warmer), before the conventional cooler and drier conditions, can improve subsequent storage longevity of rice and soybean seeds harvested while still moist (Whitehouse *et al*. 2015, 2017, 2018a, b).

Several separate investigations were carried out, using more than 20 accessions of rice (*Oryza sativa* L.) from 5 variety groups, across a 3-year period (Whitehouse *et al*. 2015, 2017, 2018a). Freshly harvested seeds, at different stages of maturity, were subjected to a two-stage drying procedure. All seeds experienced an initial drying period at 45 °C under different conditions, before final drying in a controlled environment room, set at 15 °C and 15 % relative humidity. Analysis confirmed that the improvement in subsequent longevity observed, following the two-stage drying procedure, was not limited to a specific period in relation to seed maturity, or affected by the total exposure time but rather, appeared to be dependent on the moisture content of the seeds at harvest (Figure 5.3). The critical seed moisture content at harvest was found to be 16.5 %; below which there was little, or no benefit from high temperature drying. However, above 16.5 %, seeds showed a positive relation with harvest moisture content, with substantial benefit from drying at 45 °C to subsequent longevity. Hence, it was concluded that there are **temporal** and water discontinuities in the effect of temperature on subsequent seed longevity (Whitehouse *et al*. 2018a). It is thought that the loss in moisture is a critical factor controlling the maturation process, by inducing the stress response and other protective mechanisms, which significantly increase seed quality. Therefore, it is likely that the high temperature exposure is inducing a similar stress response within seeds that are still in a pre-desiccation state, similar to that experienced during maturation drying, allowing the continuation of accruement in longevity (Leprince and Buitink 2010; Angelovici *et al*. 2010; Chatelain *et al*. 2012).

Research like this is pertinent as improvements in seed quality could have huge implications on how seeds are handled prior to, and managed during, long-term conservation. It is possible that similar benefits could be seen in other species grown in humid, tropical climates, especially those with poor seed storage longevity, or where harvesting seeds at a range of maturities and hence, perhaps moisture content, is inevitable.

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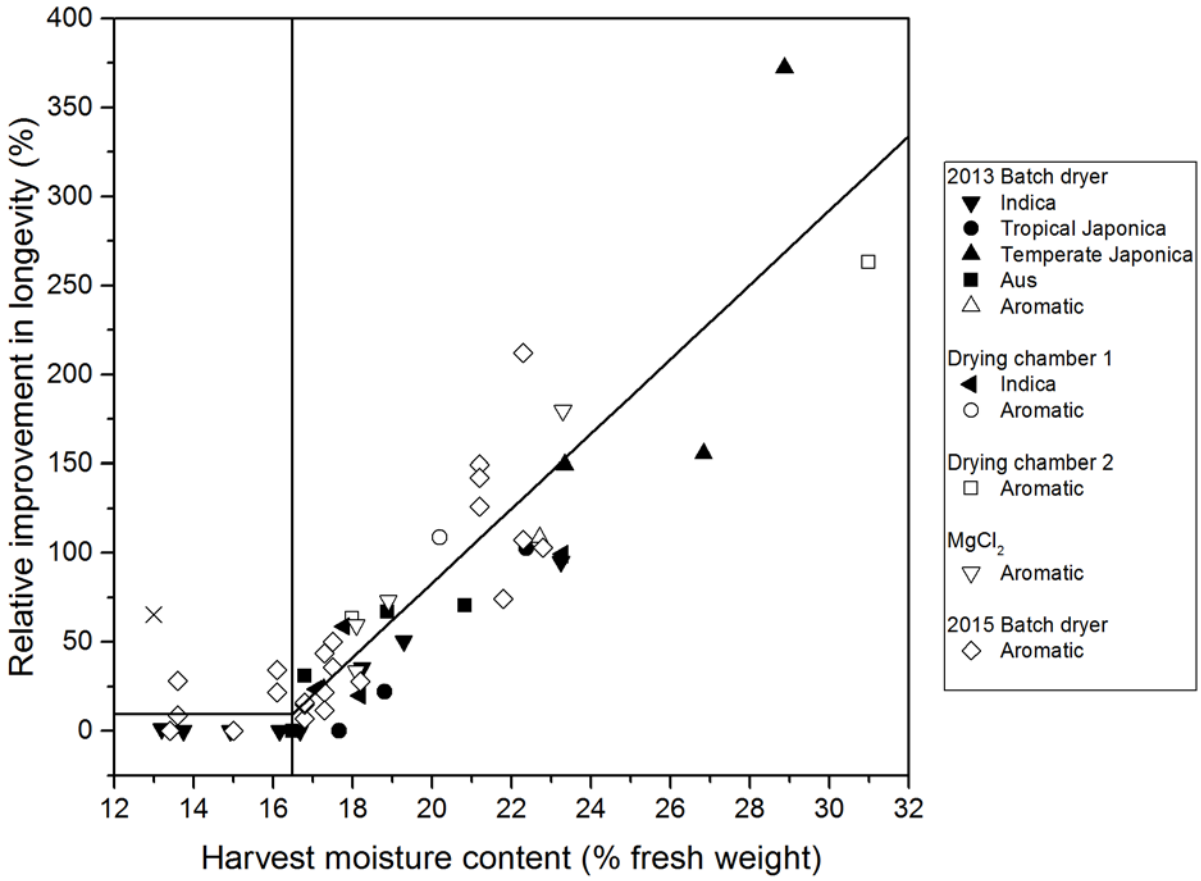

Figure 5.3: Relationship between the improvement in longevity of rice (*Oryza sativa* L.) seeds dried initially at 45 °C under different conditions relative to that when dried in a drying room (15 °C, 15 % RH) throughout and harvest moisture content (% fresh weight) (refer Whitehouse *et al*. 2018a for details of drying conditions at 45 °C). Reproduced with permission from Cambridge University Press.

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5.3.3 Seed cleaning

Seed cleaning is the removal of extraneous plant material (chaff, leaves, pods, sticks), insects, inert material (sand, soil), damaged, shrivelled or empty seeds, and seeds of other species to improve the purity of samples for storage. Good seed cleaning reduces bulk, facilitates drying, optimises storage space and reduces costs associated with storage and utilisation.

Seeds come in such a wide variety of shapes and sizes that every collection must be assessed individually to determine the best method of cleaning. During cleaning, the seed lot should be checked regularly to ensure seeds are not being damaged. Wilson and Wilson (2006) provide a good overview of seed and fruit diversity common to Australian species. For a list of techniques and useful equipment for seed cleaning, refer to Terry and Sutcliffe (2014), and Sweedman (2006). The essence of seed cleaning is to utilise any physical characteristic that differentiates the seeds from the surrounding non-seed material – size, shape, weight, and surface texture, for example. Any consistent difference is potentially exploitable for seed cleaning, as long as it doesn't damage the seeds. Examples of seed cleaning techniques include crushing collections in a calico bag, shaking seeds from capsules, sieving through different mesh sizes, use of a seed **aspirator** (see Case Study 5.2), hand sorting, use of a gloved hand or rubber bung to roll seeds over a rubber mat (Terry and Sutcliffe 2014), or, where no damage to seeds will occur, a mechanical threshing unit.

5.3.3.1 Wet collections

Wet or fleshy fruits (or outer fruit layers) need to be treated immediately to avoid both degradation of the seeds inside and any potential progress towards germination. Fruit pulp is usually removed via washing in a sieve and the seeds (or woody **endocarps**) are then dried and further processed. It should be noted that seeds of some fleshy-fruited species are recalcitrant, and desiccation tolerance will need to be assessed (Chapter 6). Fleshy fruits may alternatively be soaked for a few hours in an enzyme solution, to speed up the process of pulp removal (Tieu *et al*. 2008, Box 5.4).

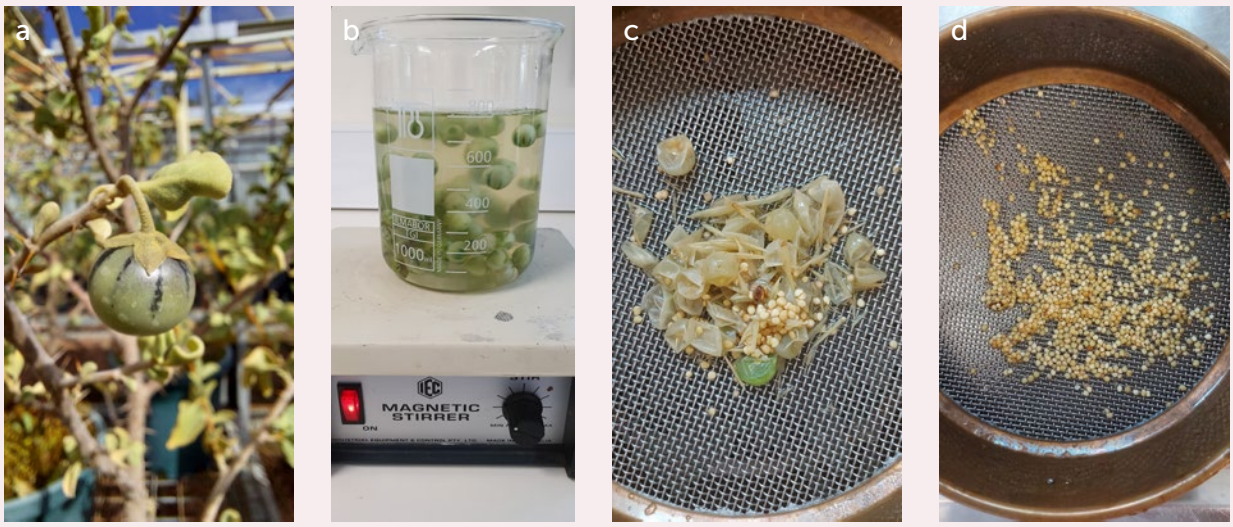
5.3.3.2 Dry collections

Dry fruits may open (dehisce) naturally to release seeds. These **dehiscent fruits** benefit from drying prior to cleaning, as the drying process will often open the fruits and allow the seeds to fall out. In certain cases where not all seeds readily fall from the dried material, it is worth examining whether this intransigent cohort is worth extracting. In certain taxa this retentive element can be non-viable (e.g., *Lepidosperma* (Turner 2013)) and more cleaning can reduce the quality of the collection. Drying these fruits at room temperature or in a drying room is recommended. Although drying at high temperatures may speed up dehiscence, it is best avoided due to the potential loss of seed viability and the adverse effects on longevity until it is more closely studied in Australian native species. In some species (e.g., *Acacia* spp.) shaking or threshing is required after drying to separate the seeds from the opened fruit. Note that some plants (e.g., in the family Rutaceae) have explosive seed dispersal and during drying containers need to be covered, whilst still allowing air circulation. Some dry fruits, such as those of *Astroloma*, *Eremophila*, *Persoonia* and *Scaevola*, do not open to release seeds.

Box 5.4: Enzymatic de-pulping of fleshy fruits

The seeds of many native Australian species are enclosed in fleshy drupes, berries, sarcotestas and arils. These covering structures are common in the fruits and seeds of *Eremophila, Leucopogon, Persoonia,* and *Solanum*, for example, that may contain large amounts of pectins, sugars and water which can hinder seed cleaning and drying, hence the need for their removal prior to storage. De-pulping techniques include soaking in water for several days to promote natural breakdown, then manual removal of the remaining material. This can be a slow and laborious process. Commercially available enzymes used in the wine, olive, and fruit juice industries, are an effective tool for removing these outer pulpy layers (Tieu *et al*. 2008). Instead of taking days to process, fruits can be depulped in a few hours.

To begin the cleaning treatment, freshly collected fruits are quickly macerated and placed into the enzyme broth (one commercially available product is Lafazym Extract). The mixture is then placed onto a magnetic stirrer and heated to around 40 °C. Constant agitation is important to improve the speed of depulping, and complete removal of the flesh can be achieved within 3–4 hours. These depulping methodologies can be effective for a range of species with fleshy fruit including those of *Dianella, Leucopogon*, *Lomandra*, *Myoporum, Nitraria, Rhagodia* and *Solanum*.



Box 5.4 **(a)** *Solanum orbiculatum* fruit prior to collection; **(b)** fruits circulating in enzyme; **(c)** de-pulped seeds ready for final cleaning; **(d)** cleaned seeds. (Images: R. Campbell, D. Merritt)

5.3.3.3 Care and caution

It is important to process collections with care to avoid either harming the collection and/or harming oneself. It is recommended to examine a small sample of unfamiliar material to determine a) the target and the waste; b) what techniques can be reasonably applied to clean the collection, and; c) whether the plant material has any irritant, allergenic or toxic properties (e.g., *Anigozanthos*, *Brachychiton*, *Grevillea*, and *Isotoma* (Sweedman and Brand 2006)). It is advisable to avoid vigorous threshing or shaking as this may cause percussion damage, which could impact viability. Seeds should be cleaned to a reasonable end point. For some species, storage of natural **dispersal units** is recommended, as extracting seeds from fruits or covering structures is likely to cause damage or may be too time consuming and costly. Examples include species of *Atriplex*, *Ptilotus* and some Poaceae. In these cases, a bulkier collection may be stored.

5.3.3.4 Equipment

Equipment suitable for seed cleaning is listed in both Terry and Sutcliffe (2014) and Sweedman (2006). The most useful items include a set of sieves of various mesh sizes and a seed aspirator. Beyond that, a number of cheap and readily available items can potentially be used for seed cleaning and it can pay to be creative in what you use. One thing to be mindful of is the issue of static electricity. When dealing with small- seeded collections static can be highly problematic, with seeds either sticking to, or jumping out of, trays during processing. To this end it is advisable to avoid plastic items as much as possible and look for alternatives of metal, glass, paper or card. Plastic trays are readily available but steel steam pans from a commercial kitchen supplier are a good alternative. Trays can also be made from newspaper, paper or card, and these can be particularly useful to dry washed seed collections or for makeshift trays if processing in the field. Wood (2020) has online instructions on making paper trays and envelopes for seed bank purposes.

Case Study 5.2: Seed cleaning of *Pomaderris lanigera* at the NSW Seedbank

1. Fruits of *Pomaderris lanigera* in situ.

2. Seeds transported to drying room in calico bag (note label in collection with collector number and species name).

3. Fruits are stripped from branches and crushed using a rubber bung and rubber mat.

4. Collection sieved to remove sticks and leaves.

5. Collection cleaned in aspirator to remove more debris.

6. Collection after first pass through aspirator and further sieving.

7. Collection after second pass through aspirator to obtain pure seed.

8. Collection ready for storage and testing (note label with collector number and name has remained with collection throughout cleaning process).

(Images: Leahwyn Seed, Andrew Orme and Simone Cottrell, Royal Botanic Gardens and Domain Trust.)

5.4 Storage of orthodox seeds

The aim of seed banks is to maintain the viability of high-quality seed collections until the seeds are required for their end use. Drying is a critical first step in limiting seed deterioration of orthodox seeds. The importance of drying seeds prior to storage cannot be stressed enough since small changes in seed moisture content have a large impact on seed longevity in storage (Box 5.5). Drying also enables seeds to survive the necessary sub-zero storage temperatures without suffering damage from lethal ice crystals, and minimises damage caused by insect or fungal attack. Seed drying is recommended as soon as possible after collecting, keeping in mind that immature seeds may benefit from slow drying (Section 5.3.2) and some species may not survive drying (refer Chapter 6).

Once appropriately dried, the cooler the orthodox seeds are stored, the greater their longevity. Standards for genebanks recommend storage of seeds at a temperature of -18 ± 3 °C and relative humidity of 15 ± 3 % (FAO 2014). Depending on the species, orthodox seeds of initial high viability and quality are estimated to have a longevity of several decades, up to a few hundred years when stored under these conditions (Walters *et al*. 2005; Walters 2015). Recommended storage conditions for the medium-term are refrigerated temperatures of 5–10 ± 3 °C and relative humidity of 15 ± 3 %. These conditions are suitable for storage for up to 10 years for wild species (Walters *et al*. 2005; FAO 2014; Colville and Pritchard 2019). Storage conditions don't need to have humidity control if the seeds are dried and sealed before storage. Short-term storage is expected to provide high quality seeds for at least five years and may be accomplished at ambient temperatures (under as cool and stable temperatures as possible, but not more than 25 °C).

Table 5.1: Storage conditions for seeds banked for different time frames and for different purposes. Note that significant variation in seed longevity will exist depending on the species being conserved.

Time frame	Conditions	Suitable for:
Short term (≤ 5 years)	Air-conditioned room c. 23 °C, or refrigerator or cool room (5–15 °C) Ambient relative humidity if <50 %	Restoration seed banks (See Merritt *et al*. 2021)
Medium term (≤ 10 years)	Temperature 5–10 °C Relative humidity 15–20 % Seed moisture content c. 3–7 %	**Active collections**, including those for plant breeding and research
Long term (>10 years)	Temperature ≤ minus 18 °C Relative humidity 15–20 % Seed moisture content c. 3–7 %	Conservation seed banks including wild species and agricultural genebanks; **base collections**.

Box 5.5: Harrington's 'Rule of Thumb'

Orthodox seeds lifespan is approximately doubled for every 10 °C drop in temperature and 1 % drop in seed moisture content.

5.4.1 Seed drying

Most orthodox seeds are **hygroscopic**, meaning they absorb or lose water until reaching equilibrium with their surrounding air. This is the basic principle of seed drying. The international standard for seed drying is derived from the expectation that seed longevity in storage is maximised when seeds are dried to a moisture content that is in equilibrium with 5–20 °C and 10-25 % relative humidity (RH), and subsequently stored at -18 °C or less (Rao *et al*. 2006; FAO 2014). A low temperature is also preferred, over higher temperatures; typical of heated-air dryers, to reduce the risk of seed deterioration, especially when seeds have a high moisture content. Studies have confirmed that these drying and storage conditions are suitable for many Australian species (Offord *et al*. 2004; Crawford *et al*. 2007). A seed drying and storage environment can be easily monitored using temperature and RH dataloggers, for example, TinyTag™ or Thermocron™ loggers, or automated wireless network and Bluetooth based systems.

A seed collection often undergoes drying and cleaning concurrently, in which case seeds are cleaned inside a controlled environment drying facility, or simply returned to drying conditions in between cleaning. After cleaning, seeds may undergo more controlled drying. Before packaging seeds for sub-zero temperature storage, seed moisture status (ideally 15–20 % equilibrium RH (eRH) or c. 3–7 % moisture content) should be measured and recorded (Box 5.2). When seeds reach equilibrium, they are not able to dry further and are ready to be packaged. Therefore, in controlled drying conditions it is not strictly necessary to know the seed moisture content (Whitehouse *et al*. 2020), but rather monitor and record eRH.

For optimal results, it is worth understanding the factors that affect seed drying:

- Temperature: Water evaporates more rapidly at high temperatures. However, the use of high temperatures to accelerate seed drying is not recommended as it will speed up the seed aging process and may unacceptably reduce viability. Temperatures of 10–25 °C are recommended for seed drying.
- Relative humidity: Aim for c. 15–20 % RH.
- Seed size and structure: Small seeds dry fast, relative to larger seeds. Seed coat permeability affects the rate of moisture loss. Seeds with physical **dormancy** (refer Chapter 7) can lose moisture but not absorb moisture.
- Air velocity: Drying time is approximately halved when the velocity of air around each seed is doubled, so drying can be more rapid if seeds are spread into a thin layer on trays.
- Seed moisture content: Water is lost more rapidly from seeds at the beginning of drying than towards the end.

Source: Schmidt and Thomsen (2003)

While drying, mature seeds are usually placed in porous bags (e.g., calico, paper or woven polypropylene bags), particularly if they have an explosive seed release mechanism (e.g., *Daviesia* or other legume species, or Rutaceae), or open paper envelopes, or spread out in trays. The time required to dry seeds is largely dictated by their size, the thickness of the seed coat, and the total volume of the seed batch. Four to six weeks is generally sufficient for seeds of most species. For orchid and other micro-seeds, drying for one week is preferable due to their small size and shorter-lived nature.

Several options for drying mature seeds are available. If weather conditions are warm and dry, air drying of seeds in ambient conditions is possible, providing the RH is less than 50 %, and noting that these conditions are not suitable for medium- or long-term storage. Drying in the shade is preferable. Drying without shade is possible, but care must be taken to avoid overheating which may cause, for example, cracking (Probert *et al*. 2003). Ideally, seeds should be laid out in a single or thin layer, or mixed regularly, for even drying. Seeds should be placed on open racks with enough space to allow for the free circulation of air. On a larger scale, seeds can be laid out on tarpaulins or shade cloth (if outside, these should be overlaid by shade cloth to prevent seed removal by wind).

In humid and tropical climates, the ambient RH may be >75 % in which case seeds should be dried using forced ventilation or moisture extraction, with a domestic fan or air conditioner aiding air circulation. In such scenarios, seeds should be sealed overnight (e.g., in plastic food containers or plastic drums) to prevent moisture uptake as the air temperature drops and RH increases.

Artificial drying methods are preferable as they achieve a lower seed moisture content in a more consistent and repeatable manner. Ideally, seeds are dried in a purpose-built drying room maintained at 15–20 % RH and 15–20 °C. Similarly, dehumidifiers installed into a well-sealed space can achieve an RH of ≤25 %. Such facilities are common in conservation seed banks and, under these conditions, the seed moisture content will reduce to approximately 3–7 % of fresh weight. However, artificial drying methods can be costly, and the method(s) used should be guided by the anticipated use of the seeds and length of storage. Artificial drying may not be warranted for seeds that will be used within five years of collection (e.g., in the commercial or landcare sector; see Merritt *et al*. 2021 for more details on drying methods suited to these sectors).

Drying over silica gel or beads (silicon dioxide) is a practical solution for small quantities of seeds, as well as for drying seeds in the field during collection trips of a few days or weeks in regions of high humidity. However, it is preferable to air-dry the seeds first to avoid drying moist seeds too quickly. Place the silica gel in an airtight container with the seeds. Separation is important due to the rapid drying induced by silica gel, therefore, enclose the seeds and/or silica gel in porous bags inside the airtight container, or suspend seeds over the silica gel on a porous rack. Use a 1:1 weight ratio of seeds to silica gel, place the container at c. 15 °C and mix the seeds periodically to ensure even drying (a datalogger may also be enclosed to monitor the RH). The gel usually has an indicator dye that changes colour when saturated, at which point it is replaced or regenerated. Other possible desiccants include zeolite beads, calcium chloride, rice, and charcoal.

Alternative means of achieving artificial drying include propagation igloos or greenhouses, solar tumblers, solar drying boxes, and drying cabinets with forced venting set to 30–35 °C. There may also be opportunities to use an industrial drying service at agricultural premises.

More details can be found in Linington (2003), Sweedman (2006) and Merritt *et al*. (2021).

5.4.2 Packaging dried seeds

Packaging of seed collections is crucial to maintain the low moisture content achieved during drying. Containers for long-term storage need to be airtight to avoid water reabsorption from the surrounding air when storage conditions are not humidity controlled. Packaging also keeps seed batches separate and prevents insect and disease contamination in storage. Ideally, dried seeds should be packaged quickly in a controlled humidity environment and storage containers equilibrated to the same RH as the seeds (usually 15 %) before packing the seeds and sealing.

A variety of containers are used in seed banks, for example heat-sealed tri-laminate foil bags (Figure 5.4) or glass jars with a rubber seal. Seed collections vary in size so a range of containers may be required, preferably with a wide aperture relative to volume. Containers with a wide neck allow easier access for large and irregular shaped seeds. Containers with a square cross-section pack effectively on shelves. Containers should be resistant to punctures from sharp seeds. Advantages and disadvantages of different storage containers are presented in Table 5.2. (see Gold and Manger 2014b for more details). Storage containers for cryostored seeds are discussed in Chapter 10.

It is recommended that containers are tested for potential moisture leaks over four weeks (at least) under cold storage conditions prior to use. Include self-indicating silica gel sachets in replicates of the test containers to detect the presence of moisture once containers are retrieved and opened or check the silica gel colour each week if the container is transparent (Gomez-Campo 2006). If the seal of the container fails, the silica gel will absorb moisture and the indicator will change colour. Any colour change should begin at c. 20 % RH. The self-indicating silica gel sachets can be permanently added to detect leaks during storage, by monitoring for colour change inside transparent containers, or by checking when seeds are removed from the container for use.

Figure 5.4: Seeds stored in heat sealed foil bags in the walk-in cold room of the Australian PlantBank, Australian Botanic Garden Mount Annan. Seeds are stored in the same manner in the freezer but are more difficult to photograph due to the effect of low temperature on photography equipment and lighting. (Image: Simone Cottrell)

Table 5.2: Options for packaging dried seeds (from Gold and Manger 2014b).

Container material	Advantages	Disadvantages
Glass containers (manufactured to high standard)	Transparent – seeds can be seen and moisture indicators can be added to check integrity of seal	Heavy – potential for breakage Not space efficient Not suitable for large quantities of seeds
Tri-laminate foil bags	Lightweight Space efficient Can be vacuum sealed	Need to be properly sealed, which is easily overlooked Can be punctured by sharp objects and seeds e.g., grasses Do not allow seeds or moisture indicators to be seen Not practical for large quantities of seeds (e.g. revegetation collections)
Sealed plastic containers or bags (non-woven)	Lightweight May be transparent	Rarely seal effectively Potential effect of plasticisers on seeds Not space efficient
Metal containers (Not recommended)	Robust	Seal poorly Do not allow seeds or moisture indicators to be seen May be prone to corrosion

Container lids and seals:

- To seal foil bags use a heat sealer with a serrated sealing bar of at least 10 mm. Vacuum sealing can be used to remove air in the bag but may increase the risk of puncturing and can lead to creasing across the weld seam. Place sharp seeds in a dry card envelope prior to sealing.
- Lids with natural or synthetic rubber seals, clamped or crimped onto the container are much more effective than screw lids. A torque meter should be used to check screw lids are properly sealed.
- Effective sealing compounds include natural rubber, bromobutyl, chlorobutyl and flurotec seals (not silicone). Glass bottles fitted with polypropylene lids out-performed similar bottles with metal caps and butyl rubber seals in tests. Well-designed plastic lids without seals can perform well.
- Clamped containers (e.g., glass storage jars) and crimped containers (e.g., glass freeze drying vials) are very effective but may not be available in sizes needed for diverse seed collections.
- Screw lids or clamped seals enable regular access to seeds. However, screw lids may loosen during use due to thermal expansion/contraction during freezing and thawing which can significantly weaken the seal.

Source: Gold and Manger (2014b)

5.4.3 Seed storage

Once seeds are dried and packaged, they should be placed directly into storage to further slow the seed aging process. The temperature at which seeds are stored depends on the desired storage life of the seed collection and the facilities available (Table 5.1). Conservation collections may be divided into active and base collections (see Chapter 2.6.1, Chapter 15.3) prior to storage. In some seed banks, for example, those used in plant breeding, the active collection may be held at a higher temperature (e.g., 5–10 °C) than the base collection (e.g., -18 °C) (FAO 2014). The proportion of seeds held in active and base collections varies from seed bank to seed bank and depends on the **taxon** and the intended usage.

5.4.4 Safety duplication of banked collections

Safety duplication refers to a genetically similar sub-sample of an accession being stored at another location to provide insurance against the loss of material. Duplicate seed lots should be maintained under the same, or better, storage conditions as the main collection (FAO 2014). To save time, samples for duplication can be split from the main collection and prepared for storage at the same time. It is important to duplicate both the seed material and the accompanying information. Whether, and how, the duplicate collections are used depends on the formal agreement made between staff at the main and duplicate sites. If a formal agreement is not in place (see Chapter 2.6.2, Chapter 15.4.2), a memorandum of understanding is worthwhile to ensure that seed use is discussed before collections are sent to duplicate sites.

5.5 Testing banked collections

The testing of banked seed collections is necessary to assess the proportion of seeds that are available to regenerate into healthy plants. Critical components of this assessment are **seed fill** (5.5.3), viability (5.5.4), and germination (Chapter 7). Initial seed viability is also a significant factor in seed longevity (5.7.1). General suggestions for sampling and testing are presented in Box 5.6 and in Commander *et al.* (2021).

Box 5.6: Some reminders about sampling for seed-fill, viability, and germination tests

- Randomly select seeds after mixing the collection well, to ensure representative sampling.
- Larger samples give more accurate results (>50 seeds). If possible, use a minimum of 3 replicates with ≥10 seeds in each, under the same conditions (to give a measure of variability).
- All methods are estimates only and depend on the experience and ability of the operator to interpret the results.

For germination tests:

- Always use a control when testing germination treatments to ensure that the treatment is effective compared to non-treatment.
- If monitoring viability, always use the same method as previous tests.

Check and record seed germination regularly and share information on successful and unsuccessful test methods. For rare or threatened species, or very small seed collections, consider whether resources are available to grow on the germinants produced in a germination test.

5.5.1 Seed quality

After a seed collection has been cleaned and dried, it can be quantified and assessed for quality. Some aspects of seed quality relate to collection information and species identity and these are covered in Chapter 4. The second component of seed quality relates to physical and biological measures of the collection such as seed purity, seed fill, and seed viability. Knowledge of these quality parameters is necessary to assess the number of viable seeds in a collection and to set a baseline against which the quality of the collection can be compared through time.

Before assessing seed quality, it is essential to determine whether the collection comprises seeds or (**indehiscent**) fruits. Some fruits open to release the seeds (dehiscent fruit), such as *Acacia, Eucalyptus* and *Grevillea*, and hence, seeds are stored. For other species, fruits don't open to release the seeds (indehiscent), and the fruits represent the material stored. This fruit may contain one seed (e.g., Apiaceae, most Asteraceae, Poaceae) or many seeds (e.g., many genera of Ericaceae). The type of **diaspore** (i.e., seed or fruit) will determine how quality is measured.

5.5.2 Seed quantification

The first step in assessing seed quantity is to determine the number of diaspores in a collection. If a collection is small e.g., < 1,000 diaspores, then this may be done by hand. For larger collections, the number of diaspores in a collection can be estimated by weight. In some instances, (e.g. some species of *Melaleuca*), the seeds may be indistinguishable from non-seed material. In these instances, the non-seed material will be quantified as if it is a seed, with the exact proportion being accounted for during follow up seed fill and viability tests. The procedure to quantify a collection is described in Box 5.7.

5.5.3 Seed-fill

After a collection has been quantified, the next step is to determine seed fill, that is how many seeds contain an **embryo**/**endosperm** within a random sample of diaspores. Some families are frequently affected by **predation** (e.g., Fabaceae) or tend to have low seed-fill (e.g., some Rutaceae) (e.g., Martyn *et al*. 2009). Seed fill can also be lower than expected due to production of diaspore mimics (for example, *Callitris* and *Casuarina* species), predation, or environmental stresses during seed development.

There are two main methods for determining seed fill: a cut test and an x-ray.

5.5.3.1 Cut test

A cut test is a simple and inexpensive test that involves cutting diaspores in half to check for the presence of a filled seed, or seeds in the case of multi-seeded indehiscent fruits. As well as determining the presence of seed, a subjective assessment of seed viability can be made to distinguish between obviously dead seed and potentially viable seed. Generally, a potentially viable seed is firm, fresh and healthy in appearance with an embryo (and endosperm if present) that are white to ivory in colour, sometimes ranging to yellow. A green embryo may be an indication of immaturity, but some mature and viable seeds have green embryos (Wright *et al*. 2000). Alternatively, a green embryo may be an indicator of desiccation sensitivity (refer Chapter 6). Disadvantages of the cut test are the labour intensive and destructive nature of the test (i.e., the seed is killed in the process).

5.5.3.2 X-ray imaging

The use of an x-ray machine to image seeds is a quick method by which seed fill can be determined, with accuracy dependent on seed size and structure. This is a non-destructive technique, meaning that seeds can be used for other purposes after this assessment, though it should be noted as a 'pre-treatment' if seeds are subsequently stored or tested for germination. The photographic record can also be saved for future reference. A disadvantage of the x-ray test is the high cost of the equipment and a diminished ability to determine seed maturity (e.g., based on embryo colour). Assessing seed fill in very small seeds, or those within certain fruits or with deeply textured seed coats, can also be difficult through x-ray analysis. In such cases a cut test may be useful to aid interpretation of the x-ray image.

5.5.3.3 Seed fill calculation

Seed fill can be calculated as follows:

$$Seed\ fill\ (\%) = \frac{Number\ of\ filled\ seeds}{Number\ of\ diaspores\ in\ sample} \times 100$$

If the diaspore is a fruit, seed fill will be the number of apparently viable seeds contained within the fruit and may be a number > 1. In this case the average number of viable seeds per fruit may also be calculated and recorded. When the diaspore is a seed, seed fill will be a number ≤ 1.

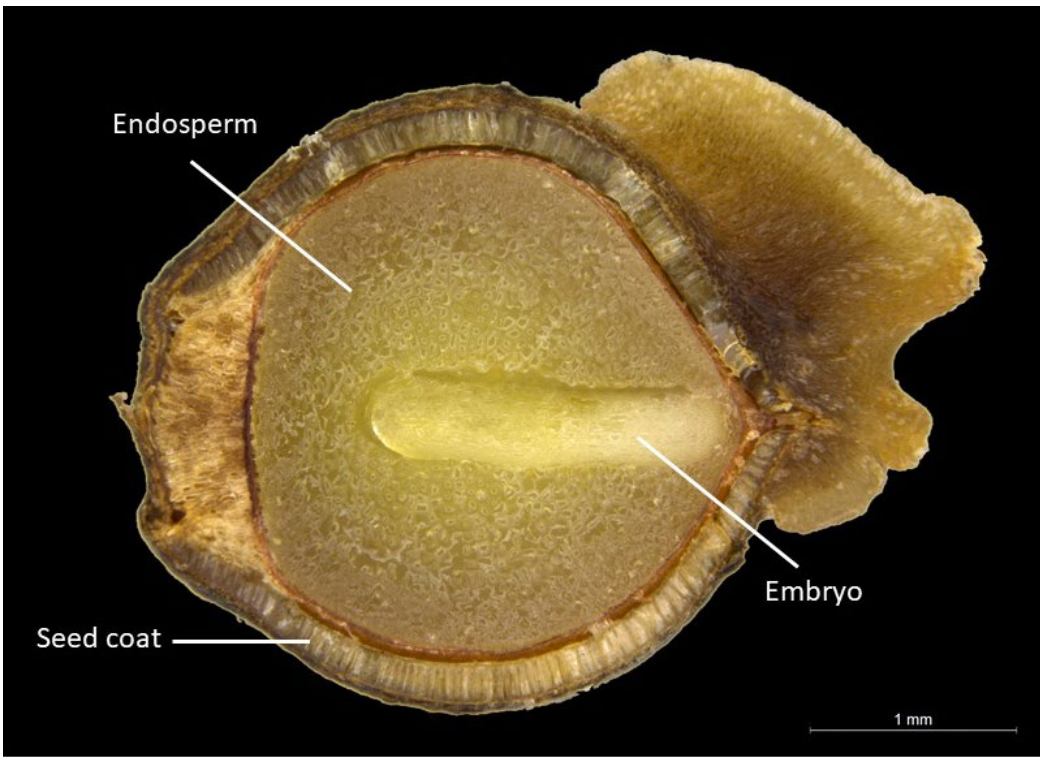


Figure 5.5: Cut test of a *Hybanthus floribundus* seed, showing a firm, white embryo and endosperm surrounded by a thick seed coat. (Image: David Symons)

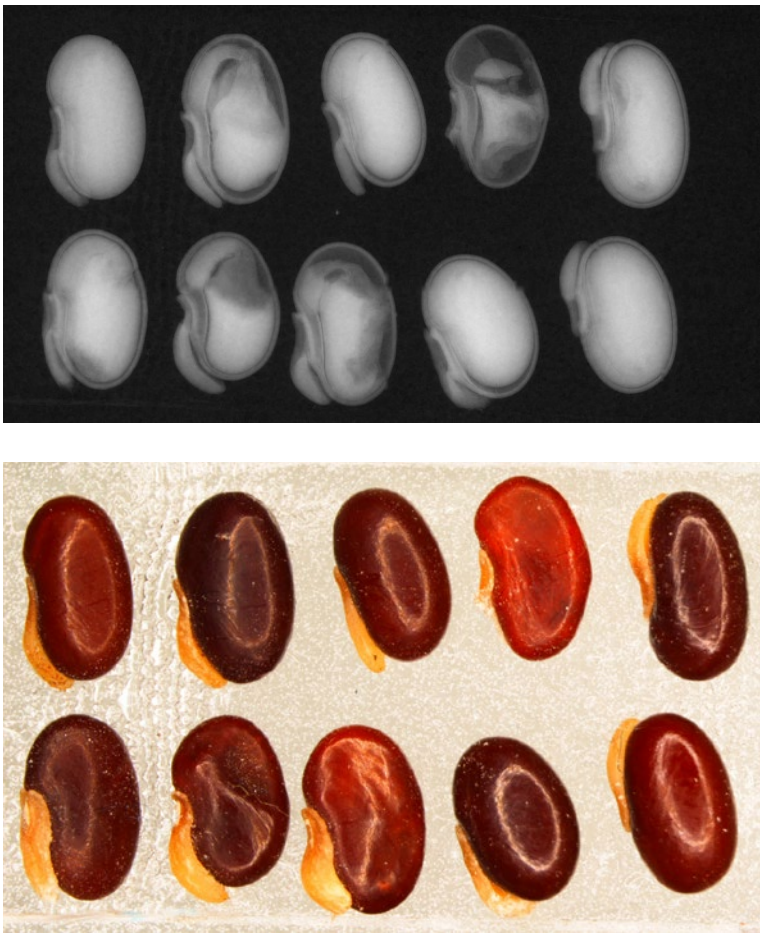


Figure 5.6: *Daviesia cordata* seeds (top) and x-ray image (bottom) to determine seed fill. The x-ray image shows four seeds are clearly non-viable, and two (top right and bottom left) with minor damage. (Images: D. Merritt)

5.5.4 Seed viability

Even if seeds are filled, it is important to determine whether they are also alive (viable). This can be difficult in practice, as living seeds can often look the same as dead seeds. Ideally, conservation collections should have a high viability when initially placed in the seed bank and this viability should be monitored during storage (Section 5.7.2). Seed viability can be affected by a range of factors including environmental conditions during maturation on the parent plant, seed maturity at collection, and post-harvest cleaning and storage conditions (Sections 5.3 and 5.4). Every effort should be made to maximise seed viability by following collecting guidelines and handling seeds carefully after harvest. But it should also be noted that initial seed viability can be inherently low for some wild species regardless of careful handling practices; reinforcing the need to quantify initial viability to inform future use.

5.5.4.1 Germination

The most accurate test of viability is the germination test, as seeds that germinate are clearly alive. This works well if the conditions required for germination are known. A germination test has the added benefit of producing plants which may then be used for research or translocation. However, if the optimal germination test conditions are not known, or if the seeds are dormant, there may be a proportion of viable seeds that do not germinate. This can lead to a significant underestimate of viability. To address this, a destructive cut test (5.5.3.1) or a viability stain (5.5.4.2) should be conducted at the conclusion of a germination test to provide data on the proportion of dormant versus dead seeds. If the germination test is expected to last for several months or more, a small sample of seeds may be used for viability staining at the beginning of an experiment and compared to the viability staining of seeds at the end of the experiment, to account for loss of viability during the experiment. For more information on germination and dormancy, see Chapter 7.

5.5.4.2 Viability staining

Biochemical tests are available that can be used to assess seed viability. The most frequently used test is a **tetrazolium (TZ or TTC) test**. This test involves soaking seeds in a 0.5 to 2 % (w/v) solution of a tetrazolium salt (*2,3,5-Triphenyltetrazolium Chloride*). After the seeds have been imbibed for a suitable length of time (usually 12–36 hours) viable tissue stains red, while dead tissues stain a pale pink colour, or remain unstained. Whilst the TZ test can provide a rapid assessment of viability compared to the germination test, there can be issues of applying the test outside of crop species. Many seed preparation techniques are used, including piercing of the seed coat to allow penetration of the stain, cutting the seed longitudinally or transversely to expose the embryonic tissue, or removing the seed coat or excising the embryo entirely (ISTA 2021). Seed preparation and staining time may differ markedly between wild species and crop or ornamental species (Miller 2005; ISTA 2021). Standardised tests have been developed for many crop species (ISTA 2021) but standard methods for testing Australian species have not been widely developed and experimentation is likely required for protocol development for the species of interest as interpretation can be subjective and sometimes misleading. For example, fungal infection of seeds can lead to false red staining, as the fungal mycelium is also alive (Miller 2005). If biochemical tests are to be used for testing a species, steps should be taken to correlate final germination and viability indicated by staining (e.g., Ooi *et al*. 2004) to address these issues. Examples of tetrazolium testing for Australian species are provided by Gravina and Bellairs (1999) and Thompson *et al*. (2001) and Commander *et al*. (2021) provides further useful information on TZ testing for Australian species. This method is also destructive.

5.5.4.3 Embryo culture

Another test to determine seed viability is to extract **zygotic embryos** from seeds and culture in vitro under sterile conditions (Chapter 9). In many cases, seed embryos will grow rapidly once removed from the seed, even if the seed does not germinate. This method provides an effective way to assess seed viability (based on the growth and development of the embryo), mostly without the complications of seed dormancy or other factors. However, while this technique is very effective, it also requires a high degree of technical skill, sufficient resources and time, as extracting embryos can be a slow and laborious process and should only be considered as a last resort or when the seeds are rare, unusual or from a threatened species. The medium used for culturing extracted embryos can be as simple as water agar (effective for developed embryos e.g. those of *Eucalyptus* or *Acacia* species) or more complex, such as Murashige and Skoog medium (Murashige and Skoog 1962), with added vitamins, sugars and plant growth regulators (Chapter 9). Prior to embryo-extraction, seeds need to be surface-sterilised and the embryos are then gently removed without damage under sterile conditions. This method has the advantage of potentially producing plants for research or translocation, following a similar process to tissue culture (see Chapter 9).

5.5.4.4 Reporting viability results

When reporting results of a viability test, it is important to do so in a consistent manner. The results reported should be relevant to the diaspore type, and the quality of the batch. If inert non-seed material has been stored and included in the quantification of the original collection, then all "seed units" need to be accounted for in the viability assessment. If an x-ray machine is used, the viability test can use only filled and apparently viable seeds, otherwise the viability test may assess both filled seeds and inert non-seed material (e.g., empty seeds, or chaff or frass if indistinguishable from seeds). Two calculations may therefore be produced from a viability test: the viability of the sample, and the seed viability.

1. $$Sample\ viability\ = \frac{Number\ of\ viable\ seeds}{Number\ of\ 'seed\ units'}$$

2. $$Seed\ viability = \frac{Number\ of\ viable\ seeds}{Number\ of\ apparently\ viable\ seeds}$$

If a germination test has been used the term 'viability' can be replaced by 'germinability' in the above metrics. Germinability is arguably a more useful measure of the utility of a seed collection as it gives an indication of how many germinants could potentially be produced from a given amount of seeds. This can be useful when determining seeding rates.

It should be recognised that there is no agreed standard on how wild seed quality is reported. Unlike most crop groups, non-crop species have a larger diversity of storable units which means a consistent reporting system across collections needs to be carefully considered. The seed practitioner also needs to ensure that the viability results reflect the nature of the collection being examined.

Box 5.7: Calculation of seed purity and the total number of seeds in a seed batch

1. Weigh the entire collection batch.
2. Remove a small sub-sample of c. 1–5 g and weigh.
3. Separate the seeds from the non-seed debris in the sub-sample and count the seeds.
4. Weigh all the seeds in the sub-sample.

From the recorded data the following can be now determined:

1. $$Seed\ purity\ of\ batch\ (\%) = \left(\frac{Weight\ of\ pure\ seeds\ in\ subsample}{Weight\ of\ subsample}\right) \text{x } 100$$
2. $$Weight\ of\ seeds\ in\ batch = \left(\frac{\%\ Seed\ purity}{100}\right) \text{x } Total\ weight\ of\ batch$$
3. $$Individual\ seed\ weight = \frac{Weight\ of\ pure\ seeds\ in\ subsample}{Total\ number\ of\ seeds\ in\ subsample}$$
4. $$Total\ number\ of\ seeds\ in\ seed\ batch = \frac{Weight\ of\ seeds\ in\ batch}{Individual\ seed\ weight}$$

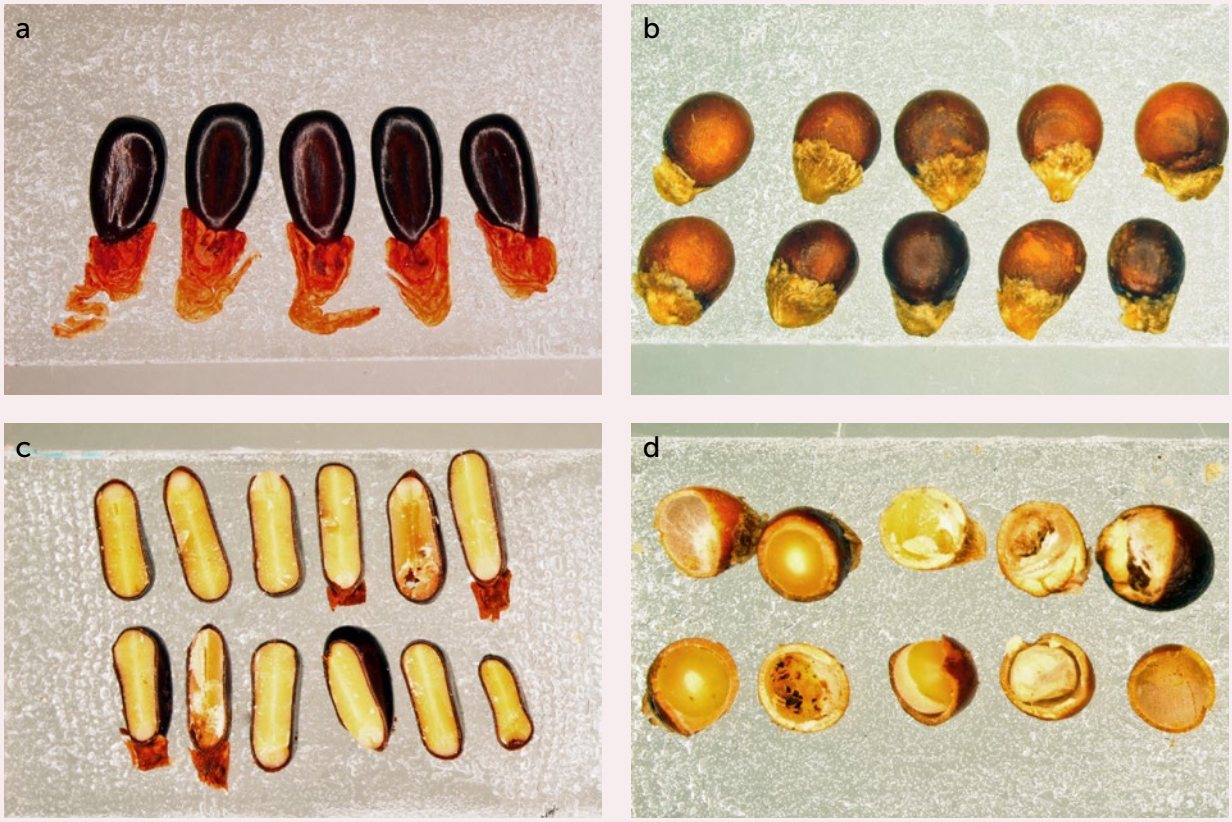


Figure 5.7: Comparison of cut test results from two different seed lots showing intact seeds of *Acacia cowleana* **(a)** and *Hibbertia commutata* **(b)** and cut tests. For *A. cowleana* **(c)** all seeds are considered potentially viable. For *H. commutata* **(d)** only two seeds are considered potentially viable; the remainder are empty or contain shrivelled or damaged tissues. (Images: D. Merritt).

5.6 Data management

Conservation collections have three equally important key components: the germplasm material (seed or vegetative); a correct identification, usually through an herbarium **voucher specimen**; the collection data (see Chapter 15, section 15.2).

There are various standards for data management. The Millennium Seed Bank Conservation Standards, for example, include a section on data management, but as a minimum a data management system should be capable of managing and exporting and exchanging the following;

- Field data relating to the original collection.
- Herbarium specimen location.
- Initial seed quantification, quality and viability/germination assessment, including baseline data for future reference.
- Pre-storage handling procedures including cleaning.
- Propagation treatments including success/failure rates.
- Storage conditions.
- Distribution of material.
- Re-testing data of viability/germination.

For more information, see Chapter 15.

5.7 Management of stored collections

Once viable collections of seeds are stored with accompanying collection, testing and storage information, their longevity needs to be monitored by periodic assessments of viability (5.7.2) and possibly seed vigour (5.7.3). Storage environments need to be monitored to ensure that containers maintain seeds at the correct moisture content and fridges or freezers maintain the correct temperature (5.4). Seeds may also be distributed for various purposes (5.7.5), and particular caution should be taken with the small collections that are often present in conservation seed banks (5.7.4).

5.7.1 Seed longevity

Orthodox seed longevity increases in a predictable manner with a reduction in temperature and humidity (seed moisture content) and seeds of many species are predicted to survive for decades, or longer, under appropriate cold storage conditions. Storing seeds under optimal conditions significantly reduces the aging process, but deterioration still occurs, and all seeds eventually die. Aging is a very complex biological process that includes a cascade of events, many of which are discrete in the dry state, and which transition seeds from being alive to dead (Walters 1998). It is well known that **lipid peroxidation** and **free radicals** are major contributors to seed aging, and research has focussed on identifying cellular and chemical changes that occur within deteriorated seeds – however the temporal pattern from the origin of deterioration to death is still unknown. To date, germination tests are the most used method to assess seed viability. **DNA** markers and QTL mapping have aided the identification of genomic regions which could potentially control longevity and may be future tools to show interspecific variability in longevity (Fu *et al*. 2015).

Temperature and seed moisture content during storage have a profound effect on the longevity of seeds (Figure 5.8A) and must be carefully controlled and monitored to prevent rapid deterioration

and unnecessary loss of seeds. There are well-established models describing the effects of temperature and seed moisture content on the longevity of orthodox seeds (Ellis and Roberts 1980). These models contribute to the basis of internationally accepted storage practices (FAO 2014), as well as to our understanding of the underlying physiological phenomena influencing seed aging. But the maximum longevity of orthodox seeds stored under conservation storage conditions depends on many factors and is a topic of ongoing research. **Genotype**, and pre-storage factors including the environmental conditions during seed development, the maturity of seeds at the point of collection, and post-harvest handling, all affect longevity (see Case Study 5.1). Whilst there are few long-term studies completed for Australian species examining the longevity of seeds stored under conditions appropriate to conservation storage, data exists to demonstrate seeds of many Australian Fabaceae, Myrtaceae and Proteaceae can be stored at 5–10 % moisture content and 5 °C for up to 10 years (Gunn 2001; Offord *et al*. 2004), and many other Australian species can survive for at least 12 years at about 5 % moisture content and -20 °C (Crawford *et al*. 2007).

Studies that present seed viability data over multi-decadal timescales under cold storage (Walters *et al*. 2005; Chau *et al*. 2019; Liu *et al*. 2020) are very valuable. But they are relatively scarce due the difficulties in studying seed longevity under genebank storage conditions because of the long timeframes required. As an alternative, a widely used technique to study longevity in a reasonable timeframe is to rapidly age seeds of diverse species in a common, experimental storage condition of high temperature and seed moisture content (e.g., 45 °C or 60 °C, and 60 % RH). The resultant seed viability data generated can be used to compare the relative longevity of seeds of different collections or species (Probert *et al*. 2009). This rapid aging approach is useful for the relative ranking of seed longevity between species (Table 5.3) and can assist in identifying potentially short- and long-lived seeds to inform seed bank management (e.g., to inform viability testing schedules). But it should be noted that predicting from rapid aging data the actual longevity of seeds stored under genebanking conditions cannot be done with a high degree of certainty due to the fundamentally different physiological processes that occur in seeds under these vastly different storage conditions (Walters *et al*. 2004).

Across both long-duration (i.e., storage under genebanking conditions) or shorter-duration (i.e. storage under rapid aging conditions) studies of seed aging, a common technique to model seed viability data is to calculate the time for seed viability to fall to 50 % ($p_{50;}$ Fig. 5.7B; Box 5.8). For Australian species, rapid aging experiments have identified relatively long-lived seeds within species of the Casuarinaceae, Fabaceae, Myrtaceae, Rhamnaceae, and Sapindaceae, and relatively short-lived seeds within species of Asteraceae, Brassicaceae, Poaceae, and Orchidaceae (Hay *et al*. 2010; Merritt *et al*. 2014; Erickson *et al*. 2017). Note, however, that variation in seed longevity between species can be vast (spanning four orders of magnitude in rapid aging experiments) and there are examples of plant families (e.g., Fabaceae, Proteaceae) containing some species that produce relatively long-lived seeds, and some species that produce relatively short-lived seeds (Merritt *et al*. 2014; Table 5.3). Based upon data from rapid aging studies, it has been proposed that seeds with p_{50} values (at 45 °C and 60 % RH) < 10 days could be considered short-lived, and species with p_{50} values > 100 days long-lived (Mondoni *et al*. 2011).

Rapid aging data can be used to examine species characteristics, as well as seed, plant, and environmental traits that may be associated with longevity. Such studies have identified correlates of seed longevity including seed mass, composition, relative embryo size, taxonomy, and climate (Walters *et al*. 2005; Probert *et al*. 2009; Nagel and Borner 2010; Mondoni *et al*. 2011; Merritt *et al*. 2014). Although these correlations are not supported by all studies, there has been a significant advance in understanding the influence of taxonomy and climate. For example, Walters *et al*. (2005) proposed the seeds from some families were inherently short-lived (e.g., Apiaceae,

Brassicaceae) and others long-lived (e.g., Chenopodiaceae, Malvaceae). Furthermore, species originating from cool, temperate climates (Walters *et al*. 2005) or cool, moist environments (particularly those with small embryos) tend to produce shorter-lived seeds (Probert *et al*. 2009). Species from warm and arid climates (Walters *et al*. 2005; Erickson *et al*. 2017), particularly non-endospermic seeds (Probert *et al*. 2009) and seeds of **serotinous** species (Merritt *et al*. 2014), commonly produce long-lived seeds. Seeds from alpine environments (Table 5.4) commonly produce shorter-lived seeds (Mondoni *et al*. 2011; Satyanti *et al*. 2018). Mondoni *et al*. (2011) also presented a highly significant relationship between longevity and mean annual temperature and rainfall with seeds from cooler, wetter climates having shorter lifespans.

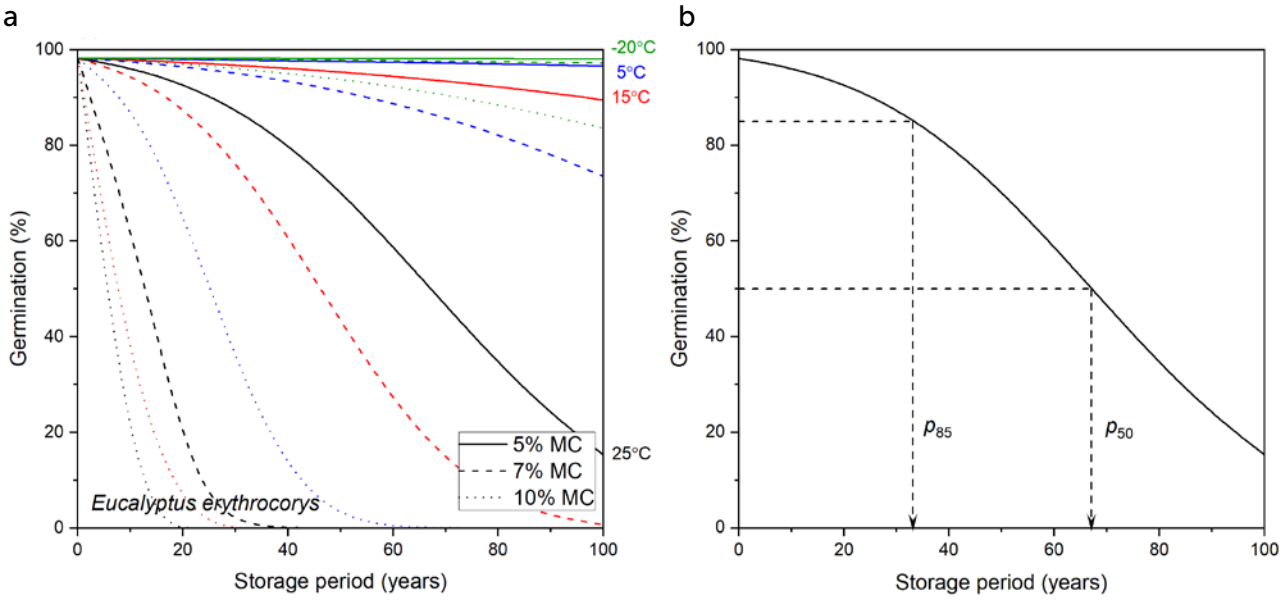


Figure 5.8: (a) Predicted survival curves for seeds of *Eucalyptus erythrocorys* stored at different moisture contents (5, 7 and 10 % fresh weight) and constant temperatures (-20, 5, 15 and 25 °C). **(b)** Seed survival curve with dashed lines indicating the time for viability to decline to 85 % (p_{85}) and 50 % (p_{50}). The predicted survival curves are based on calculations using the seed viability constants from Crawford *et al*. (2013) in the Seed Information Database (Royal Botanic Gardens Kew 2021).

Table 5.3: Examples of the longevity (p_{50}) of seed collections of selected Australian genera stored under rapid aging conditions of elevated temperature and relative humidity.

Genus	Family (-aceae)	p_{50} (days)	No. species tested	Climatic zone	Plant life-form	Refs*
Acacia	Fab-	49.5–588.6	29	Various	Shrub/tree	5, 6
Allocasuarina	Casuarin-	110.4–351.2	5	Temperate	Shrub/tree	2, 5
Angophora	Myrt-	64.2–289.7	2	Temperate	Tree	5
Atriplex	Chenopodi-	17.4–116.1	5	Temperate or grassland	Shrub/forb	2, 5
Banksia	Prote-	183.6–250.4	4	Temperate	Shrub/tree	5
Brachyscome	Aster-	18.7–32.6	6	Various	Herb/forb	5, 7
Caladenia	Orchid-	0.5–13.6	3	Temperate	Terrestrial orchid	4
Callitris	Cupress-	3.0–79.0	3	Temperate	Shrub/tree	5
Calothamnus	Myrt-	256.3–771.0	4	Temperate	Shrub	2

Genus	Family (-aceae)	p_{50} (days)	No. species tested	Climatic zone	Plant life-form	Refs*
Chloris	Po-	42.2–43.9	2	Temperate	Grass	5
Corymbia	Myrt-	58.1–394.9	6	Various	Tree	5
Dodonaea	Sapind-	56.4–365.0	6	Temperate or grassland	Shrub/tree	5
Epacris	Eric-	22.4–81.6	2	Alpine	Shrub	7
Erigeron	Aster-	10.9–20.6	3	Alpine	Herb	7
Eucalyptus	Myrt-	41.4–364.0	12	Various	Tree	2, 5, 6
Ficus	Mor-	27.8–45.0	2	Rainforest	Tree	8
Grevillea	Prote-	14.7–93.2	4	Various	Shrub/tree	5, 6
Hakea	Prote-	14.9–181.8	7	Various	Shrub/tree	2, 5, 6
Juncus	Junc-	21.7–79.0	5	Temperate	Rush/herb/ sedge	5
Melaleuca	Myrt-	159.3–420.0	4	Temperate or rainforest	Tree/shrub	2,8
Myriophyllum	Halorag-	7.0–7.2	2	Vernal pool	Aquatic	3
Parsonsia	Apocyn-	1.5–2.8	2	Rainforest	Vine/ climber	8
Plantago	Plantagin-	6.8–14.1	3	Various	Annual/ perennial herb	1,5
Poa	Po-	3.8–71.2	3	Alpine	Grass	7
Pterostylis	Orchid-	5.5–9.5	2	Temperate	Terrestrial orchid	4
Ptilotus	Amaranth-	30.0–69.0	3	Arid	Herb	6
Ranunculus	Ranuncul-	4.2–18.0	6	Alpine	Herb	7
Solanum	Solan-	19.0–97.4	4	Subtropical rainforest or grassland	Shrub	2, 5, 8
Stylidium	Stylidi-	22.8–57.0	3	Temperate or arid	Herb	5, 6
Thelymitra	Orchid-	10.3–17.1	2	Temperate	Terrestrial orchid	4
Trithuria	Hydatell-	24.2–44.3	2	Vernal pool	Aquatic	3
Wahlenbergia	Campanul-	9.8–48.0	6	Various	Annual/ perennial herb	1, 6, 7
Wurmbea	Colchic-	32.3–53.5	2	Temperate	Herb	5
Xanthorrhoea	Xanthorrhoe-	61.1–65.7	2	Temperate	Shrub/tree	5

*References: (1) Kochanek *et al*. 2009; (2) Probert *et al*. 2009; (3) Tuckett *et al*. 2010; (4) Hay *et al*. 2010; (5) Merritt *et al*. 2014; (6) Erickson *et al*. 2017; (7) Satyanti *et al*. 2018; (8) Sommerville unpublished.

Table 5.4: Comparison of seed longevity (expressed as p_{50}) between alpine and non-alpine Australian species indicates that alpine seeds are shorter lived than their non-alpine relatives. Number of species tested for each family is indicated in brackets. Data was summarised from Satyanti *et al*. (2018) and Merritt *et al*. (2014).

Family	p_{50} (days)	
	Alpine	Non-alpine
Asteraceae	20.8 (14)	32.4 (20)
Brassicaceae	4.1 (1)	10.1 (2)
Caryophyllaceae	28.0 (2)	43.7 (1)
Cyperaceae	25.2 (5)	87.0 (3)
Ericaceae	37.9 (3)	60.0 (2)
Juncaceae	19.9 (2)	42.6 (5)
Myrtaceae	36.2 (2)	176.0 (19)
Poaceae	21.4 (8)	30.6 (5)
Ranunculaceae	10.2 (7)	8.0 (1)
Stylidiaceae	15.7 (1)	28.4 (3)

5.7.1.1 Longevity testing to assess seed quality

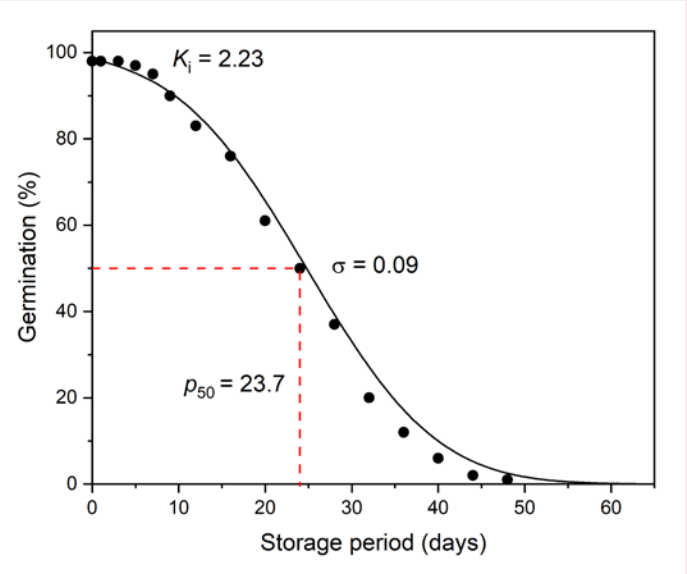
Longevity can differ substantially between different seed collections of the same species, even for crop species (Kochanek *et al.* 2009; Yamasaki *et al.* 2020). This is attributed in part to differences in the initial quality of different seed collections. Seeds need to be at their maximum quality when collected to maximise longevity (usually as close to the point of natural dispersal as possible; Chapter 4). Longevity is acquired during the latter stages of seed development, but the temporal pattern of seed quality development, including the timing of maximum quality, as well as how long it is maintained thereafter, is dependent on both genotype and the environment (i.e., temperature and humidity). This makes it very challenging for seed practitioners to assess the storage potential of incoming material using only a standard germination test (Hay and Whitehouse 2017). A more robust method for assessing the storage potential of a seed lot is to carry out an aging experiment in a similar manner as described for comparative longevity (Box 5.8). The decline in seed viability during storage is initially slow, with seeds showing a lag phase (i.e., a period of viability maintenance) before viability starts to decline rapidly. Seed lots that are closer to that "tipping point", and/or show a lower initial viability, will show poorer longevity compared with a seed lot that is still in the lag phase of high viability (Hay and Whitehouse 2017).

Box 5.8: Seed aging experiments

- *Assesses initial seed physiological quality (storage potential)*
- *Can be used to compare longevity between species/seed lots*

Method:

1. First elevate seed moisture content (MC) to a MC equivalent to 60 % RH (at the required storage temperature) by suspending seeds over a non-saturated lithium chloride (LiCl) salt solution within an airtight container at a cool temperature (e.g., 20 °C). Elevating MC at a cool temperature prevents seed viability loss prior to commencement of the aging experiment.
2. Once seed MC is elevated, store the seeds at a high temperature (e.g., 45 °C or 60 °C, depending on the anticipated lifespan) within an airtight container over a non-saturated LiCl solution providing an RH of 60 % at the storage temperature.
3. Remove seeds at regular intervals to test their ability to germinate
   - If longevity is unknown, adhere to the Kew protocol* (1, 2, 5, 9, 20, 30, 50, 75, 100, and 125 days). Aim to capture the greatest number of data points during the period of declining germination.
4. Check the moisture content of the seeds (60 % RH) prior, during, and at the end of experimental storage either using a hygrometer or gravimetrically (high/low constant oven method, ISTA, 2021)
5. Plot the germination results against storage time
6. Analyse the data using probit analysis; fitting the Ellis and Roberts (1980) viability equation to give you *Ki* (initial viability), sigma (standard deviation of seed deaths over time) and p_{50}; which can either be read off the graph or calculated. The p_{50} values or sigma are used to compare the longevity between species or seed lots.



*References: Newton R, Hay F, Probert R (2014) Protocol for comparative seed longevity testing. Technical Information Sheet_01. (Millennium Seed Bank Partnership: Ardingly); ISTA (2021) International rules for seed testing. (International Seed Testing Association: Basserdorf)

5.7.2 Monitoring seed viability in storage

The regeneration standard for crop genebanks is 85 % of the initial viability, or lower for certain species such as wild species that show a low initial viability (FAO 2014). Seed viability monitoring is costly and so re-test schedules are ideally set based on the expected seed longevity, and in a manner that will not overly consume seeds through unnecessarily frequent testing (particularly for threatened species), but that will nevertheless identify the early onset of viability decline. Inherent seed longevity has been assessed through extensive modelling of longevity across a wide range of storage environments to develop species-specific seed viability equations (Ellis and Roberts 1980) for approximately 70 species (mostly agricultural crops). However, the extent of experimentation required to develop these viability equations requires large numbers of seeds, is resource-intensive, and quite difficult for seed banks conserving diverse and large numbers of seed collections from all kinds of plant species. Although this is the case for the majority of Australian taxa stored in wild-source seed banks, it is still important that the viability of seed collections is monitored during storage to ensure timely regeneration, and/or recollection of a species before germplasm is lost (Rao *et al*. 2006; see also Chapter 15).

An initial germination test, for each new collection, should be carried out as close to the acquisition date as possible. The collection should then be re-tested every 5 years if the seeds are expected to be short-lived, or every 10 years if the seeds are expected to be long-lived (FAO *et al.* 2014; see also Figure 15.3 for a flow diagram to assist with viability monitoring decisions). However, the frequency of testing will also depend on staff and resource availability, the number of seeds available for testing, and the seed storage conditions. Variation in germination before storage may be influenced by the length of time from harvest to testing including time spent in the drying room and in order to obtain a representative result, it is important that testing is carried out as close to storage as possible (Hay and Whitehouse 2017). Furthermore, its equally as important to ensure the germination test is carried out under optimum conditions (temperature, light), and to provide treatments that alleviate dormancy, if required, to allow seeds to reach their full germination potential, and to use consistent testing methods (e.g., dormancy breaking treatments and incubation conditions (Chapter 7: (Whitehouse *et al*. 2020). Using the same methods each time allows an accurate assessment of whether seed viability has been maintained or whether it has fallen since last testing. If viability is declining, seedlings may be regenerated from the original batch and used to multiply seed numbers, or plans made for timely re-collection (Chapter 15). For threatened species with low seed availability, consult with appropriate allied institutes or nurseries to ascertain if the germinants from viability monitoring can be grown on for planting out or retained as living collections.

5.7.3 Seed vigour

Seed vigour encompasses those seed properties which determine their potential for rapid, uniform germination and emergence and development of normal seedlings under a wide range of environmental conditions, including field conditions (ISTA 2021). Seed vigour includes a number of characteristics of seed performance including speed and uniformity of germination and seedling growth, germination and seedling emergence under unfavourable environmental conditions, and storage potential (ISTA 2021; Hay and Probert 2013).

Seed vigour testing can be used to identify and differentiate the quality of seed batches in a manner that is more sensitive than a germination test alone. Measures of seed vigour can identify a decline in seed quality earlier than standard germination testing. Because a major cause of differences in seed vigour is aging, a vigour test can provide a useful early indicator of seed

deterioration in storage (Whitehouse *et al*. 2020). Comparing time to germination in initial and subsequent tests can be one method of identifying seed accessions beginning to decline.

Many methods of seed vigour testing have been developed (Table 5.5), and new techniques continue to be explored (Fu *et al*. 2015). Direct tests of seed vigour include measures of germination and seedling performance under environmental stress, such as the speed and extent of germination under adverse conditions of temperature or water availability, or the evaluation of storage potential through determining the rate of viability decline under accelerated aging conditions (Box 5.8). Indirect tests of seed vigour include biochemical tests that correlate to germination performance, such as solute leakage, respiration rate, and identification of biomarkers of vigour through proteomics (Black *et al*. 2006; Fu *et al*. 2015; ISTA 2021).

Table 5.5: Common seed vigour tests and the properties of high and low vigour seed batches. Modified from Black *et al*. (2006).

Seed batch property	Method	Test outcome	
		High vigour	Low vigour
Germination speed and uniformity	Germination test at sub-optimal temperatures; low water potentials	Rapid and uniform germination	Slow and variable germination
Seedling size	Germination test under standard or sub-optimal conditions	Large and uniform; normal seedlings	Small and variable; normal or abnormal seedlings
Seedling emergence potential	Field emergence test	High in most soil conditions	Low in suboptimal soil conditions
Storage potential	Rapid aging at high temperature and RH	Good longevity	Poor longevity
Solute leakage	Electrical conductivity test	Low solute leakage	High solute leakage

5.7.4 Handling small collections

Storage of relatively small quantities of seeds is one feature that differentiates conservation seed banks, particularly for threatened species, from agricultural and restoration seed banks. Small collections (fewer than 1,000 seeds) may be stored for a variety of reasons. For example, the species in question may set limited seeds, even in good conditions, or drought or predation may reduce the amount of seeds available for collection. Collections over several years are recommended to accumulate a larger collection of seeds and improve the genetic representation within the collection for a particular species, especially if poor conditions have contributed to reduced seed availability. However, seeds collected in different seasons should be accessioned and stored separately. Once a small collection is banked, it may not be available for distribution or duplication at a second site. Curators should also use smaller sample sizes for germination testing and viability monitoring. If germination is problematic, research may focus on developing successful dormancy-breaking techniques for closely related analogue species before applying the most successful treatment to the small collection (Chapter 7).

5.7.5 Seed distribution and end-use

Seed samples from banked collections (usually active collections only) may be distributed to users. People using seed bank collections are generally involved in restoration of plant diversity, research, or plant breeding. Distribution of seed samples may depend on how the seeds are to be used, whether the collections can easily be replaced, and whether conventions or legislation govern the supply and use of seeds (for example, quarantine restrictions, collecting permit restrictions or threatened species legislation). See Chapters 2 and 15 for more details.

If seeds are to be distributed, the supplier needs to decide which accession(s) to send, if more than one is available. Suppliers should ensure that seeds arrive in good condition, shipped in sealed foil bags, for example, with care taken not to crush or over-heat the seeds. Depending on the reason for supply, the user may benefit from information on provenance (see Chapter 3), data on the most recent viability test, and germination protocols.

An agreement between the user and the seed provider stating the reason for distributing the seeds and any limitations on their use is usually necessary (Chapter 15). A copy of this agreement should be supplied with the seeds to be despatched. For example, many agricultural genebanks operate under the International Treaty for Plant Genetic Resources for Food and Agriculture, whereas distribution of most wild species, especially rare and threatened species, is covered by various state, territory and federal laws alongside international treaties such as CITES and the Nagoya Protocol (Chapter 15.4). Seeds are distributed under a Standard Material Transfer Agreement to ensure the fair and equitable sharing of germplasm, associated passport and collection data, and potential benefits arising from their use. Records of seed distribution must be kept stating the accession number, date and quantity of seeds supplied, details of the user (name, address and organisation), copies of any agreements made and permits required such as a phytosanitary certificate and export permit number. These records also serve to ensure a minimum number of seeds are retained at the donor bank, and/or provide a trigger for recollection or regeneration activities. The agreement may also require the user to provide feedback to the seed provider, in the form of data gathered during the utilisation of seeds.

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Chapter 6
Identifying and conserving non-orthodox seeds

Karen D. Sommerville, Graeme Errington, Bryn Funnekotter, Zoe-Joy Newby

6.1 Introduction

In practical terms, **non-orthodox seeds** are those that cannot be stored under standard seed banking conditions (Chapter 5). This includes seeds that do not tolerate drying, seeds that tolerate some drying but not to low **moisture content**, and seeds that tolerate drying but not freezing. Non-orthodox seeds are most common in warm, wet **habitats** such as tropical and subtropical rainforests (Tweddle *et al*. 2003; Wyse and Dickie 2018) but are also found in cooler, drier and more seasonal habitats (Berjak and Pammenter 2008). Estimates of the global proportion of species with seeds that don't tolerate drying range from 5–10 % (Walters *et al*. 2013; Wyse and Dickie 2017) but the total proportion of non-orthodox seeds may be somewhat higher when other categories are included. An awareness of the type of storage behaviour that can be expected from different types of seeds is essential to understanding whether the seeds can be stored at all and, if so, how long they might survive in storage. In this chapter we describe the different types of non-orthodox behaviour, direct and indirect methods for identifying non-orthodox seeds, and alternative methods for conserving them.

6.2 Categories of non-orthodox seed storage behaviour

Seeds from different species exhibit a wide variety of responses to drying that range from extreme **desiccation tolerance** to extreme **desiccation sensitivity** (Berjak and Pammenter 2008; Walters 2015). They can also show a variety of responses to different storage temperatures, ranging from tolerance of freezing at -20 °C to sensitivity to temperatures below 10 °C (Hong and Ellis 1996). Broadly categorising those responses can be helpful to determining the most appropriate ways to store non-orthodox seeds from wild species, particularly when seed supply for in-depth research is limited.

6.2.1 Recalcitrant (desiccation sensitive) seeds

Recalcitrant seeds are very sensitive to desiccation. The term 'recalcitrant', in this sense, was first used by Roberts (1973) to distinguish these seeds from **orthodox** seeds. Recalcitrant seeds may tolerate a small amount of water loss, but they cannot tolerate sufficient drying to survive standard seed banking (i.e., storage at ~15 % RH and -18 to -20 °C). The moisture content below which further drying causes damage (the threshold water content) is above 20 % for these seeds compared to 3–7 % for orthodox seeds (Walters and Maschinski 2019). The water remaining in recalcitrant seeds after they've been dried to the threshold water content is sufficient to form damaging ice crystals during storage at sub-zero temperatures (Walters and Maschinski 2019).

Recalcitrant seeds have been identified in species from 297 genera and 84 families (Subbiah *et al*. 2019). They are most common among species of tropical and subtropical rainforest but also occur in species from drier tropical habitats and temperate forests and woodlands (Tweddle *et al*. 2003). In Australia, the majority of recalcitrant species identified to date grow in tropical or subtropical rainforests (see Case Studies 6.1 and 6.2). Well-known recalcitrant species include tropical **fruit** crops such as avocado and mango. Australian native species in this group include *Araucaria bidwillii* (Bunya Pine), *Cryptocarya rigida* (Forest Maple), *Syzygium* spp. (Lilly Pillies), *Davidsonia pruriens* (Davidson's Plum) and *Diploglottis australis* (Native Tamarind) (Table 6.1; Hardstaff *et al*. 2020; Hamilton *et al*. 2013; Sommerville *et al*. 2021).

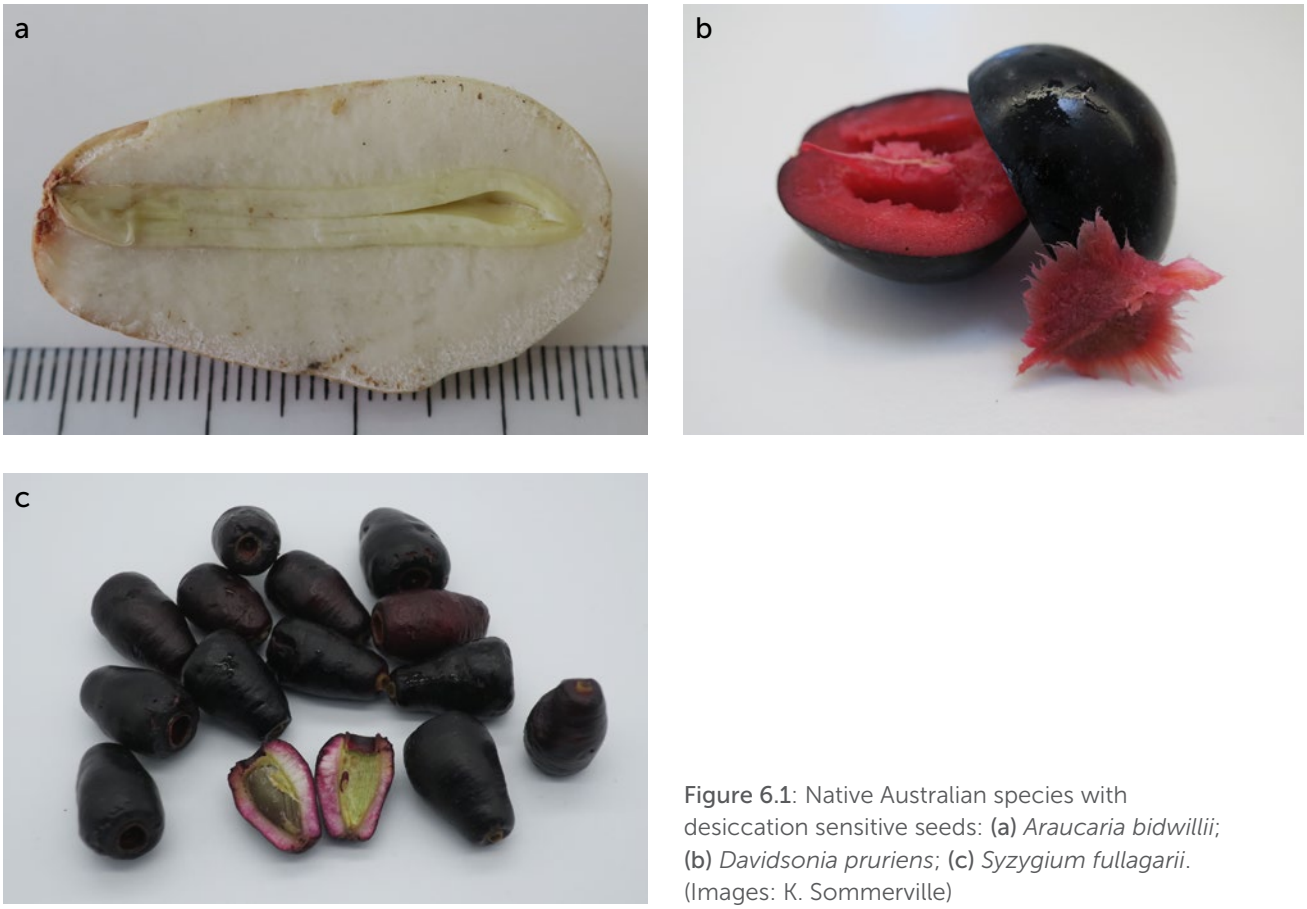

Figure 6.1: Native Australian species with desiccation sensitive seeds: **(a)** *Araucaria bidwillii*; **(b)** *Davidsonia pruriens*; **(c)** *Syzygium fullagarii*. (Images: K. Sommerville)

Table 6.1: A selection of Australian species identified as desiccation sensitive. Note that these species were sensitive to desiccation to the low moisture content (3–7 % MC) required for standard seed banking (or to 10 % MC for species studied by Thusitana *et al*. 2018). Most species have not been tested for tolerance of drying to higher moisture content so may be 'intermediate' rather than truly desiccation sensitive.

Family	Species	Source
Araceae	*Gymnostachys anceps*	1
	Pothos longipes	1
Arecaceae	*Archontophoenix alexandrae, A. cunninghamii*	2
	Linospadix monostachyos	1
	Livistona decora	2
Cunoniaceae	*Davidsonia pruriens*	1
Ebenaceae	*Diospyros australis, D. mabacea*	1
	Diospyros compacta	3
Elaeocarpaceae	*Elaeocarpus obovatus*	1
	Sloanea australis	1, 2
Fabaceae	*Archidendron hendersonii*	1
	Castanospermum australe	2
Flacourtiaceae	*Scolopia braunii*	2
Lauraceae	*Cinnamomum oliveri*	1
	Cryptocarya rigida	1
	Endiandra introrsa	1
	Endiandra pubens	2
	Neolitsea dealbata	1
Malvaceae	*Argyrodendron actinophyllum, A. trifoliolatum*	1
	Sterculia quadrifida	1, 3
Meliaceae	*Dysoxylum mollissimum, D. rufum*	1
	Synoum glandulosum	2
Menispermaceae	*Carronia multisepalea*	1
Monimiaceae	*Wilkiea huegeliana*	1
Myrtaceae	*Acmena ingens, A. smithii*	2
	Pilidiostigma glabrum	1
	Syzygium australe, S. fullagarii, S. moorei, S. paniculatum, S. pseudofastigiatum, S. wilsonii	2
	Syzygium unipunctatum	1
Opiliaceae	*Opilia amentacea*	3
Pennantiaceae	*Pennantia cunninghamii*	1
Piperaceae	*Piper hederaceum*	1, 2
Proteaceae	*Helicia ferruginea*	1
	Hicksbeachia pinnatifolia	1
Rubiaceae	*Atractocarpus chartaceus, A. fitzalanii, A. stipularis*	2

Family	Species	Source
Rutaceae	*Clausena smyrelliana*	1, 2
	Glycosmis trifoliata	2, 3
	Micromelum minutum	1, 2, 3
Sapindaceae	*Castanospora alphandii*	1
	Cupaniopsis flagelliformis	1
	Diploglottis australis	1
	Diploglottis campbellii	2
	Guioa coriacea	2
	Harpullia arborea, H. pendula, H. ramiflora	2
	Lepiderema pulchella	1
	Mischarytera lauteriana	1
	Rhysotoechia robertsonii	2
	Toechima dasyrrhache	2
Sapotaceae	*Planchonella australis*	1

[1] Sommerville *et al*. (2021); [2] Hamilton *et al*. (2013); [3] Thusitana *et al*. (2018)

6.2.2 Intermediate seeds

The term '**intermediate**' was introduced by Ellis *et al*. (1990) to describe species with storage behaviour neither truly recalcitrant nor truly orthodox. The 'intermediate' category includes species with a broad range of responses to desiccation and cold storage, but these may be grouped into two subcategories: desiccation-intermediate and freezing-intermediate (or temperature-intermediate). An early estimate of the incidence of intermediate behaviour suggested around 10–15 % of the world's **angiosperm** species may fall in this category (Dickie and Pritchard 2002). This proportion is likely to vary across different environments and has been found to be much higher, for example, for species from the tropical islands of Hawaii (Chau *et al*. 2019).

6.2.2.1 Desiccation-intermediate seeds

Desiccation-intermediate seeds do not survive drying to the low moisture content tolerated by orthodox seeds but can be dried considerably more than recalcitrant seeds. In general, these seeds may be dried to equilibrium with 40–50 % **relative humidity** (equivalent to around 7–12 % MC depending on species; Hong and Ellis 1996). The threshold water content is very variable among species and can only be determined by testing how well seeds germinate following drying to progressively lower moisture contents. Crop species in this category include *Coffea* spp. (Coffee) and *Carica papaya* (Papaya). Australian native species in the category include *Eidothea hardeniana* (Nightcap Oak), *Alectryon subdentatus* (Hard Alectryon) and *Meiogyne stenopetala* (Sommerville *et al*. 2021; Table 6.2).

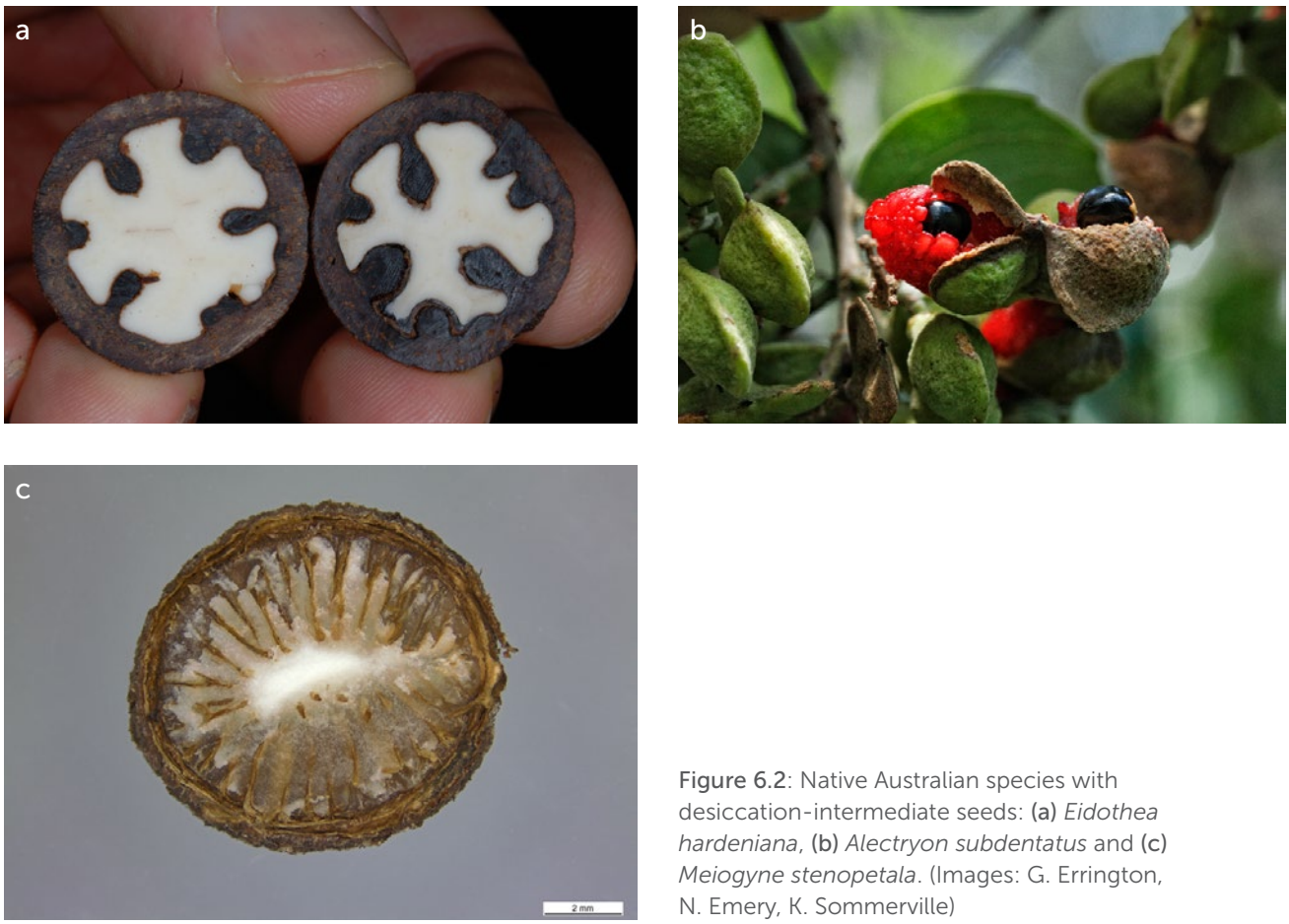

Figure 6.2: Native Australian species with desiccation-intermediate seeds: **(a)** *Eidothea hardeniana*, **(b)** *Alectryon subdentatus* and **(c)** *Meiogyne stenopetala*. (Images: G. Errington, N. Emery, K. Sommerville)

6.2.2.2 Freezing-intermediate seeds

Freezing-intermediate seeds survive drying to the low moisture content of orthodox seeds but do not survive freezing at -20 °C or begin to lose viability within 12 months of storage at that temperature (Hong and Ellis 1996). Chau *et al*. (2019) refer to these seeds as 'temperature-intermediate' as a poor response to cool storage can actually occur at temperatures between 10 and -30 °C (Walters 2015). Examples of Australian native species in this category include *Archirhodomyrtus beckleri* (Rose Myrtle), *Gmelina leichhardtii* (White Beech) and *Rhodomyrtus psidioides* (Native Guava; Sommerville *et al*. 2021) and *Nymphaea* species (Dalziell *et al*. 2019; Table 6.2).

Figure 6.3: Native Australian species with freezing-intermediate seeds: **(a)** *Archirhodomyrtus beckleri*, **(b)** *Gmelina leichhardtii* and **(c)** *Rhodomyrtus psidioides*. (Images: G. Errington, K. Sommerville)

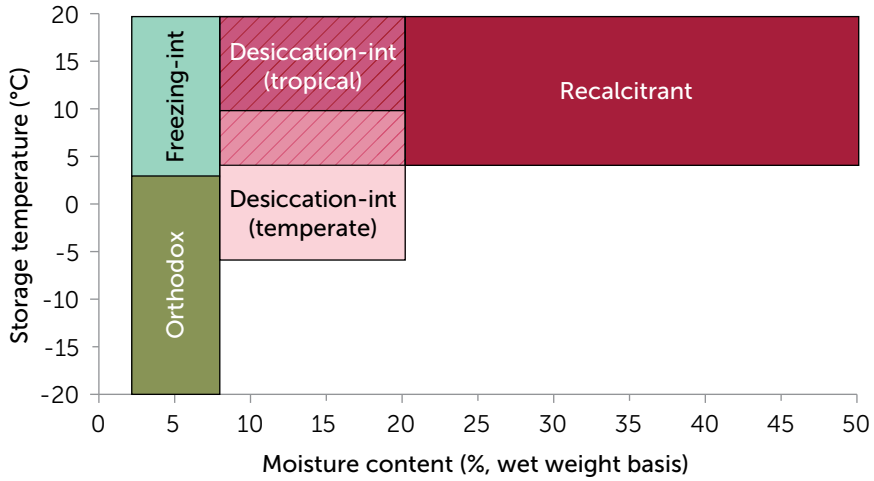

Figure 6.4: Simplified conceptual diagram illustrating the range of moisture contents to which recalcitrant, intermediate (-int) and orthodox seeds may be dried, and the range of temperatures at which the dried seeds may be stored. In practice, ranges may overlap between categories. Data derived from Hong and Ellis (1996).

6.2.2.3 Short-lived seeds

Seeds from a range of different plants – including orchids (Merritt *et al*. 2014a), alpine species (Satyanti *et al*. 2018), and species from a variety of other families and habitats (Merritt *et al*. 2014b) – are known to be comparatively short-lived in storage (Chapter 5). It has generally been assumed that these seeds are orthodox and simply short-lived; however, Walters (2015) included these as an additional category of intermediate storage behaviour. By definition, orthodox seeds should

have their **longevity** extended by storage at sub-zero temperatures compared to storage at temperatures above zero (Chapter 5). Testing the response to freezing for 12 months is therefore part of the process for determining whether a species has orthodox or intermediate storage behaviour; seeds that begin to lose viability within 12 months of storage at -20 °C generally fit the criteria for classification as intermediate (Hong and Ellis 1996; Section 6.3.1). Some seeds previously identified as short-lived, then, may actually be freezing-intermediate. In practice, both short-lived and freezing-intermediate seeds are likely to be best conserved by **cryopreservation** (see Sections 6.5.3 and 10.6.2).

Table 6.2: A selection of Australian native species identified as 'intermediate' in storage behaviour. These species may be desiccation-intermediate (DI, tolerant of some drying but sensitive to drying to 3–7 % MC) or freezing-intermediate (FI, tolerant of drying but sensitive to freezing at -20 °C). Species designated FI/SL (freezing-intermediate/short-lived) have been found to have reduced viability after storage for several years at -20 °C but have not been tested to determine whether freezer storage decreases longevity compared to storage at higher temperatures. Data derived from Dalziell *et al*. 2019 for Nymphaeaceae and Sommerville *et al*. (2021) for all other families.

Family	Species	Behaviour
Annonaceae	*Meiogyne stenopetala*	DI
Apocynaceae	*Melodinus australis*	FI
Asteliaceae	*Cordyline* sp. Mt Banda Banda	FI/SL
Asteraceae	*Vittadinia tenuissima*	FI/SL
Atherospermataceae	*Doryphora sassafras*	FI/SL
Cunoniaceae	*Ceratopetalum apetalum*	DI
Ebenaceae	*Diospyros fasciculosa*	DI
Euphorbiaceae	*Fontainea australis, F. oraria*	DI
Lamiaceae	*Callicarpa pedunculata*	FI/SL
	Clerodendrum floribundum	DI
	Gmelina leichhardtii	FI
Lauraceae	*Cryptocarya laevigata, C. microneura*	DI
Lomandraceae	*Lomandra spicata*	DI
Melastomataceae	*Melastoma affine*	FI/SL
Myrtaceae	*Archirhodomyrtus beckleri*	FI
	Rhodamnia maideniana	FI/SL
	Rhodomyrtus psidioides	FI
	Uromyrtus australis	FI/SL
Nymphaeaceae	*Nymphaea immutabilis*	FI/SL
	Nymphaea lukei	FI/SL
	Nymphaea macrosperma	FI/SL
Proteaceae	*Eidothea hardeniana*	DI
Rutaceae	*Acradenia euodiiformis*	FI/SL
Sapindaceae	*Alectryon subdentatus*	DI
	Jagera pseudorhus	DI
Simaroubaceae	*Quassia* sp. Mount Nardi	FI/SL

Case Study 6.1: Investigating seed storage options for *Syzygium* and *Lophomyrtus* threatened by Myrtle Rust in New Zealand

Karin van der Walt

Syzygium maire (Swamp Maire, Maire Tawake, Waiwaka) is the only native *Syzygium* species in New Zealand. This 16 m tall glabrous tree is **endemic** and mostly found in waterlogged ground or margins of streams in lowland and cloud forests. Similar to other *Syzygium* species, *S. maire* has fleshy berries which develop over 8–11 months, maturing during summer and autumn (Figure 6.5a). The endemic genus *Lophomyrtus* comprises two species; *L. bullata* (Ramarama) and *L. obcordata* (Rohutu). Both species can be a locally conspicuous component of the understory in coastal, lowland or montane forest and shrubland (Dawson and Lucas 2012). *L. bullata* fruits are dark red or black berries containing 10–14 seeds, maturing between late summer and autumn (Figure 6.5b). *L. obcordata* fruits mature between January and May and, similar to *L. bullata,* are bright or dark red berries containing 7–10 seeds (Figure 6.5c). All three species have been listed as Nationally Critical due to the predicted **population** decline associated with Myrtle Rust (de Lange *et al*. 2018). Although **ex situ conservation** strategies to prevent extinction of Myrtaceae species due to Myrtle Rust have been highlighted, limited information on seed development, effective **germination** and seed storage behaviour is hampering conservation efforts in New Zealand.

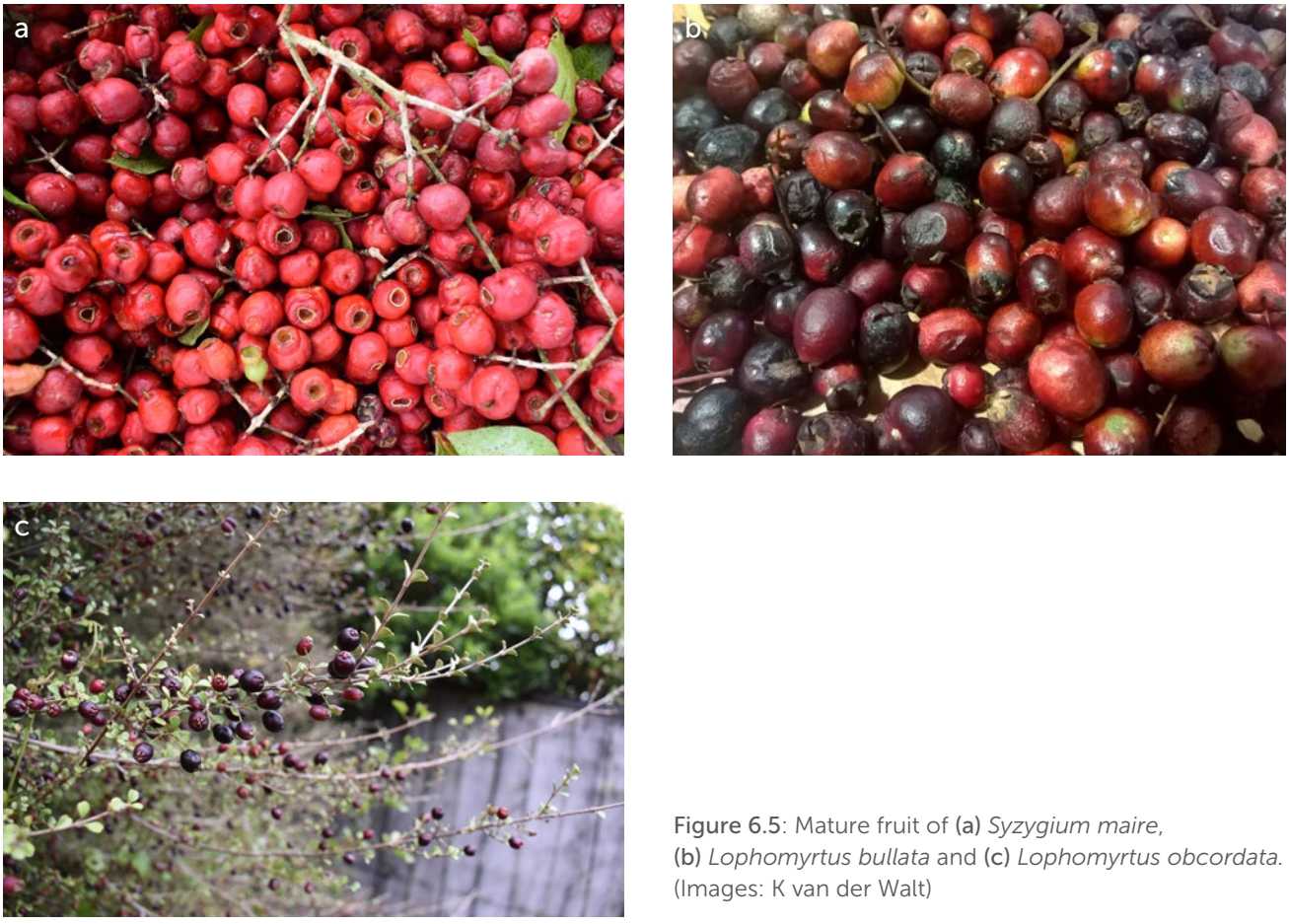

Figure 6.5: Mature fruit of **(a)** *Syzygium maire*, **(b)** *Lophomyrtus bullata* and **(c)** *Lophomyrtus obcordata.* (Images: K van der Walt)

S. maire seeds were screened for desiccation sensitivity by equilibrating seeds in three relative humidity chambers (15, 30 and 50 % RH) created using lithium chloride. Upon equilibration, seed viability was assessed through a germination test. To determine the role of storage temperature and substrate on *S. maire* seed longevity, freshly collected fruits (containing pulp and seeds) were mixed with two parts medium grade vermiculite and placed in airtight containers stored at 5 °C. Samples were randomly selected for viability tests at 4-week intervals and storage experiments were terminated after 12 weeks.

The seed storage behaviour of the two *Lophomyrtus* species was investigated by comparing viability of (a) fresh seed, (b) seed desiccated to 5–7 % seed moisture content (MC) and (c) desiccated seed stored for six months at 5 °C, -18 °C and -196 °C (in liquid nitrogen). Seed viability was assessed through a germination test conducted in an incubator at 15/25 °C with a 16 h dark 8 h light cycle. Viability of ungerminated seeds was assessed using a **tetrazolium (TZ) test** or a **cut test**. The MC of fresh and desiccated seeds was determined gravimetrically using the Rules of the International Seed Testing Association (ISTA 2021) by drying samples for 17 h at 103 °C.

Syzygium maire fruits are shed at high moisture content (80 %) with 98 % of the seeds germinating within seven to ten days. *S. maire* seeds did not survive desiccation to below 20 % MC and were therefore excluded from further storage experiments at sub-zero temperatures. *S. maire* fruits stored hydrated at 5 °C retained viability but started to germinate after eight weeks in storage. Moisture content of fresh *L. bullata* and *L. obcordata* seeds was 39.5 % and 40.6 % respectively while viability was 70.3 % (*L. bullata*) and 91.5 % (*L. obcordata*). Desiccating seeds to 5–7 % MC had no significant impact on viability of *L. obcordata* but resulted in more than 35 % viability loss in *L. bullata*. Storage of desiccated *Lophomyrtus* seeds at 5 °C, -18 °C and -196 °C resulted in further viability loss in *L. bullata* while *L. obcordata* seed showed no significant difference in viability of seeds stored for six months, irrespective of the temperature (Figure 6.6).

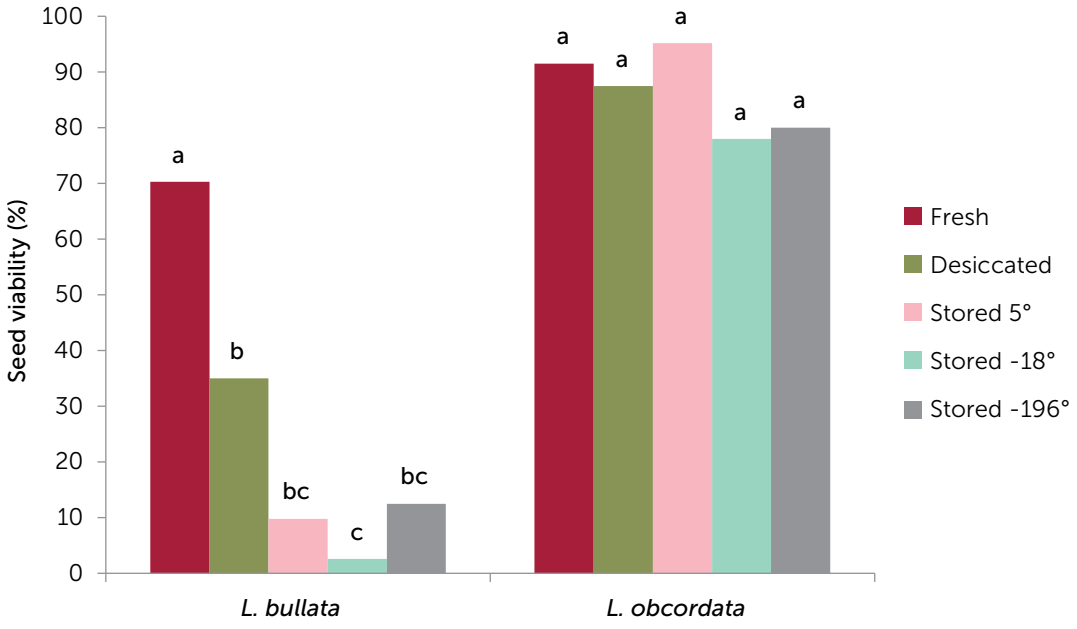

Figure 6.6: *Lophomyrtus bullata* and *Lophomyrtus obcordata* viability for fresh seed, desiccated seed, and seed stored for six months at 5 °C, -18 °C and -196 °C. Values in each graph with different letters are significantly different at $P < 0.05$ based on Kruskal Wallis test.

These results demonstrate that *S. maire* seeds are highly recalcitrant, but not cold sensitive, and can be stored fully hydrated for up to six weeks at 5 °C. However, cryopreservation is likely to be the only suitable long-term storage option for *S. maire* (van der Walt *et al*. 2020). Our study found that *L. bullata* seeds lost significant viability following desiccation; this contrasts with a study conducted by Nadarajan *et al*. (2020) which found that *L. bullata* was not sensitive to desiccation. **Provenance** and individual genetic traits can influence seed desiccation tolerance within species (Pereira *et al*. 2017) and this is possibly also the case for *L. bullata,* although further research is required. *L. obcordata* seeds retained viability after desiccation and storage at 5 °C, -18 °C and -196 °C, and it is therefore likely that standard seed banking is a suitable option for long term storage.

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Case Study 6.2: Non-orthodox seed storage behaviour of Australian *Nymphaea*

Emma Dalziell

Waterlilies (genus *Nymphaea*, family Nymphaeaceae) are spectacular aquatic flowering plants that occur across the wet-dry tropics of Northern Australia (Figure 6.7). While the majority of *Nymphaea* species are not currently listed as being of conservation concern, freshwater wetlands are increasingly threatened by the impacts of climate change, saltwater intrusion in coastal areas, agriculture and development (Dalziell 2016; Dalziell *et al.* 2020). Such changes to freshwater wetlands are predicted to detrimentally impact upon populations of *Nymphaea,* thus necessitating the potential need for ex situ conservation for these species.

In order to determine whether seed banking is a viable ex situ conservation option for seeds of *Nymphaea* we assessed:

1. Fresh seed moisture content and desiccation tolerance by drying to *ca.* 5 % internal seed moisture content (15 % RH) for 28 days.
2. Storage behaviour of seeds stored under a range of temperatures (25, 5, -20 and -190 °C and moisture contents (15, 30, 50, 70 and 95 % RH) for up to 12 months.

3. Comparative longevity using rapid aging at 60 % RH and 45 °C for seeds of *N. violacea*.

These experiments were conducted on multiple collections of seeds from four *Nymphaea* species: *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*.

Fresh seed moisture content was high across all species and ranged between 90–99 % RH and subsequent drying did not detrimentally affect seed viability. For seeds stored under a range of experimental storage conditions, seed viability tended to decrease with increasing RH, temperature and time in storage (Figure 6.8). However, the impact of storage temperature and moisture content on seed viability varied significantly between species and collections, and in some instances colder storage temperatures did not result in improved longevity (Figure 6.8). For seeds of *N. immutabilis, N. lukei, N. macrosperma* and two of four collections of *N. violacea*, seed viability declined significantly under all temperature and RH treatment combinations across the 12-month storage period, and storage at -196 °C did not improve longevity. For seeds from the two collections of *N. violacea* that retained high viability in the storage experiment, the comparative longevity experiment showed a 50 % reduction in seed viability (p_{50}) after 19.5–24.8 days at 60 % RH and 45 °C (Dalziel *et al*. 2019).

Most seeds from Australian *Nymphaea* do not display 'orthodox' storage behaviour, whereby drying to 3–7 % internal moisture content (MC) and storage below 0 °C results in a predictable increase in storage longevity. Rather, seeds display complex responses to storage temperature and MC and are generally considered short-lived. This type of storage response is becoming increasingly apparent globally for diverse, wild species as existing collections stored in conservation genebanks are being retested (e.g., Walters 2015; Chau *et al.* 2019; Colville and Pritchard 2019). For Australian *Nymphaea,* further refinements of cryostorage protocols will be required for successful ex situ seed storage. Alternatively, practitioners may consider the ex situ storage of bulbs, rhizomes or tubers.

Figure 6.7: **(a)** *Nymphaea violacea* and *macrosperma* in Marlgu Billabong, Parry Lagoons Nature Reserve, Western Australia, **(b)** colour variation in flowers of *N. violacea* from the Kimberley region in Western Australia, **(c)** cleaning seeds of *N. violacea* from the fruit. (Images a and b: Emma Dalziell, c: Todd Erickson)

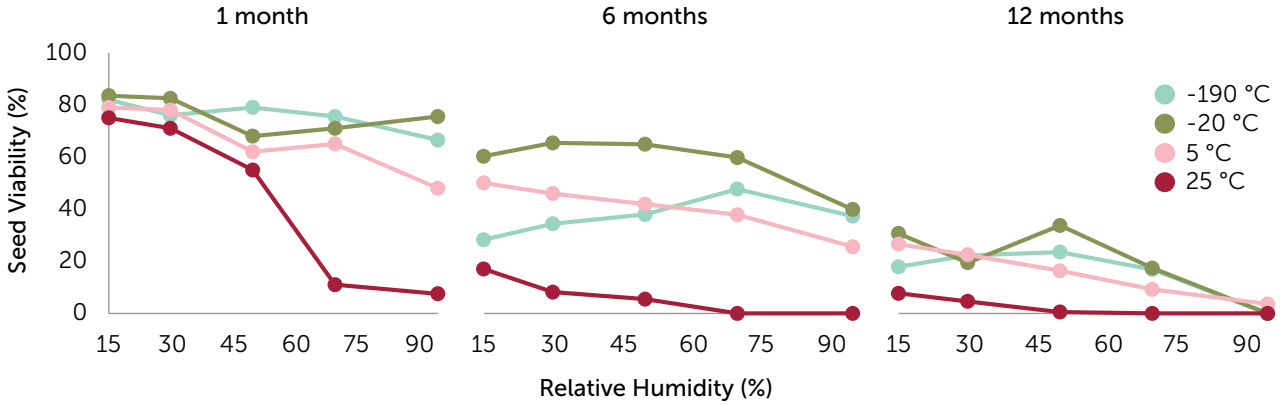

Figure 6.8: Seed viability (determined via cut-testing and tetrazolium staining) of *Nymphaea lukei* stored under a range of experimental storage conditions for 1, 6 and 12 months. This figure is a derivative of Figure 5 by Dalziell *et al*. (2019) used under CC BY 4.0.

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6.3 Direct methods for identifying non-orthodox seeds

The most accurate way to assess seed storage behaviour is by germinating seeds after drying and storage. This involves taking subsamples of a fresh seed collection and testing germination after drying to progressively lower moisture contents followed by storage at progressively lower temperatures. The aim is to identify the lowest moisture content and lowest storage temperature at which the material remains viable. The number of treatment combinations used to determine this will depend on the number of available seeds as well as the time and other resources available to conduct the tests. Ideally, tests for a given species should be completed on samples from multiple populations as the response to drying and storage can be variable (see Section 6.3.4.1; Case Study 6.2). Many species for which storage behaviour has already been determined are listed in the online Seed Information Database maintained by The Royal Botanic Gardens, Kew (see Section 6.4.1) and this should be consulted as a first step to avoid duplication of effort.

6.3.1 Hong and Ellis protocol for determining seed storage behaviour

A protocol published by Hong and Ellis (1996) provides the most accurate determination of seed storage behaviour. This protocol is applied in two steps: an initial assessment of desiccation tolerance, followed by an assessment of survival in cold storage. The precise storage treatments applied differ according to whether the seeds are suspected of being orthodox, intermediate or recalcitrant. The full protocol requires several thousand seeds, and therefore is unsuitable when seed numbers are limited.

Step 1: Assessing desiccation tolerance

As soon as the seeds are received, a subsample is taken to assess fresh seed moisture content (MC). An initial viability assessment is made by germinating a second subsample of the fresh seeds. Further subsamples are then re-tested following drying to 15 % MC in steps of 5 %, then from 15 % MC to 2.5 % MC in steps of 2.5 %. For example, if the initial moisture content was around 50 %, then 13 subsamples would be tested at 50, 45, 40, 35, 30, 25, 20, 15, 12.5, 10, 7.5, 5 and 2.5 % MC. The moisture content of the subsamples can be reduced by holding the seeds in airtight containers over solutions of lithium chloride (or other salts) that generate a range of different relative humidities (Hay *et al*. 2008). Alternatively, the subsamples can be dried over silica gel, or in a low humidity drying room, and weighed at regular intervals till a target weight representing the desired moisture content is achieved. The target weights are set by determining the initial weight and moisture content of the fresh seeds and using those to calculate the seed weight expected for the target moisture content (Hong and Ellis 1996).

$$Target\ weight\ (g) = \frac{(100 - initial\ MC\%)}{(100 - target\ MC\%)}\ x\ initial\ subsample\ weight\ (g)$$

Hong and Ellis (1996) recommend using 400 seeds for each subsample which, in the above example, would require a total of 5,200 seeds for germination testing and additional seeds for assessing moisture content at each step (~130 if 10 seeds are used per test). If seed availability is limited, the authors recommend reducing the number of seeds tested at each step before reducing the number of steps. Plotting germination % against MC in a graph (Figure 6.9) should then identify seeds as: a) able to survive drying to 3–7 % MC (likely orthodox), b) able to survive drying to 10–12.5 % MC (likely intermediate); c) most seeds killed by drying to 15–20 % MC (likely recalcitrant).

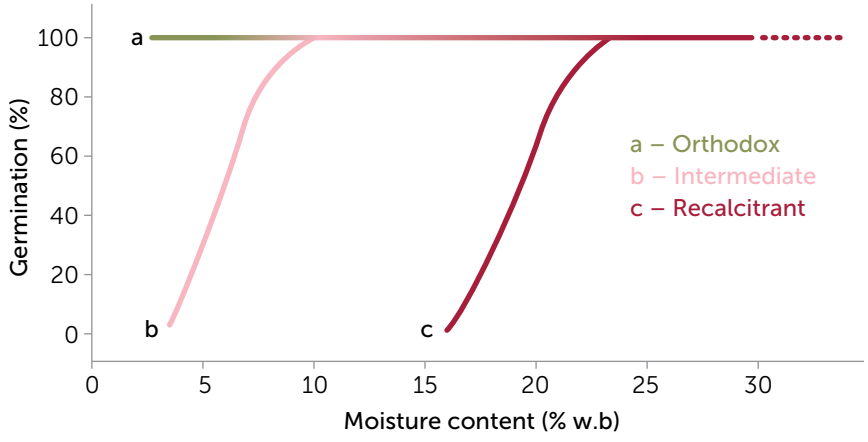


Figure 6.9: Typical patterns of germination following desiccation plotted against seed moisture content (% wet basis) for species with orthodox **(a)**, intermediate **(b)** and recalcitrant **(c)** seed storage behaviour. Chart adapted from Hong and Ellis (1996).

Step 2: Assessing response to storage

To confirm seed storage behaviour, and determine optimum storage conditions, multifactorial experiments testing MC x storage temperature x storage duration are conducted. The treatments applied are based on the expected seed storage behaviour established at Step 1, the number of seeds available, and the origin of the species. The recommended treatment combinations are summarised in Table 6.3.

Table 6.3: Experimental treatments to determine optimum storage conditions for seeds expected to show orthodox, intermediate or recalcitrant storage behaviour based on their response to drying (Hong and Ellis 1996).

	Likely storage behaviour based on initial test of desiccation tolerance		
Storage treatments	**Orthodox**	**Intermediate**	**Recalcitrant**
Moisture content (%)	10, 7.5, 5	Threshold MC ± 2 % and 4 % MC	Fully imbibed and fully imbibed less 2–5 % MC
Temperature (°C)	10, 5, 0, -20	20, 15, 10, 5, 0, -10, -20	Tropical lowland spp.: 20, 15, 10, 5 Tropical highland spp.: 15, 10, 5, 0 Temperate spp.: 10, 5, 0, -5
Duration (mths)	3, 12	3, 12, 24	1, 3, 6, 12
No. treatments	24 + controls [a]	105 + controls	32 + controls

MC = moisture content; Threshold MC is the moisture content below which seed viability is significantly reduced.
[a]Control samples for each MC level are tested prior to storage.

6.3.1.1 Simplified protocol for determining seed storage behaviour

Recognising that a large number of seeds may not be available for testing species collected from the wild, Hong and Ellis (1996) also suggested a simplified protocol that requires far fewer seeds. This protocol advises testing germination after drying to around 10–12 % and 5 % MC, then testing germination following storage at -20 °C for 3 months.

6.3.2 The 100-seed test

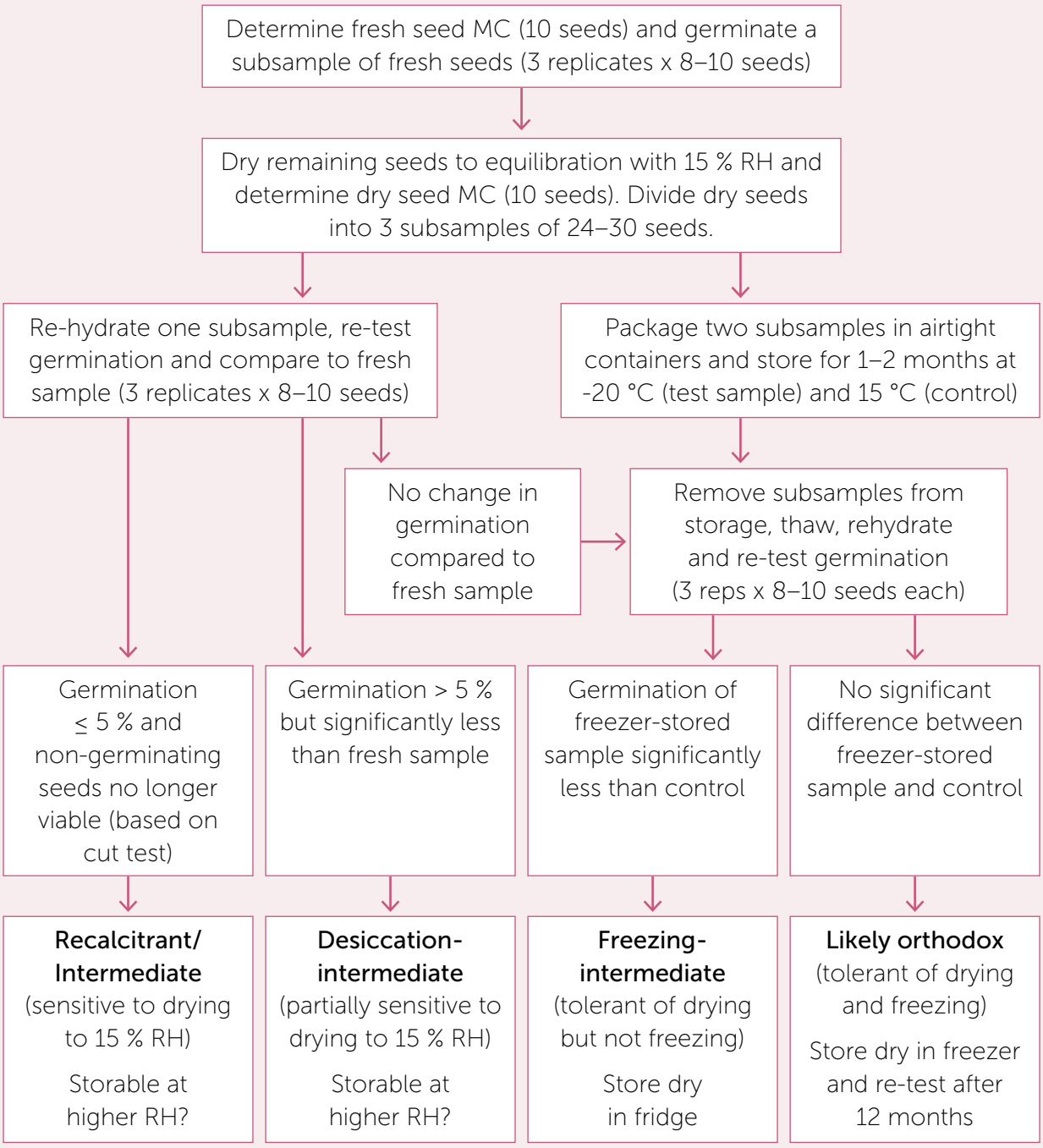
An alternate method published by Pritchard *et al*. (2004) focusses on the desiccation component of storage behaviour. This method is known as the '100-seed test' and, as the name suggests, uses around 100 seeds to distinguish desiccation tolerant seeds from those that don't survive desiccation to low moisture content. In this method, subsamples of seeds are tested for germination when fresh, after moist storage (an experimental control), and after drying over silica gel to the point that no further weight loss occurs. This method is useful for identifying seeds that are sensitive to desiccation to 3–7 % MC but does not distinguish between recalcitrant and desiccation-intermediate or freezing-intermediate seeds. The authors also note that, given the high degree of heterogeneity in wild seed collections, the lowest safe MC may be overestimated because individual seeds with a higher degree of desiccation tolerance may still germinate.

6.3.3 Australian PlantBank variation

The '100-seed test' was the method initially used by staff at the Australian PlantBank to assess seed storage behaviour for the Rainforest Seed Conservation Project (Hamilton *et al*. 2013). However, difficulties encountered with control samples held in moist storage (germination in storage or fungal contamination and viability loss), and subsequent loss of viability in some desiccation tolerant seeds stored at -20 °C for several years, led to the adoption of the modified protocol outlined in Box 6.1. This method (which utilises aspects of the methods described in 6.3.1.1 and 6.3.2) tests both desiccation and freezing tolerance and can be applied using around 120 seeds.

Box 6.1: Diagram of the procedure employed at the Australian PlantBank for identifying non-orthodox seeds

This procedure is a modified version of a protocol published by Hong and Ellis (1996) and the 100-seed test published by Pritchard *et al*. (2004). The protocol can be used to identify sensitivity to standard seed banking conditions using 120–140 seeds. If additional seeds are available, the number of replicates may be increased and a step testing the response to drying at a higher relative humidity (e.g., 50 %) may be added to better distinguish between recalcitrant and intermediate-seeded species. The protocol is dependent on the ability to germinate the seeds; techniques for relieving **dormancy** should be developed beforehand if required. All seedlings should be monitored to the point of hypocotyl and cotyledon emergence as drying and freezing may damage the shoot but not the root. MC = moisture content.



Case Study 6.3: Identifying non-orthodox seeds of coastal dry seasonal rainforest in Darwin, Australia

Vidushi Thusithana, Sean Bellairs, Christine Bach

Dry seasonal rainforest (DSR) **restoration** projects tend to under-utilise species which produce non-orthodox seeds because of a lack of knowledge about their storage after collection. This leads to over-representation of species with orthodox seeds. It is important to identify DSR species with non-orthodox seeds to determine appropriate ex situ conservation options. In this study, we identified five DSR species with a seed moisture content (MC) higher than 15 % and assessed whether they were sensitive to desiccation and non-orthodox (Hong and Ellis 1996).

Diospyros compacta, *Glycosmis trifoliata*, *Sterculia quadrifida*, *Opilia amentacea* and *Micromelum minutum* (Figure 6.10) were selected for the study. Seeds were checked for initial MC by oven drying at 120 °C for 3 hrs (ISTA 2008). Four lots of seeds of each species were weighed and air dried in open Petri dishes at ambient laboratory conditions. The replicate samples of seeds were re-weighed at 1-week intervals until the seeds had reached 10 % MC (Thusithana et al. 2018; Jayasuriya et al. 2013). When four replicates of the seeds reached 10 % MC, the seeds were subjected to a germination test on moist filter paper in Petri dishes in a 30 °C incubator. Optimum pre-germination treatments were applied as per Thusithana *et al*. (2018). Another four replicates of seeds were stored at 10 °C for two months in sealed Ziplock plastic bags, and then checked for germination as above.

The moisture content of dispersed seeds of these five species seeds ranged from 19 % to 53 % (Table 6.4). For all the species, drying the seeds to 10 % MC, with or without subsequent storage, resulted in no germination (Table 6.4). All these species are therefore desiccation sensitive and non-orthodox, meaning long-term (or even short-term) storage is challenging. The extent to which these 'non-orthodox' seeds can be dried before viability is lost needs to be tested for dry storage. However, from our results it is evident that seeds may not survive when dried to ≤ 10 % MC. Possibly these seeds can be wet stored in a medium (sawdust, coir, peat or vermiculite) that is slightly moistened with distilled water and then kept moist while stored at ambient temperatures (Sanjeewani et al. 2013; Walters et al. 2013). However, these results show that seeds will lose viability when stored at ≤ 20 °C. Future research should focus on determining the exact temperature range to store seeds or the MC that seeds can be dried to without losing viability.

Table 6.4: Seed moisture content and germination (mean ± SE) of fresh, dried, and dried plus stored seeds of five species that had an initial seed moisture content greater than 15 %. Reproduced from Thusithana *et al*. (2018) with permission from CSIRO.

Species	Moisture content (%)	Germination (%)		
		Fresh seeds	After storage at 10 °C for two months	After desiccation to 10 % moisture content
Diospyros compacta	19.1 ± 0.3	96.0 ± 3.1	0.0 ± 0.0	0.0 ± 0.0
Glycosmis trifoliata	53.2 ± 1.0	92.0 ± 2.3	0.0 ± 0.0	0.0 ± 0.0
Micromelum minutum	40.7 ± 0.7	100.0 ±0.0	0.0 ± 0.0	0.0 ± 0.0
Opilia amentacea	54.3 ± 0.2	70.4 ± 3.7	0.0 ± 0.0	0.0 ± 0.0
Sterculia quadrifida	28.4 ± 1.1	96.0 ± 1.3	0.0 ± 0.0	0.0 ± 0.0

Figure 6.10: Fruits of climax species tested for storage behaviour. Clockwise from top left: **(a)** *Micromelum minutum,* **(b)** *Sterculia quadrifida*, **(c)** *Glycosmis trifoliata*, **(d)** *Diospyros compacta.* (Images: V. Thusithana)

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6.3.4 Factors to consider when assessing storage behaviour

There are several factors affecting desiccation tolerance and germination that can influence the outcome of an assessment of seed storage behaviour and may potentially lead to incorrect conclusions. These include intrinsic factors, such as seed dormancy, and extrinsic factors such as collection timing or **post-harvest** handling.

6.3.4.1 Seed quality

Seed quality can vary between years, between locations and even between individuals for a variety of reasons, including the conditions in the environment during seed development and the level of **genetic diversity** in the population. Seeds of lesser quality may germinate fairly well when fresh but respond poorly to desiccation and storage (Chapter 5), so testing seeds from a single collection may not give a realistic assessment of storage behaviour for the species (Sommerville *et al*. 2021). If possible, seeds should be collected from multiple individuals in a population, and separate tests should be carried out on collections from several populations, to gain an understanding of the true storage behaviour for the species (Hong and Ellis 1996).

6.3.4.2 Seed maturity

The desiccation tolerance of seeds increases as they mature on the plant (Finch-Savage and Blake 1994; Vertucci and Farrant 1995) therefore collecting and using immature seeds for assessing storage behaviour can result in classifying seeds as recalcitrant or desiccation-intermediate when they are actually desiccation tolerant at maturity. The point of maturity generally corresponds with the timing of natural **dispersal** but, for seeds that aren't readily dispersed when mature (e.g., *Macadamia* species), some preliminary research to identify indicators of maturity may be needed.

6.3.4.3 Seed dormancy

The use of direct methods for evaluating seed responses to drying and storage is dependent on a high level of germination from fresh seed. In tropical and subtropical rainforest, the habitat with the highest proportion of non-orthodox seeds, more than 50 % of species have been found to have some form of seed dormancy, with physiological dormancy being the most common (Baskin and Baskin 2014). The presence or absence of dormancy should therefore be established, and techniques for breaking dormancy determined, before setting up extensive tests to assess seed storage behaviour. Tetrazolium staining (Chapter 5, Section 5.5.4.1) could potentially be used in place of germination testing where dormancy is an issue, but staining can be difficult to interpret if the procedure hasn't been optimised for a given species and the results standardised against germination of that species.

The likelihood of seed dormancy may be determined from a review of available literature (e.g., by looking at time to seedling emergence in Floyd 2008; Zich *et al*. 2018; Dunphy *et al*. 2020 for rainforest seeds), or from the initial germination response of fresh seed incubated at temperatures approximating those in the natural environment at the time of dispersal. Physical dormancy is easily identified by an **imbibition** test and overcome by scarification (Chapter 7); this dormancy type is also strongly associated with orthodox storage behaviour (Tweddle *et al*. 2003). Physiological, **morphological** and morphophysiological dormancy types require more complex experimental work to identify and relieve; this can consume considerable time and resources and may require many hundreds of seeds. Refer to Chapter 7 for a discussion of suitable methods.

6.3.4.4 Fungal contamination

Fungal contamination during germination experiments is a problem often encountered when working with seeds collected from moist environments (Berjak and Pammenter 2008). Fungal growth can rapidly overgrow seeds sown on agar, leading to deterioration of the seeds and inconclusive test results. Surface sterilising the seeds before sowing can reduce the onset of fungal growth, but often does not resolve the problem. An alternative used at the Australian PlantBank is to sow the seeds in seed raising mix and house each replicate in a separate mini-glasshouse (Figure 6.11). A mild fungicide can also be applied to reduce fungal growth.

Figure 6.11: Sowing seeds in seed raising mix and incubating them in a mini-glasshouse can help reduce issues with fungal contamination. (Image: K. Sommerville)

6.3.4.5 Extended germination periods

Germination tests can take a long time to complete for some rainforest species (e.g., 22 months for *Diospyros pentamera* and 29 months for *Eidothea hardeniana*; Sommerville *et al*. 2021). Germination of these seeds can also be very sporadic, with sometimes 2–3 months between germination events. Where possible, then, Hong and Ellis (1996) recommend continuing tests until all seeds have either germinated or rotted. Ungerminated seeds can be tested to see if they remain **viable** by squeezing gently with a pair of forceps, or by dissecting a subsample. Drying or cold storage may also cause a delay in germination compared to that of fresh seeds and this should be considered if stored seeds fail to germinate within the same time frame as fresh seeds (Hong and Ellis 1996).

6.3.4.6 Collection method and timing

Under the dense, closed canopy of evergreen rainforests, it can be very difficult to collect fruit directly from a plant, particularly from the tallest trees and vines. In this case, the fruit may need to be collected from the ground after it has been dispersed. Seeds from ground-collected fruit, however, can be more sensitive to drying or freezing than those collected directly from the plant due to deterioration caused by infestation with fungi (Berjak and Pammenter 2003). Collections made directly from the plant well after the seeds have matured can also deteriorate quickly (Ellis and Hong 1994) as the seeds have already begun to age. To avoid these issues, fruit should only be collected from the ground if it appears to be freshly fallen (with no visible signs of damage to the flesh), and mature fruit collected directly from plants should be collected as close to maturity as possible.

6.3.4.7 Post-harvest handling

Prolonged soaking, fermentation or moist seed storage can encourage seeds to transition into the germination phase. Once seeds have started to shift into this phase, subsequent drying can reduce viability (Hong and Ellis 1996), giving the appearance of desiccation sensitivity when the seed may actually be tolerant.

6.3.4.8 Pre-treatments

Methods that are intended to break dormancy and encourage germination can increase desiccation sensitivity if applied before the seeds are dried. For example, in temperate regions, seeds are commonly stored in moist and cool conditions (cold stratified) to break dormancy; this may improve germination but can also lead to a reduction in desiccation tolerance (Hong and Ellis 1996).

6.3.4.9 Inappropriate drying regimes

The temperature, humidity and rate at which seed drying occurs can all have an effect on a seed's tolerance of desiccation (Leprince 2003). For example, drying seeds in a hot shed, especially if the humidity is high, will cause seeds to deteriorate rapidly compared to drying them in an air-conditioned room with low humidity. Seed drying must therefore be carefully controlled to provide the most accurate interpretation of seed storage behaviour (Berjak and Pammenter 2003). Refer to Chapter 5 for appropriate seed drying techniques.

6.3.4.10 Imbibition injury

Re-wetting seeds after they have been dried can cause injury to the seeds if it occurs too quickly. This may occur, for example, if the dry seeds are soaked in water, a sterilant (such as bleach) or a dormancy-alleviating solution (such as **gibberellic acid** or **smoke water**) before sowing. To avoid imbibition damage, Dickie and Stuppy (2003) recommended equilibrating seeds to 100 % relative humidity, before they are put in contact with free water, by holding the seeds over a reservoir of water in an airtight container for 24–48 hours. Hong and Ellis (1996) noted that equilibration to 100 % relative humidity can also be achieved without injury by placing the seeds on solid water agar. Seed banks in Australia generally just allow seeds to equilibrate overnight to ambient humidity – which averages 50–70 % in the major cities[38] – before treating or sowing the seeds.

6.4 Indirect methods for identifying non-orthodox seeds

In situations where optimal germination is not possible or practical to achieve in a suitable time frame, or where seed numbers are very low, the following characteristics may be used to assess seed storage behaviour indirectly (see also Box 6.2).

6.4.1 Known behaviour of related species

Recent modelling by Wyse and Dickie (2018) has shown that the storage behaviour of other species in a genus is a good predictor for the behaviour of an untested species in the same genus, able to predict desiccation sensitivity with an accuracy of 89 %. This can be a useful starting point for assessing a new species, particularly if that species has a similar seed structure to previously tested species in the same genus and if storage behaviour in that genus is consistent among species (Sommerville *et al*. 2021). While there are still many genera for which no data on storage behaviour are available (see, for example, Sommerville *et al*. 2018), data for more than 17,500 species and 3,900 genera (Wyse and Dickie 2018) may be accessed through the online Seedbank Information Database[39] (SID; Royal Botanic Gardens Kew 2020).

38 http://www.bom.gov.au/watl/humidity/
39 https://data.kew.org/sid/

Data on Australian rainforest species not yet in SID may be found in Hamilton *et al*. (2013), Thusitana *et al*. (2018) and Sommerville *et al*. (2021).

6.4.2 Collection environment and plant habit

While non-orthodox seeds can occur in any environment, they are much more common in tropical and subtropical rainforests than in drier habitats such as sclerophyll forest or woodland (Tweddle *et al*. 2003; Wyse and Dickie 2017). Within rainforest habitats, woody species are more likely to produce desiccation sensitive seeds than herbs (Wyse and Dickie 2018) and tree species are significantly more likely to produce desiccation sensitive seeds than shrubs or climbers (Sommerville *et al*. 2021). For example, in a study of 162 Australian rainforest species, 42 % of trees were found to have desiccation sensitive seeds compared to 7–17 % for herbs, climbers, shrubs and shrub/trees (Sommerville *et al*. 2021). Environment and plant habit can therefore provide useful flags for potentially desiccation sensitive seeds.

6.4.3 Seed characteristics

Several studies on storage behaviour have now identified seed characteristics that can provide a useful indication of storage behaviour.

6.4.3.1 Seed structure

Seeds that have a large well-developed **embryo** combined with a papery thin seed coat are often recalcitrant (Daws *et al*. 2005, Hamilton *et al*. 2013, see also Chapter 10 Box 10.2). 'Large' in this case may refer to seeds ranging in diameter from around 1 cm (e.g., some *Syzygium* species) to 5 cm or more (e.g., *Castanospermum australe*). Rainforest seeds with a green embryo (e.g., *Archidendron hendersonii*) have also generally been found to be non-orthodox (either recalcitrant or intermediate) in two studies of Australian species (Hamilton *et al*. 2013; Sommerville *et al*. 2021). In contrast, seeds with a water-impermeable seed coat (e.g., many species in Fabaceae and Malvaceae, see Chapter 7) are usually orthodox (Tweddle *et al*. 2003).

6.4.3.2 Seed mass

Seed mass was found to be a useful predictor of desiccation response in recent modelling by Wyse and Dickie (2018). In particular, the great majority of seeds with very low oven-dry weight (for example, < 20 mg) are desiccation tolerant (Sommerville *et al*. 2021).

6.4.3.3 Fresh seed moisture content

The moisture content of fresh seeds can also provide a useful indication of storage response. The majority of rainforest seeds with a fresh moisture content < 20 % (fresh weight basis) have been found to be desiccation tolerant while those with a fresh moisture content > 50 % are largely recalcitrant (Sommerville *et al*. 2021). Fresh seed moisture content can be assessed within 24 hours using a simple oven-drying technique. Fresh seeds are weighed individually (or in 3–5 replicates of 5–100 seeds for small seeds), dried in a pre-heated oven at 103 °C for 17 hours, and re-weighed after cooling (following ISTA 2021; see also Chapter 5). Note that only the seed components should be used for this test; woody fruits and all fleshy material surrounding the seeds, including **mesocarps** and arils, should be removed prior to drying. Individual seeds can be placed in aluminium weighing boats and then into an aluminium oven tray for ease of handling. Large seeds should be cut in half to ensure complete drying.

Fresh seed moisture content can then be determined using the following formula:

$$Moisture\ content\ (\%) = \frac{(Fresh\ weight - Oven\text{-}dried\ weight)}{Fresh\ weight}\ \text{x}\ 100$$

6.4.3.4 Seed coat ratio and $P_{D\text{-}S}$

The seed coat ratio (SCR) is a measure of the mass of the dried seed coat compared to the mass of the whole seed. $P_{D\text{-}S}$ is a measure of the probability of desiccation sensitivity based on seed coat ratio and seed dry weight (Daws *et al*. 2006). Large seeds with a very thin seed coat (e.g., *Castanospermum australe*) usually have a low seed coat ratio and a high $P_{D\text{-}S}$ value and tend to be desiccation sensitive.

A sample of ten seeds is used to determine SCR but, rather than drying the whole seed intact, each seed is separated into two parts: 1. the seed **embryo** and **endosperm**; 2. all covering structures (e.g,. seed coat, **endocarp**). All fruit structures (e.g., capsules) are removed and only the seed components are used.

The two components of each seed are weighed separately, dried in an oven at 103 °C for 17 h, then re-weighed.

The seed coat ratio for each seed is then calculated using the following formula:

$$Seed\ Coat\ Ratio\ (SCR) = \frac{Oven\text{-}dried\ weight\ of\ endocarp\ and\ testa}{Oven\text{-}dried\ weight\ of\ whole\ seed}$$

The likelihood of desiccation sensitivity ($P_{D\text{-}S}$) can then be calculated using a formula developed by Daws *et al*. (2006):

$$P_{D-S} = \frac{e^{3.269 - 9.974a + 2.156b}}{1 + e^{3.269 - 9.974a + 2.156b}}$$

where a = SCR, and b = $\log_{10}$(oven-dried seed weight [in grams]).

Where the value of $P_{D\text{-}S}$ is greater than 0.5, the species is very likely to be desiccation sensitive.

6.4.4 Differential scanning calorimetry

At present there are no simple indicators that can be used to identify freezing-intermediate seeds other than re-testing germination after storage (Chau *et al*. 2019); however, a device known as a differential scanning calorimeter (DSC) has been used to resolve apparent freezing sensitivity in *Cuphea carthagenesis* (Crane *et al*. 2006) and to identify *Brassica* species that may be short-lived in storage at sub-zero temperatures (Mira *et al*. 2019). The DSC is a device that records energy changes in a seed sample, and the temperatures at which they occur, as the sample temperature is lowered from ~30 to -150 °C and back. Staff at the Australian PlantBank are currently testing the usefulness of the device for identifying rainforest species that are likely to be sensitive to storage at -20 °C. This research has the potential to enable the identification of species that will be sensitive to, or short-lived at, -20 °C while at the same time providing an indication of more suitable storage temperatures for those species[40].

40 https://www.rbgsyd.nsw.gov.au/Science/Rainforest-Conservation-Research/Rainforest-Seed-Conservation-Project/What-are-we-testing/Freezing-sensitivity

Box 6.2: Assessing storage behaviour when seeds are scarce or hard to germinate

Determining conclusively whether a species' storage behaviour is recalcitrant, intermediate or orthodox requires a series of germination tests with progressively drier seeds stored at progressively colder temperatures. When seeds are scarce or difficult to germinate, the following (developed by Sommerville *et al*. 2021) will help you to identify the most likely response to drying. DS = desiccation sensitive, DT = desiccation tolerant, PtDT = partially desiccation tolerant

Step 1: Check for information on seed storage behaviour for other species in the genus

- Storage behaviour in the genus is consistently orthodox and the untested species has fruit and seed similar in appearance to previously tested species ..... Likely DT
- Storage behaviour in the genus is consistently recalcitrant and the untested species has fruit and seed similar in appearance to previously tested species ..... Likely DS
- Storage behaviour in the genus is inconsistent or no species have previously been tested ..... Step 2

Step 2: Dissect seed with a scalpel and examine seed coat

- Seed coat hard, difficult to dissect ..... Step 3
- Seed coat soft, easy to dissect ..... Step 4

Step 3: Determine whether seed coat is impermeable by conducting an imbibition test

- Seed coat impermeable ..... Likely DT
- Seed coat permeable ..... Step 4

Step 4: Determine oven dry weight and fresh seed moisture content (fresh weight basis).

- Dry weight < 20 mg **OR** moisture content < 20 % ..... Likely DT
- Dry weight ≥ 20 mg **AND** seed moisture content ≥ 50 % ..... Likely DS
- Dry weight ≥ 20 mg **AND** moisture content 20 % to < 50 % ..... Step 5

Step 5: Determine seed coat ratio and calculate $P_{D\text{-}S}$ (following Daws *et al.* 2006).

- $P_{D\text{-}S} < 0.01$ ..... Likely DT
- $P_{D\text{-}S} > 0.3$ ..... Likely DS
- $P_{D\text{-}S} \geq 0.01$ and < 0.3 ..... Step 6

Step 6: Inspect structure of dispersal unit and seed embryo

- Seed encased in a woody endocarp ..... Likely DS or PtDT
- Embryo green in colour ..... Likely DS or PtDT
- Neither of the above ..... Step 7

Step 7: Conduct seed storage behaviour experiments as outlined in Section 6.3.

6.5 Conservation options

6.5.1 Recalcitrant (desiccation sensitive) seeds

Seeds that cannot be dried are not suitable for storage using standard seed banking techniques. Options for conserving these species include very short-term storage of seeds in a fridge (several days to 1–2 years; Hong and Ellis 1996), germination of the seeds and maintenance of the seedlings in a shade house (1–3 years; Dunphy *et al*. 2020), establishment and maintenance of the species in living collections (Chapter 11) or **tissue culture** (Chapter 9), or cryopreservation of the embryo or other plant parts (Chapter 10).

6.5.1.1 Short-term storage in the fridge

A temporary storage option for recalcitrant seeds is to use low, non-freezing, temperatures (4–15 °C), to slow their metabolic rate as much as possible without inducing ice formation that would damage the seeds. However, these seeds will slowly continue development, aging and potentially germinating in storage, limiting the time the species can be stored this way. Seeds of chilling-sensitive species such as Cacao, for example, show significant declines in viability when stored below 10 °C (Chin and Roberts 1980). When the appropriate conditions are used, storage in the fridge can extend the seeds' life span from a few days to a few months or even 1–2 years. Seeds will need to be carefully monitored for fungal and bacterial contamination, which can rapidly reduce the viability of the collection (Berjak and Pammenter 2008).

To store seeds in this way:

- Collect fresh fruits and extract the seeds
- Clean and surface sterilise the seeds, for example:
  - 5 min exposure to 70 % ethanol OR
  - 5–30 min exposure to a 2–3 % solution of sodium or calcium hypochlorite OR
  - Exposure to an appropriate fungicide
- Assess initial seed viability
- Store on moist paper towelling or in a moist medium (such as sphagnum moss) in an airtight container to limit dehydration during storage
- Store at a temperature appropriate for the species (e.g., 4–15 °C)
- Monitor for contamination (container may need to be opened periodically to remove condensation and limit fungal growth)
- Assess viability of seeds often as decline can happen rapidly.

6.5.1.2 Germination and storage as seedlings

An alternative conservation option is to germinate and conserve the species as seedlings. This is a viable mid-term storage option that can extend the window for utilisation of the collection by 1–3 years. Many seedlings can be maintained in a small area (such as a single germination tray) by reducing light and nutrient levels to limit their growth (Dunphy *et al*. 2020).

- Collect fresh fruits and extract the seeds
- Sow seeds into a suitable growing medium
- Provide sufficient water for germination
- Once seedlings have established, reduce light and nutrient levels to keep growth to a minimum
- Monitor for pests and diseases

6.5.1.3 Living collections

Desiccation sensitive seeds can also be used to establish potted or in-ground living collections (discussed further in Chapter 11). These have the disadvantage of requiring considerable space and on-going maintenance but can be useful for generating seeds and vegetative material for tissue culture, cryopreservation and **translocation** projects.

6.5.1.4 Tissue culture

Tissue culture utilises an artificial growth medium to provide all the necessary nutrients and conditions for plant growth, allowing the clonal propagation of plants from minimal starting material. Tissue culture provides a longer-term alternative to storage using seedlings and is a valuable conservation tool for threatened species that produce recalcitrant seeds. However, tissue culture requires access to more specialised equipment and technical expertise, increasing the cost of conservation. Chapter 9 covers the processes involved in initiating and maintaining tissue cultures.

6.5.1.5 Cryopreservation

Cryopreservation (storage in liquid nitrogen or its vapour) provides the only long-term ex situ conservation option for recalcitrant species. Whole seeds (excluding larger recalcitrant seeds), embryonic axes from the seed, and tissue cultured material are all sources of **germplasm** that can be used to conserve recalcitrant species when appropriately treated. This option requires considerable expertise and often a lengthy research program to adapt the basic techniques to each new species. Cryopreservation processes are covered in detail in Chapter 10.

6.5.2 Desiccation-intermediate seeds

Desiccation-intermediate seeds may be stored in the short to medium term (e.g., 5–6 years for Coffee and Papaya; Hong and Ellis 1996) once their desiccation and storage temperature limits have been identified (Section 6.3.1). The amount of drying that the seeds can survive is influenced by the rate and temperature of drying; more rapid dehydration (for example, at lower relative humidity) can result in tolerance of a greater reduction in MC, likely because the seeds spend less time exposed to intermediate water contents where damage can occur (Berjak and Pammenter 2002). Their ability to tolerate some degree of desiccation without any pre-treatment makes these seeds potential candidates for cryopreservation. Whole seeds, or at least their embryonic axes, may be cryopreserved after drying by cooling the seeds so rapidly that any remaining water in the cells forms a kind of '**glass**' rather than damaging ice crystals. See Chapter 10 for more information.

6.5.3 Freezing-intermediate seeds

Freezing-intermediate seeds may be stored for many years in a fridge, rather than a freezer, however the longevity in storage is very variable among species (Chau *et al*. 2019). These seeds may also potentially be suitable for cryopreservation, but testing is required to confirm their tolerance. For some species, a poor response to freezing is associated with the occurrence of long-chain fatty acids which undergo constant phase changes at standard storage temperatures (Crane *et al*. 2006; Mira *et al*. 2019). Storage at temperatures outside the range where these phase changes occur (e.g., in cryopreservation at -150 to -196 °C) may improve the response of the seeds to freezing. Revival of these seeds may also depend on a 'heat blast' in which seeds are exposed to a high temperature (e.g., 45 °C) for a small period of time to allow lipid crystals to melt before the seeds are allowed to fully rehydrate (Crane *et al.* 2006).

6.6 Summary

Non-orthodox seeds are challenging to conserve and techniques for identifying and conserving these seeds are constantly evolving. Progress has been made in developing techniques that allow the identification of desiccation sensitive seeds without extensive germination testing, but on-going research into techniques to conserve these species is necessary to improve conservation outcomes. Work on improving the identification and handling of Australian seeds that display intermediate or short-lived storage behaviour is also required.

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Chapter 7
Seed germination and dormancy

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7.1 Introduction

Seeds form the primary means of reproduction and species persistence for most flowering plants. **Germination** is a critical process in the lifecycle of plants, so to maximise seedling survival after germination, seeds have evolved a range of **dormancy** and germination timing mechanisms to sense the external environment and germinate during periods most conducive to seedling establishment. Dormancy is best described as a seed characteristic that defines the environmental conditions that must be met before germination is possible (Willis *et al*. 2014). A dormant seed is one that though viable, does not have the capacity to germinate (within a specific time) when exposed to normal environmental conditions suitable to support germination of the non-dormant seed (Baskin and Baskin 2004a, b). In an ecological context, dormancy exists to prevent seed germination during transient periods when environmental conditions are suitable for germination, but the probability of seedling survival is low (Black *et al*. 2006). Given that up to 70 % of species worldwide possess some type of seed dormancy (which is likely to be similar in Australia) and require a specific set of conditions to germinate, it is essential that **conservation** practitioners learn to recognise when seeds are dormant, rather than non-viable (Erickson *et al.* 2016). This means that germination cues need to be determined, or likely to be, to overcome dormancy and to provide the right conditions for germination. Otherwise, the success of seed-based conservation programs will be limited, and resources and time wasted.

Many types of dormancy have evolved, reflecting the diversity of **habitats** into which seeds are shed (Willis *et al*. 2014). Dormancy types are classified based on physical, physiological, or morphological attributes (Baskin and Baskin 2004a). In an Australian context, seeds have adapted to harsh and variable environments to take advantage of the few opportunities for plant recruitment – as with the flush of germination after fire, for example. To germinate a seed in the laboratory or soil seedbank, it is necessary to first define the presence and type of dormancy or lack thereof, determine the conditions necessary to alleviate the dormancy, and then resolve the conditions for germination. This chapter covers the basic approach to identifying seed dormancy type(s) and developing germination techniques for species for which little or no information is available.

7.2 Gathering species-specific information relevant to germination and seed dormancy

Prior to commencing germination experiments it is well worth reviewing literature to gather as much information as possible. Useful information includes environmental and biological data for related species, genera and families and similar seed types. Useful sources of literature (Commander 2021) are:

- Scientific journals and papers published online, such as within:
  - Australian Journal of Botany
  - Australasian Plant Conservation
  - Seed Science Research
  - Seed Science and Technology
- Review papers, such as
  - The process of germination in Australian species (Bell 1999)
  - Seed dormancy and germination stimulation syndromes for Australian temperate species (Merritt *et al*. 2007)
  - The ecology, dormancy and germination of Australian native seeds (Commander *et al*. in prep.)
- Books, such as
  - Growing Australian Native Plants from Seed (Ralph 2003)
  - Seed Collection of Australian Native Plants (Ralph 1993)
  - Australian Seeds (Sweedman and Merritt 2006)
  - The Florabank Guidelines (Commander 2021)
  - Pilbara Seed Atlas and Field Guide (Erickson *et al*. 2016)
  - Banksia Woodlands: A restoration guide for the Swan Coastal Plain (Stevens *et al*. 2016)
  - Australian Rainforest Seeds: A Guide to Collecting, Processing and Propagation (Dunphy *et al*. 2020)
- Online databases, such as
  - Royal Botanic Gardens, Kew – Seed Information Database (SID)[41]
  - The Australian Seed Bank Partnership[42]
  - Seeds of South Australia[43]
  - Royal Tasmanian Botanic Gardens germination database[44]
- Botanic gardens, research institutions, the Australian Seed Bank Partnership and 'Friends of' groups (e.g., Kings Park Volunteer Master Gardeners[45])

41 https://data.kew.org/sid/
42 https://asbp.ala.org.au/search#tab_simpleSearch
43 https://spapps.environment.sa.gov.au/seedsofsa/
44 https://gardens.rtbg.tas.gov.au/conservation/tsccgerminationdatabase/
45 https://www.bgpa.wa.gov.au/contact-us/16-kings-park-volunteer-master-gardeners

- Native seed suppliers and native plant nurseries (e.g., Revegetation Industry Association of WA Accredited Members[46] and Australian Native Plants Society (Australia) Seed Suppliers[47]
- Native Plant Societies
- And see also publications in the references section

7.2.1 Related plants

In many cases, species that are related display similar dormancy / germination traits. For example, most species in the Fabaceae family possess physical dormancy (Section 7.4.1, Table 7.3) and require a hot-water or scarification treatment to alleviate dormancy before germination can proceed. Many species of Asteraceae respond well to a period of dry **after-ripening** (Section 7.6.2.1).

Gather other information relevant to germination biology that will assist in selecting suitable germination temperatures. Include environmental data from the species' habitat, especially:

- The time of year when the seeds mature and are dispersed
- The mean monthly maximum and minimum air and soil temperatures
- The mean monthly rainfall
- The timing of natural disturbance events that may promote germination (e.g., fire)

7.2.2 Seasonal response

Seeds naturally germinate during the wet season when water becomes non-limiting. For areas that experience seasonal rainfall, average temperatures during the wet season are useful starting points for germination. For example, species from the southern areas of Australia have optimal germination temperatures of 10–20 °C (which coincides with the winter rainfall zone), in contrast to those from the summer rainfall zone in northern areas of Australia where germination optima are often 20–30 °C.

Finally, understanding a species' seed bank type can also be useful (Box 7.1). Determine whether seeds are:

- Persistent on plants (termed **serotinous**) or,
- Shed into the **soil seedbank** (termed **geosporous**)

46 https://www.riawa.com.au/accreditation/members
47 http://www.anpsa.org.au/seedsupp.html

Box 7.1: Seed bank type – serotiny and geospory

Species that store their mature seeds in the plant canopy (termed **serotinous** or **bradysporous**) are usually non-dormant as the seeds are only released during a period conducive to germination, often after a disturbance such as fire. Common examples of serotinous species include species of Myrtaceae (*Corymbia, Eucalyptus* and *Melaleuca*), Proteaceae (*Banksia*) and Casuarinaceae (*Allocasuarina* and *Casuarina*).

Species that release mature seeds into the soil (termed geosporous) are far more common in the Australian flora. Many geosporous seeds are commonly dormant when shed as they are usually released at a time of year unsuitable for seedling establishment. Species of Apiaceae, Asteraceae, Ericaceae and Poaceae are commonly geosporous. Other characteristics of serotinous and geosporous species are shown in Table 7.1.

Table 7.1: Characteristics of serotinous and geosporous species.

Characteristic	Serotinous	Geosporous
Release true seed from fruit	most	many
Release seeds while still contained within fruits (indehiscent fruits – see Box 7.2)	few	many
Non-dormant & capable of germinating immediately given right environmental conditions	most	few
Dormant & germinate only after environmental conditions conducive to dormancy loss	few	most

7.3 Seed testing

7.3.1 Determining the natural germination unit

As the anatomical features of seeds and **fruits** can significantly affect germination characteristics and seed dormancy type, it is important to clearly find and understand these features. Seeds and fruits vary immensely in size, shape and structure, and in many cases the germination unit of a plant is not always obvious and may require further investigation under a microscope or magnifying glass. True seeds comprise an **embryo** and storage tissue (either **endospermic** tissue or cotyledons) surrounded by a **testa** (e.g., *Androcalva perlaria* – Figure 7.1). However, seeds can also be permanently encased in an **indehiscent** dry fruit (like many grass seeds – Figure 7.2a) or **endocarp** (like a macadamia nut – Figure 7.2b) that protects the seed within and acts as both the **dispersal unit** as well as the germination unit in many species (Figure 7.2). Indehiscent fruits come in many shapes and sizes and can be small and delicate (Figure 7.2a) or larger and in some cases exceptionally tough and hard (Figure 7.2b). Interestingly, the seeds of many indehiscent fruits can be difficult to germinate and, at the very least, may take longer to germinate for reasons not yet well understood.

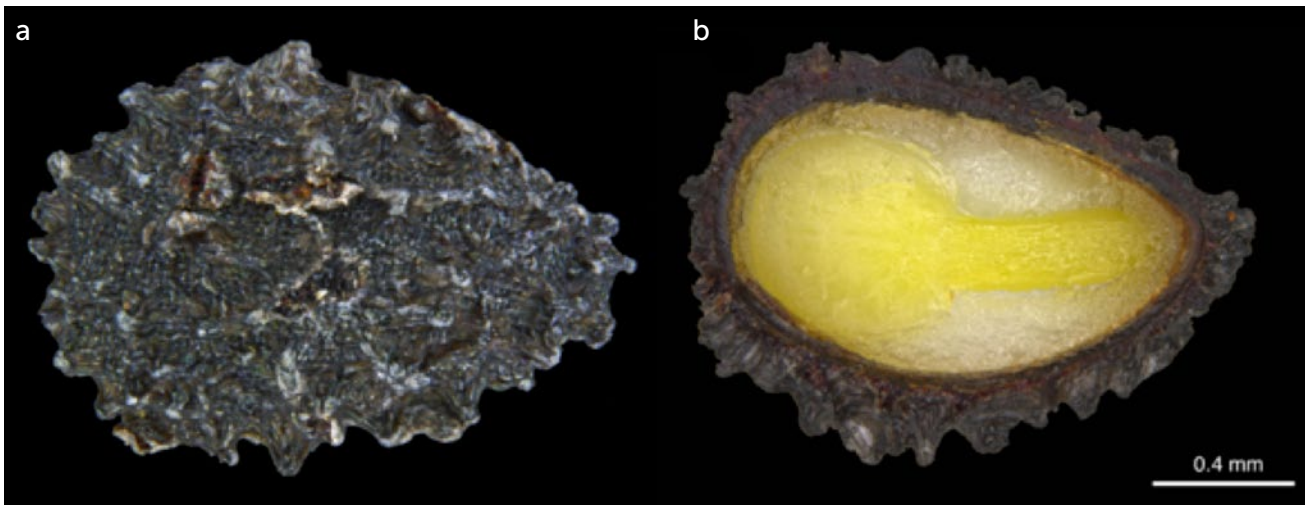

Figure 7.1: **(a)** Example of a true seed (*Androcalva perlaria*) consisting of a testa on the outside (Image: David Symons) and **(b)** On the inside an embryo (in this case yellow and spatulate) surrounded by a small amount of white endosperm. (Image: Arielle Fontaine)

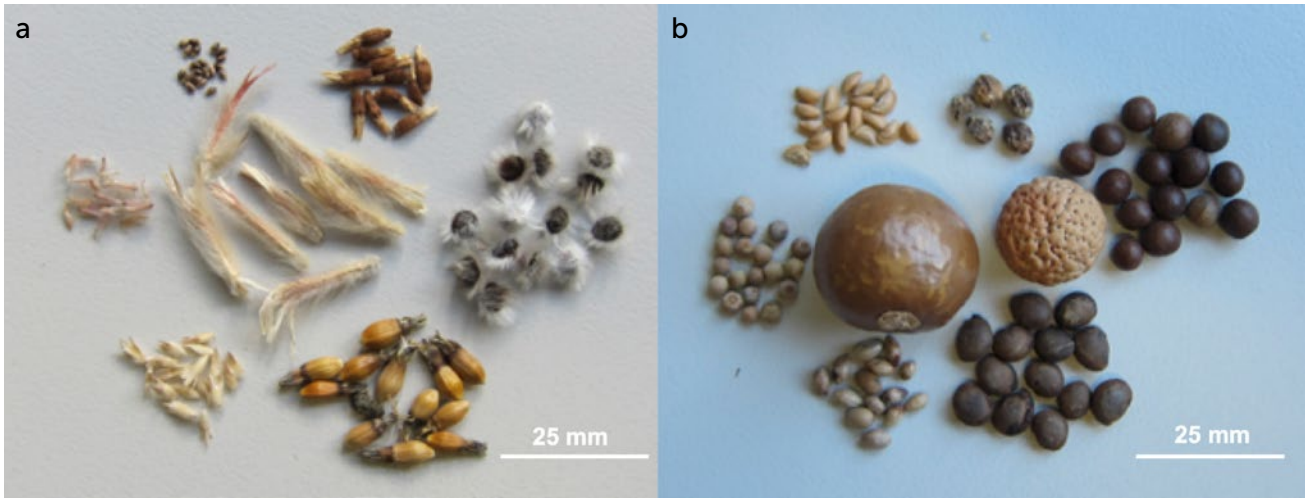

Figure 7.2: **(a)** Various examples of dry indehiscent fruits which act as both the dispersal unit as well as the germination unit. The species displayed clockwise, from left, are *Cephalotus follicularis* (Cephalotaceae), *Lepidosperma gibsonii* (Cyperaceae), *Darwinia masonii* (Myrtaceae), *Stirlingia latifolia* (Proteaceae), *Dasypogon bromeliifolius* (Dasypogonaceae), *Triodia basedowii* (Poaceae) and *Ptilotus manglesii* (Amaranthaceae); **(b)** various examples of hard indehiscent endocarps after removal of the outer (mesocarp and exocarp) layers. The species displayed are *Styphelia xerophylla* (Ericaceae), *Styphelia racemulosa* (Ericaceae), *Eremophila clarkei* (Scrophulariceae), *Stylobasium spathulatum* (Surianaceae), *Persoonia longifolia* (Proteaceae), *Styphelia nitens* (Ericaceae), *Macadamia integrifolia* (Proteaceae), and *Santalum acuminatum* (Santalaceae). (Images: Shane Turner)

Box 7.2: Indehiscent fruits

Commonly encountered indehiscent fruits include those comprising a fleshy outer **exocarp**/**mesocarp** surrounding a woody endocarp (e.g., *Persoonia longifolia* – Figure 7.3a), and those that are dry, such as an achene (e.g., Asteraceae), nutlet (e.g., *Lepidosperma* spp.) or indehiscent floret (e.g., *Triodia basedowii* – Figure 7.3b). These fruits do not open to release the seeds and have evolved to behave in a similar manner as a true seed, acting as the natural dispersal and germination unit. Once suitable environmental conditions are experienced, the seeds will germinate within the indehiscent fruit and the radicle will push through the surrounding tissue. The true seeds in these cases are usually quite fragile and protected by the covering layers but under the right conditions will readily push through these outer coverings.

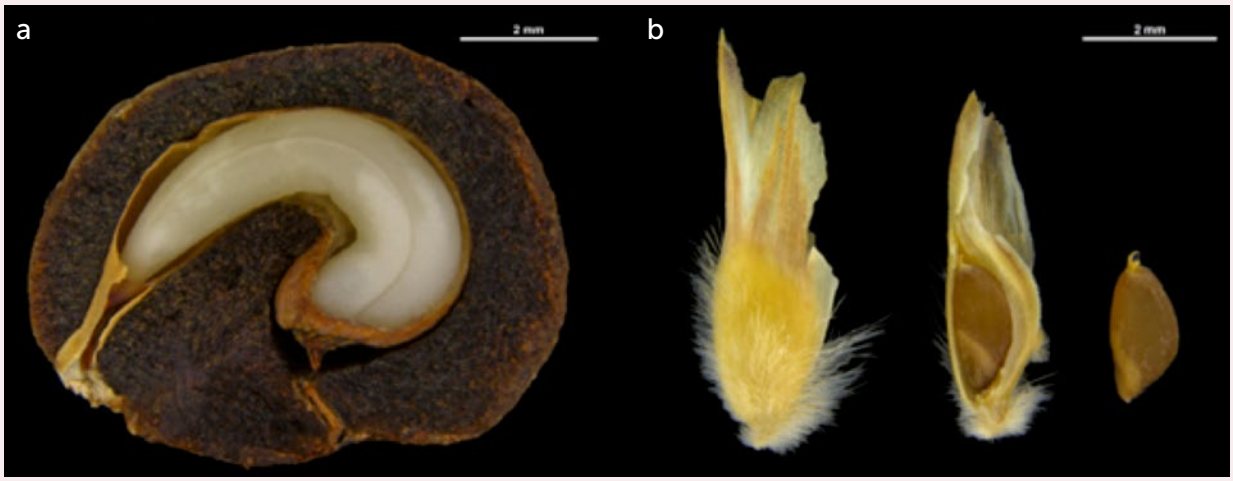

Figure 7.3: **(a)** Longitudinally dissected indehiscent endocarp of *Persoonia longifolia* showing the structure and location of the embryo within the woody endocarp. The thin papery testa surrounding the embryo has been carefully removed to reveal the two embryonic leaves (cotyledons) beneath; **(b)** Closeup example of the external and internal structure of an indehiscent fruit of *Triodia basedowii* showing the external structure, the seed positioned within the fruit and an extracted seed. (Images: David Symons)

7.3.2 Imbibition testing

For seeds to successfully germinate they must be able to take up water (imbibe). Issues with **imbibition** are encountered in seeds exhibiting Physical Dormancy (PY), a trait associated with at least 18 **angiosperm** families (see Table 7.2). However, water ingress issues can occur outside PY seeds. Most notably, large woody seeds can be slow to imbibe, particularly if surface contact with the germination media is poor. Prior to germination testing, it is good practice to observe whether the seeds are capable of imbibing water. This can be achieved by conducting an imbibition test (as detailed in Box 7.3). A more 'quick and dirty' method suitable for classic PY families encountered in Australia is a 'swelling test.' In this test two small samples of seeds (one sample of scarified (chipped or nicked) and one sample of non-scarified seeds) are sown onto agar plates and inspected seven days later. A noticeable swelling in scarified seeds (confirmed when the seeds are gently prodded and appear soft compared to non-scarified seeds) is a good indicator for PY. Sporadic swelling in non-scarified seeds may be a sign of partial dormancy expression or seed **predation** damage which is relatively common.

Box 7.3: Imbibition test

Replicate batches of dry seeds are weighed and then placed onto agar or moist filter paper. Following 1–2 minutes (long enough for the seed coat to become wet) seeds are removed, patted dry, re-weighed, and placed back onto the moist filter paper. This process is continued periodically until there is no further increase in seed weight, which usually takes between 24–96 h. If seeds have water impermeable seed coats, there will be modest increase in seed weight (< 10 %) over time. However, if seeds do imbibe then a seed weight increase > 30 % would be expected (Figure 7.4). (Note: Seed collections of many species with PY may include a mix of water impermeable and water permeable seeds so it is common for batches of seeds with PY to increase in mass to some degree).

A simplified way to undertake an imbibition test is to weigh the seeds while dry, immerse in water for 24–48 hours, pat dry and weigh again to ascertain whether there has been an appreciable increase in seed mass (>30 %).

To calculate increases in seed mass the following formula is used:

$$\%\ increase\ in\ weight = \left[\frac{(Wi - Wd)}{Wd}\right] x\ 100$$

where Wi and Wd = weight of imbibed and dry seeds, respectively.

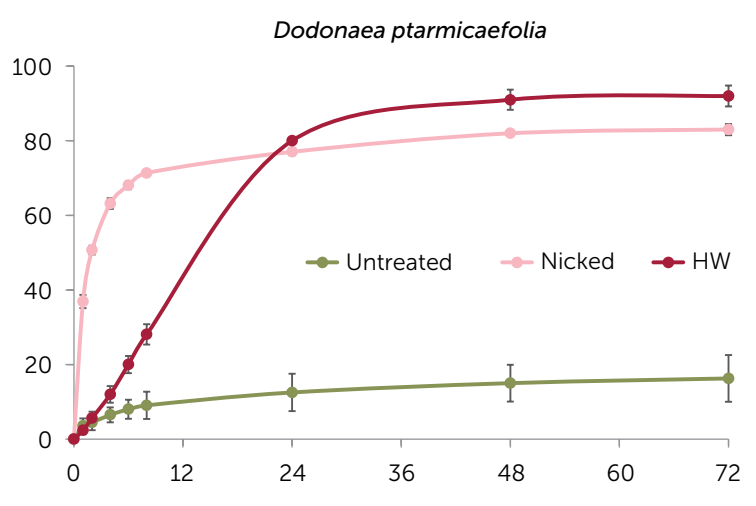

Figure 7.4: Imbibition curve for *Dodonaea ptarmicaefolia* (Sapindaceae) seed. Only a very modest increase in seed mass (~15 %) is evident in untreated (control) seeds, indicating most seeds do not imbibe water and, thus, possess physical dormancy. Seeds either scarified (nicked) or treated with hot water rapidly increase in mass (>80 %), indicating these seeds readily imbibe water within 24–48 hours, and both treatments were successful in removing physical dormancy (Image: Shane Turner – Adapted from Cook *et al*. (2008) *Annals of Botany* **101**, 1355 by permission of Oxford University Press).

7.3.3 Initial germination testing

Fundamentally, germination testing is necessary to determine the conditions required for a seed to germinate and to establish the presence or absence of dormancy. Germination is also a measure of seed viability (Chapter 5), though this can be complicated by the presence of dormancy (not all **viable seeds** may germinate). If dormancy is present, germination testing can be a critical step in classifying the dormancy type. For dormancy studies it is important to use freshly matured seeds, ideally even from several **populations** over several years (wherever possible), as seed dormancy status can change over time in storage, across source population distribution, and in response to climatic conditions during the period of seed maturation. This variation can result in difficulties in developing standardised germination protocols and repeatable results. Techniques for testing seed germination can vary widely between laboratories/nurseries and are often specific to the type of information being gathered. However, some basic principles apply – see Box 7.4.

Box 7.4: Basic principles of germination testing

To germinate, a non-dormant seed requires exposure to a suitable germination environment consisting of a suitable temperature and light regime, and ample moisture and oxygen.

When testing a seed collection, a sample size of at least 50–200 seeds is recommended (however as few as 5 seeds is acceptable where seeds are limited). Seeds may be divided into replicates for testing (Box 7.6). To produce a germination result that is representative for the entire seed collection, it is important to select the seeds at random and not preferentially select seeds that are larger or plumper.

Laboratory testing

Ideally, a germination test should be undertaken in sterile Petri dishes placed in an incubator to ensure a level of accuracy and repeatability between tests (Figure 7.5a). Several germination substrates are commonly used including:

- filter paper (either cellulose-based or the more inert glass-fibre based),
- solidified water-agar (between 0.7–1 % w/v),
- pure sand.

These substrates can be irrigated with (or include):

- de-ionised water, or
- solutions comprising a germination-promoting compound such as **gibberellic acid** (GA_3), potassium nitrate (KNO_3) (see Case Study 7.2) or smoke products (see Section 7.7).

If a treatment is applied, a control set of replicates of untreated seeds (without the added solutions) should also be set up.

Temperature regimes

The incubation temperature should be based on indicative habitat temperatures during the growing season of the species you are testing (i.e., the temperature that seeds naturally experience in the field). This approach typically uses diurnal temperatures (day/night variation) as opposed to applying a constant average temperature. For example, see Case Study 7.2 on germination testing at the Tasmanian Seed Conservation Centre, and Table 7.4 on implementing the 'move-along' experimental approach. Alternatively, consult published research on species from similar habitats. It is worth considering the temperatures used in your incubators particularly if you have a limited number of incubators, but many collections to test. To manage workload, tests may need to be staggered however, **stratification**/incubation requirements for different collections can run for weeks to months. Depending on the climate where the species is located, one approach to incubator set up is to use three incubators with set temperatures that replicate environmental conditions during winter, spring/autumn, and summer. In this regime, three incubators are set up with an approximate configuration of temperature and light at key points in the yearly cycle. However, it is important to note that replication is affected if any one of the three incubators', malfunctions at any time during the set program.

Light regimes

Light may or may not be necessary for germination to occur. The light quality (wavelength) and duration can also affect germination results, as red wavelengths promote germination and far-red wavelengths prevent germination in some species.

If complete darkness is needed, then incubators can be programmed for no light period, or Petri dishes can be:

- wrapped in two layers of aluminium foil, or
- placed inside light-excluding boxes.

Often it is useful to compare seed germination under a photoperiod regime and under complete darkness to identify a positive, negative or neutral photoblastic response (i.e., to see if germination is promoted, indifferent or suppressed by light). Note: even tiny amounts of light can elicit a response, so 'dark' treated seeds should be kept in complete darkness until the final germination count.

Usually a light/dark regime is programmed into the incubator, with photoperiod regimes right for the season when recruitment is expected to take place, for example:

- 12/12 hours, or
- 10/14 hours light/dark.

Seeds can be exposed to different types of light such as:

- artificial lighting (cool white fluorescent tubes, LED incubator lights)
- natural sunlight if being tested in glasshouses or outdoors.

Avoiding fungal contamination

For many species fungal contamination is problematic when seeds are incubated in Petri dishes.

To prevent or minimise fungal contamination, seeds may be sterilised prior to incubation by soaking in a weak solution (1–2 %) of:

- bleach (sodium hypochlorite), or
- calcium hypochlorite.

Effective soaking periods vary, but around 30 minutes is a good starting point. A surfactant (i.e., weak detergent) can be an effective addition to a sterilising/soaking solution for seeds with hydrophobic hairs. Once surface-sterilised, seeds should be washed several times in sterilised water to remove the sterilant solution prior to incubation.

Fungal contamination of seeds can also be reduced by:

- working under sterile conditions in a laboratory including
  - working in a **laminar flow cabinet**
  - using sterilised water
  - sterilising germination media before use (using an **autoclave**)
  - sterilising instruments such as forceps by flaming or dipping in bleach/ethanol solution and rinsing in sterilised water.
- using accredited nursery facilities.

Seeds that have developed fungal and bacterial contamination can be transferred to new, sterile media at any point in the germination test, taking care not to disrupt the seed surface. Note that some fungal spores such as *Aspergillus flavus* are hazardous to human health and operators need to be aware that heavily contaminated plates should not be opened and examined, but disposed of or autoclaved, to avoid exposure.

If fungal and bacterial contamination continue to be a problem, consider the use of:

- fungicides and/or
- Plant Preservation Mixture (a broad-spectrum biocide/fungicide often used in **tissue culture**).

Nursery testing

As an alternative to laboratory germination, seeds can be germinated in soil/ potting media in a nursery (Figure 7.5b). This has some benefits for species that can be adversely affected by fungal contamination (Chapter 6, section 6.3.4.4). Note that unless nursery conditions are carefully controlled, apparent germination results can be misleading due to mishaps such as drying of pots causing death of emerging radicles; or fungal gnat larvae destroying radicles as they emerge, especially in germination substrates with a high organic matter content.

Important considerations in the nursery include:

- type of media,
- seed burial depth (generally to a depth equal to the size of the seed, between 0.5–2 mm),
- watering regime (moisture loss can occur rapidly if soil pots are outside), and
- temperature.

Unless a temperature-controlled glasshouse is available, the seeds will be exposed to ambient temperatures, meaning the time of year at which the seeds are sown may have a significant impact on the germination result. It is preferable to sow the seeds at the same time of year at which they naturally germinate to ensure temperatures are not too hot or cold. If possible, record/log the temperatures seeds to which seeds are exposed to during germination.

Recording results

Record the number of seeds germinated and express as a percentage of the total sampled

- e.g., 18/25 seeds = 72 % final germination.

At the conclusion of a germination test, operators should perform a **cut test** (Chapter 5 5.5.3.1) on non-germinated seeds to determine what proportion of the sampled seeds was viable.

A cut test can be examined under a microscope, magnifying glass or with the naked eye if seeds are large enough. The aim is to determine the number of full and healthy looking seeds that have the potential to germinate but did not, and the number of empty or infested seeds that were never going to germinate.

The germination percentage can then be reported as a viability-adjusted percentage, expressed as the number of seeds germinated per number of viable seeds in the sample.

- i.e., 18 germinated, 5 empty, 2 full. Therefore 18/20 viable seeds = 90 % viability-adjusted germination.

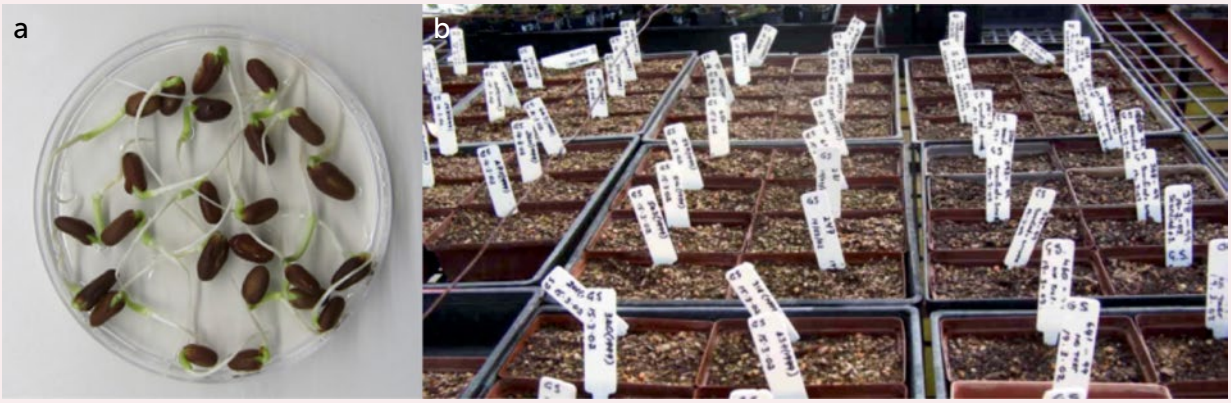

Figure 7.5: **(a)** Laboratory testing: Germination of *Acacia ramulosa* on water agar in Petri dishes after seeds were pretreated in 98 % v/v H_2SO_4 for 60 mins to break physical seed dormancy. **(b)** Nursery testing: Germination in soil filled punnets under nursery conditions for a range of native species. (Images: Shane Turner)

Box 7.5: Designing and analysing seed experiments for publication

Introduction

Design and analysis are important tools for understanding the highly variable germination responses of seeds, particularly when this variation is large as observed in many wild species. While small, non-replicated experiments can be useful to support day-to-day operations (e.g., to confirm viability or germination responses to a propagation treatment), the capacity to draw inferences from these data is limited and difficult to publish for a wider audience. Consequently, these results are unlikely to be as widely utilised as well-designed, analysed and published experimental results. Over the past decade there has been a substantial increase in the computing power and software available to present and analyse increasingly complex germination datasets. The analytical approaches currently recommended for seed-based research are outlined with reference to relevant literature.

Design

Purpose

The first step in designing an experiment is to consider the purpose of the trial and the specific questions you seek to answer. With clear questions, the experimental design can be refined to provide data that directly address the questions.

The seeds

In ecological or dormancy related studies, the experiment will ideally use the natural dispersal unit of the species, which may botanically be a fruit or seed (section 7.3.1).

Similarly, cleaned pure, seed, (**diaspore**) can also be a fruit or seed and it is useful to know which is being used, particularly in cases where fruits may contain multiple seeds. If the aim is to study a species' response without accounting for **maternal line**, population etc., ensure the collection is well mixed and a random sample is removed for testing. If within-species variation is expected and multiple collections of a species or population need to be mixed (rather than compared) consider how this will be balanced, recorded and managed.

Recording response variables

When structuring the way in which you record your results, be mindful of the analysis you want to perform and the data structure that will be required. This can save time and data handling downstream, making the analysis more efficient and minimising potential data errors.

Decide whether to record germination once at the end of the trial (e.g., 4 weeks) or periodically during the trial (e.g., weekly). At each germination check, record the time since the trial started (e.g., 5 days), the sample size (e.g., 25 seeds) and the number of seeds germinated or emerged. Once the experiment concludes, it is valuable to confirm the number of viable seeds in the sample via a cut test (see Chapter 5.5.3.1). This can then be utilised to calculate viability-adjusted germination (Box 7.5).

Recording and designing treatments

It is essential to have an untreated control for the purpose of comparing treatments. Detail not only the treatment type (e.g., heat, smoke or hormone) but also the concentration and exposure duration (e.g., soaked in 1 mM GA_3 for 48 hours). Consider if the design will include different levels of a treatment (e.g., heat for 10 mins with 3 levels of 80 °C, 100 °C and 120 °C). The design may have a combinatorial structure and therefore be orthogonal or fully factorial and allow analysis of the treatments (factors) individually, in combination, and test for interactions among them (e.g., effects of fire cues of heat singly, smoke singly, and combined heat and smoke and whether they have an additive or interactive effect).

Replication and balance

Each treatment (and all levels and combinations) should be repeated with separate sub-samples (replicates) of seed. To satisfy statistical requirements, 3 to 4 replicates of 25 seeds are often used for each treatment or level. Depending on the type of experiment and analysis there may be special approaches available for low seed availability (e.g., Davies *et al*. 2016). It is important to consider balance of the design and ensure the number of replicates are equal and the number of seeds per replicate does not vary (for some analyses number of seeds per replicate must be equal, for others minor variation is acceptable) across all treatment groups.

Beware of pseudo-replication where replicates are not statistically independent. For example, if testing the effects of a hormone on germination each replicate should be exposed to the treatment separately rather than treating one batch of seeds and then separating into (pseudo)replicates. (A surface sterilisation or smoke treatment applied to all seeds as a pre-treatment, for example, that is not the focus of the study, does not need to be applied to each replicate separately.) Another common example of pseudo-replication occurs when all four replicates are placed within one temperature-controlled chamber rather than across

four chambers. This can be managed within one chamber by randomising samples, or by incorporating blocking into the design (e.g., each replicate on a different shelf) and analysis (block as random factor e.g., Stevens *et al*. 2020).

Data summary and presentation

Summary statistics

Summary statistics are used to present the information recorded during a trial. The two most assessed components of germination are final germination and germination dynamics such as time to germination or germination rate. Summaries of mean final germination and germination dynamics can be calculated, presented in figures and statistically analysed.

Final germination is described as the proportion or percent germination (sometimes viability adjusted) (Box 7.5). It is important to also assess variation around the mean by calculating metrics such as a standard error or confidence interval.

The dynamics of germination can be described through numerous variables (e.g., time (mean time to germination (MTG)), rate (e.g., time for 50 % of final germination to be achieved, T_{50}), homogeneity and synchrony) (Ranal and Santana 2006).

Data presentation

While figures of mean and standard error are useful for interpreting germination results, a significant recent change has been the movement to options such as 'box and whisker' plots (Krzywinski and Altman 2014). Plots such as box and whisker illustrate the spread and differences of samples and are increasingly preferred for data that are often skewed/ not normally distributed, such as germination data.

When a trial seeks to investigate changes in germination over time, figures generally present the amount of germination (e.g., cumulative mean and standard error) over a series of time points, differentiating any treatments (e.g., by overlaying). Alternatively, non-cumulative germination can be plotted (e.g., the number of new germinants (rather than total) at each census). Calculated variables such as T_{50} or MTG are often presented to summarise the germination dynamics.

Statistical analysis of treatments

It is imperative to explore your data before beginning any statistical analysis. A valuable first step is to carry out a simple visual inspection of your data. This can be in the form of time series plots, histograms or 'box and whisker' plots. Understand the data structure and variation and ensure it is suitable for, or meets the assumptions of, the analysis you ultimately select. The following analysis options are commonly applicable to germination experiments, but the suitability of these approaches needs to be checked before applying them to a given dataset.

Final germination

Comparing two results

If assessing two results (e.g., a control and a treatment, samples of different ages, seeds from different storage conditions, viability versus germination) a **chi-squared test** (x^2) or a one-way ANOVA or equivalent, is used to test whether the probability of success (scored as viable or germinated) is the same for the two samples (Whitehouse *et al*. 2020), or whether the means of the two samples differ.

Factorial germination experiments

Multiple analyses are available for assessing the effect of different factors (treatments) on germination and viability. In the past, ANOVA was commonly used to compare treatment means. However, ANOVA is only appropriate for germination data that meet the assumptions of the analysis (e.g., assumptions that the residuals must follow a normal distribution across treatment groups and be independent, or that the variances must be homogeneous) (Sileshi 2012; Carvalho *et al*. 2018; Whitehouse *et al*. 2020). While wild species tend to have highly variable germination so may not to meet the assumptions, agricultural species with high germination and low variability may produce suitable data (Carvalho *et al*. 2018). For any species, ANOVA should only be utilised if the assumptions have been verified. The traditional approach of arcsine square root transforming data to better fit the assumptions is usually not sufficient. Explore the data and only use ANOVA or transformations if the assumptions can be met.

For **binomial** data (e.g., germination successes and failures), **generalised linear models** (GLM) (incorporating a logit link function for binomially distributed data) are preferred over traditional ANOVA (Hay *et al*. 2014; Carvalho *et al*. 2018; Whitehouse *et al*. 2020). This class of model incorporates terms for the fixed effects of one or more treatments. Differences among treatments can be compared using Chi or modified F statistics, though unlike ANOVA, the means will need to be back-transformed.

For more complicated experiments, it may be beneficial to employ Generalised Linear Mixed Models (GLMM). This class of model allows additional random effects to be fitted (variables likely to influence results, that are a random sample and have adequate sample size, but are not part of the question the experiment seeks to answer e.g., tests of collections from different populations, different maternal lines, or collections of different ages) (Bolker *et al*. 2009; Sileshi 2012; Gianinetti 2020; Whitehouse *et al*. 2020). For example, effects related to individual germination dishes could be fitted as random effects. For random effects a variance component is reported rather than a χ value. For this reason, effects of primary interest are usually fitted as fixed effects. An exception can be found in experiments that involve genetic partitioning, variance components may be obtained to calculate heritability or other genetic parameters – for example in a germination trial involving 80 individual families, the families could be fitted as a random effect and the heritability of the germination trait calculated. GLMM can also be used to look for patterns in treatment responses across multiple species, in which case their **phylogenetic** relatedness can be accounted for in the random component of the model. An emerging direction in ecology is to run models such as GLMMs using a **Bayesian** approach to statistics. Although not yet widely applied to seed germination data, a Bayesian approach may be beneficial as it can deal with complex models with many random effects, more easily compute the uncertainty of functions of

random variables, introduce prior external knowledge to the analysis, and make probability statements about the parameter of interest rather than the dataset (Bolker *et al*. 2009; Kéry 2010).

Germination dynamics

Repeatedly assessing germination of a sample of seeds over days or weeks can help determine dormancy alleviation and/or germination requirements, understand seed vigour (e.g., speed of germination), or understand responses of seeds to stress or stimulants (Whitehouse *et al*. 2020). If the same samples are repeatedly scored then time-to-event model fitting should be used for analysis of the germination curve (McNair *et al*. 2012; Hay *et al*. 2014; Whitehouse *et al*. 2020).

If independent samples are used, for scoring just once and then discarding (e.g., a series of samples from a stored collection, comparative **longevity** study, or seed burial experiment), a **probit analysis** (GLM or GLMM with a probit link function) might be used to analyse the data. Probit analysis is the basis of the Ellis-Roberts viability equations and comparative longevity methods (Merritt *et al*. 2014; Whitehouse *et al*. 2020; see Chapter 5).

Specialised designs

Hydrothermal time

To build an understanding of thresholds or germination niche, the germination response to many levels of a treatment, often spanning a gradient of stress or stimulants, is investigated (e.g., temperature gradients and/or water gradients). Methods such as hydrothermal time analysis can be applied to calculate base, optimum and ceiling temperature and/or water potential for germination (Alvarado and Bradford 2002; Mesgaran *et al*. 2017).

Move along

A move along experiment (section 7.6.2.2) can provide valuable insights into the germination ecology of species, particularly the season of germination and whether germination is postponed or staggered, post dispersal. These experiments apply treatments that simulate the sequence of seasons over a year or more and collect germination data over time (Baskin and Baskin 2003). Generally, one sequence or series of temperatures will begin in summer, and the other series will begin in winter. There are multiple controls because seeds must also be exposed individually to each of the temperatures in the sequences for the duration of the experiment. Depending on the specific aim of the experiment, data can be analysed using numerous pairwise comparisons (of a subset of the data from a timepoint during the experiment e.g., end of a simulated season of interest against one of the controls), a GLM treating each of the controls and series as treatments, or by fitting curves to the germination data over time and comparing those curves (Hoyle *et al*. 2015).

Analysis tools

Packages for summarising and analysing seed germination data are becoming increasingly available.

Examples include:

GerminaR (Lozano-Isla *et al*. 2019), a free R package for germination analysis.

Germinator package (Joosen *et al*. 2010), a software package that contains 3 modules to assist with experiment design for any species, automatic scoring of *Arabidopsis* germination from images, and curve fitting of cumulative germination data and extraction of variables for any plant species.

SeedCalc (Silva *et al*. 2019), a free R package for processing germination and seedling length data.

References

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7.4 Seed dormancy classification

A worldwide review of seed dormancy undertaken by Baskin and Baskin (2003) suggests the most common forms of dormancy found in Australia are likely to be physical dormancy and physiological dormancy. There is a wealth of Australian research on seed dormancy (see examples Sommerville *et al*. 2013; Commander *et al*. 2017; Cross *et al*. 2017; Kildisheva *et al*. 2018; Duncan *et al*. 2019) providing critical information on the need for seed dormancy classification to identify seed treatments that are likely to result in successful germination. A dormancy classification key has been developed by Baskin and Baskin (2004a) and is presented in Box 7.6. There are still many Australian species with dormancy mechanisms that are yet to be investigated, and others that require significant work to alleviate dormancy before seeds can be reliably utilised from banked collections (Figure 7.6).

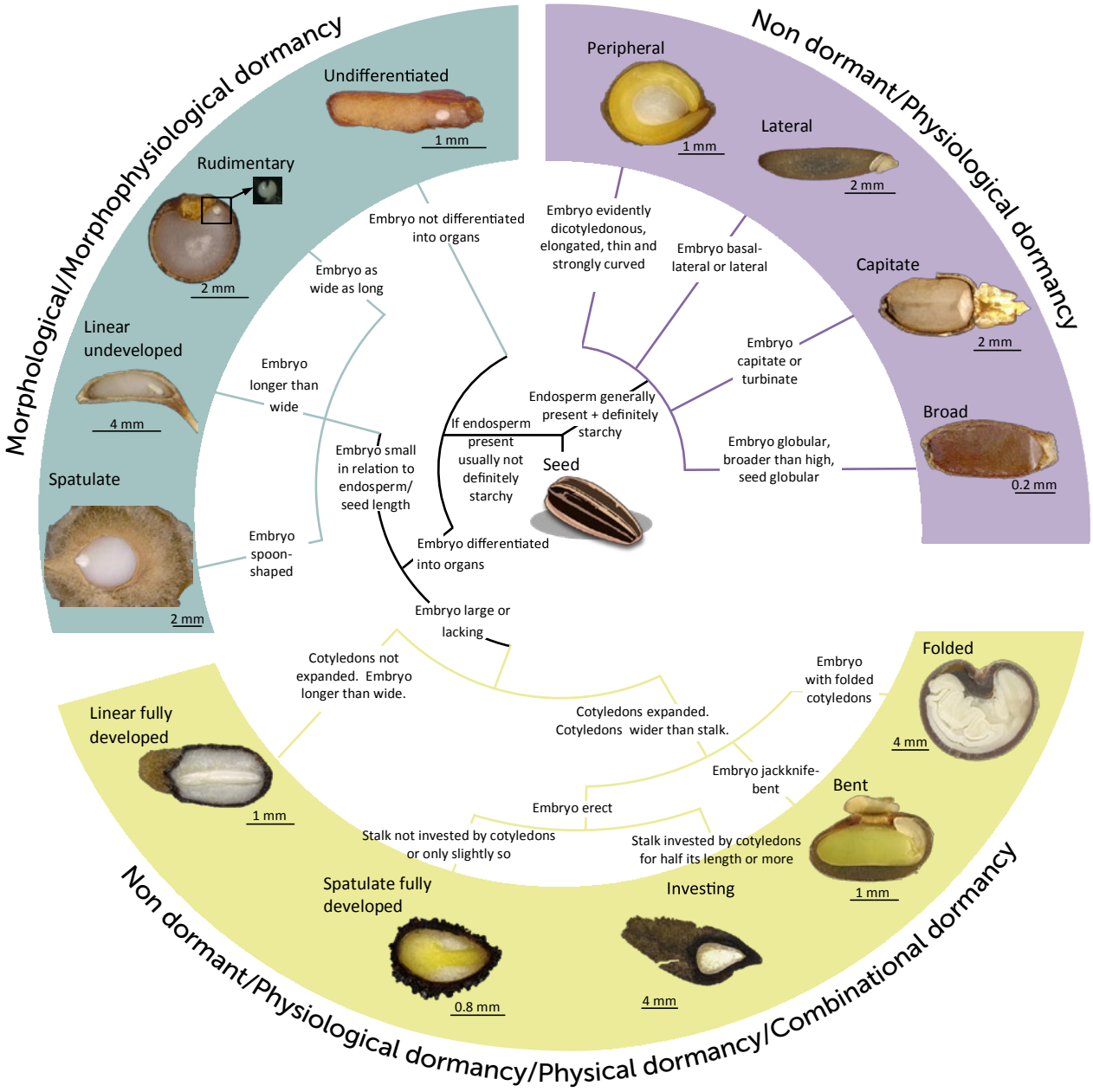

Figure 7.6: Schematic dichotomous key outlining the main embryo types using Australian examples based on a standardised set of seed and embryo attributes. Adapted from Martin 1946 and Baskin and Baskin 2007. (Developed by Shane Turner, Kerryn Chia, and Lucy Commander).

Box 7.6: Key for dormancy classes (adapted from Baskin and Baskin 2004a)

1. Seeds begin to germinate within 30 days without treatment **2**

    2. Embryo differentiated and fully developed (elongated) ..... **non dormant**

    2. Embryos small and underdeveloped and grow (elongate/develop) for a period within the seed prior to germination ..... **MD**

1. Seeds do not germinate within 30 days without treatment **3**

    3. Seed/fruit coat NOT PERMEABLE TO WATER (imbibition test), embryo fully developed **4**

        4. Germination occurs within 30 days when seed/fruit coat is scarified or nicked ..... **PY**

        4. Germination DOES NOT occur within 30 days after seeds/fruit coat is scarified, although seed becomes fully imbibed following scarification ..... **PY + PD**

    3. Seed/fruit coat PERMEABLE TO WATER: embryo either fully developed or underdeveloped **5**

        5. Embryo fully developed ..... **PD**

        5. Embryos small and underdeveloped and do not begin to grow (elongate/develop) inside the seed within 30 days ..... **MPD**

KEY

MD	**Morphological dormancy**
PY	**Physical dormancy**
PD	**Physiological dormancy**
PY + PD	**Physical dormancy + Physiological dormancy**
MPD	**Morphological dormancy + Physiological dormancy**

7.5 Seed dormancy classes

If seeds do not germinate readily within ~4 weeks (when incubated under suitable conditions), they are either non-viable, require a germination stimulant, or are dormant.

An estimated 50 to 90 % of wild species produce seeds with some form of seed dormancy (Kildisheva *et al*. 2020). Species in some large Australian plant families produce seeds with well-recognised seed dormancy attributes that can be readily overcome (e.g., Fabaceae, Poaceae, Asteraceae). However, many other dominant plant families (e.g., Cyperaceae, Dasypogonaceae, Dilleniaceae, Ericaceae, Restionaceae and Rutaceae) have dormancy attributes that are not yet well-understood. Work is in progress as seed scientists continue to study these groups and understand how best to alleviate dormancy to gain reliable germination. Seed dormancy regulates the germination process through various physical and/or physiological means that may block/suppress the capacity for seeds to imbibe water, for the embryo to grow/develop within the seed, or for the embryo to have sufficient 'push power' to overcome the constraints of the outer seed layers (Baskin and Baskin 2014). Seed dormancy is either imposed by the embryo or by the seed coat or outer coverings, and dormancy type can be assigned to one of five main classes depending on the exact mechanism(s) (Baskin and Baskin 2004a).

Once the type of seed dormancy is defined for a particular species, a targeted approach can then be adopted to select pre-treatments that may overcome the block to germination. The five main classes of seed dormancy are as follows.

7.5.1 Physical dormancy (PY)

Physical dormancy is imposed by a thin (0.1 to 1 mm in thickness) water impervious palisade layer of cells found in the outer seed coat (e.g., *Acacia* spp.) or indehiscent fruit coat (e.g., *Stylobasium* spp.) that stops all water movement into the seed until this barrier is breached in some way. Water uptake is regulated by a small, specialised region of the seed or fruit coat, termed the water gap, that opens when seeds are exposed to appropriate environmental conditions.

In the laboratory or nursery, common methods to artificially break this type of dormancy include:

- Scarification of the water-impermeable coat by either precision nicking or chipping to create a small hole in the outer seed coat (e.g., using a scalpel) or through gently rubbing the seed coat on sandpaper (see Section 7.6.2.4, and Case Study 7.4 on precision nicking).
- Heat treatment either using hot water or oven.
- Concentrated acid treatment for one to several hours. NB Take appropriate care of safety, chemical handling and appropriate PPE (personal protective equipment).
- Dry oven treatment (see Box 7.7, and Case Study 7.1 on dry heat shock).

In nature, seed coats may become water permeable by repeated heating and cooling in the soil seedbank during summer, or due to a disturbance event like the passage of a fire which can rapidly heat the top part of the soil to over 100 °C for several minutes (Tangney *et al.* 2020). Physical dormancy is known within 18 plant families world-wide (Table 7.2), including 10 found in Australia, namely the Lauraceae (e.g., *Cassytha* spp.), Nelumbonaceae (e.g., *Nelumbo* spp.), Geraniaceae (e.g., *Erodium* spp.), Convolvulaceae (e.g., *Convolvulus* spp.), Malvaceae (e.g., *Sida* spp.), Fabaceae (e.g., *Acacia* spp.), Rhamnaceae (e.g., *Pomaderris* spp.), Sapindaceae (e.g., *Dodonaea* spp.), Bixaceae (e.g., *Cochlospermum* spp.) and Anacardiaceae (e.g., *Rhodosphaera* spp.). See Figure 7.7 on the phylogenetic placement of plant families where the occurrence and location of PY has been observed.

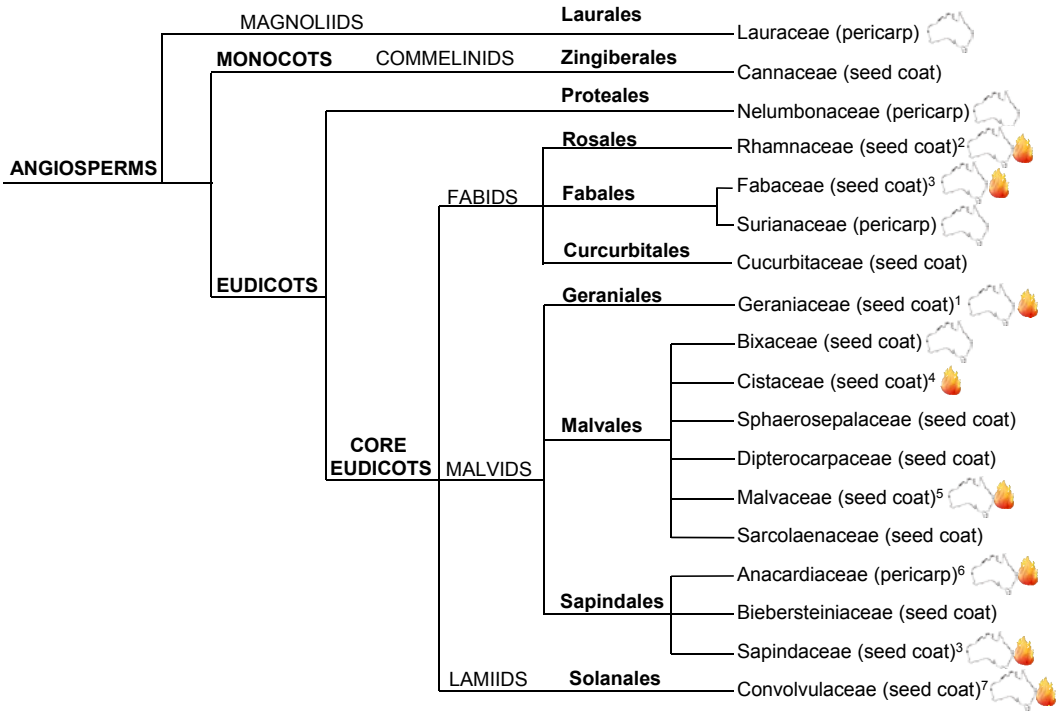

Figure 7.7: Phylogenetic tree showing the placement of plant families where the occurrence of physical dormancy has been confirmed in at least one species and the location of the water impermeable barrier i.e., seed coat (testa and/or tegumen of the seed coat) or pericarp (endocarp). Families with flames next to them indicate that at least one species within this family has been recorded germinating in situ following a fire. Australia refers to families with species native to Australia. Modified and adapted from Baskin & Baskin 1998; Baskin et al. 2000, 2006; Gama-Arachchige et al. 2013; and the Angiosperm Phylogeny Group (2016).[48] (Developed by Shane Turner, Kerryn Chia, and Lucy Commander)

References that validate the flames for fire responsive families are as follows:

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48 http://www.mobot.org/MOBOT/research/APweb/

However, it must be remembered not all **taxa** found in these families will have physically dormant seeds (Table 7.2).

Table 7.2: Physical Dormancy (PY) Families (listed in Baskin and Baskin 2014) with prevalence comments and water barrier type.

Family	PY Prevalence	Barrier Type
Anacardiaceae	*Rhus* and allied genera	Fruit coat
Biebersteiniaceae	All (single genus family)	Seed coat
Bixaceae	Dominant	Seed coat
Cannaceae	All (single genus family)	Seed coat
Cistaceae	Dominant	Seed coat
Convolvulaceae	Majority of genera	Seed coat
Cucurbitaceae	*Sicyos* only	Seed coat
Dipterocarpaceae	One subfamily reported	Seed coat
Fabaceae	Dominant, but not all taxa	Seed coat
Geraniaceae	Dominant	Seed coat
Lauraceae	*Cassytha* only	Fruit coat
Malvaceae	Dominant	Seed coat
Nelumbonaceae	All (single genus family)	Fruit coat
Rhamnaceae	Dominant	Seed coat
Sapindaceae	Dominant in a minority of genera only	Seed coat
Sarcolaenaceae	Madagascan family, one genus reported	Seed coat
Sphaerosepalaceae	Madagascan family, one genus reported	Seed coat
Surianaceae	One genus reported	Fruit coat

7.5.2 Physiological dormancy (PD)

Physiological dormancy is the most common dormancy form worldwide. Seeds with PD are permeable to water and have a fully developed embryo (as found in non-dormant seeds), however the embryo has low growth potential, and a mechanical restriction to embryo growth is imposed by the surrounding tissues (e.g., endosperm, seed, or fruit coat). To alleviate PD the seed must be exposed to appropriate environmental conditions to increase the growth potential of the embryo and allow radicle penetration through the surrounding tissues.

Environmental conditions that alleviate PD vary between species and regions.

Physiological dormancy may be alleviated by:

- Moist stratification in warm (≥ 20 °C) or cool (1–10 °C) temperatures for several weeks or months prior to incubation at a different temperature appropriate to germination (e.g., 15 to 20 °C) (see Section 7.6.2.2); or,
- A period of dry after-ripening prior to incubation at a temperature appropriate to germination (see Section 7.6.2.1); or

- Rapid cycles of wetting and drying (see Section 7.6.2.3).
- Treatment with gibberellic acid.
- Precision nicking above the radicle tip to relieve the mechanical constraints restricting radicle growth (see Section 7.6.2.4, Case Study 7.4).

Physiological seed dormancy can also be classified as either primary or secondary dormancy. **Primary dormancy** is imposed whilst seeds are maturing on the parental plant, so seeds are released in a dormant state into the soil seed bank and require exposure to specific environmental conditions to become non-dormant. **Secondary dormancy** refers to seeds which have become non-dormant due to favourable environmental conditions but that do not receive suitable germination conditions and subsequently re-enter dormancy (Baskin and Baskin 1998; Black *et al*. 2006). Seeds with PD can cycle between dormant and non-dormant states with the changing of seasons over many years in the soil seed bank. See Case Study 7.6 on the long-term burial and retrieval of seeds of a critically endangered species (*Symonanthus bancroftii*).

7.5.3 Morphophysiological dormancy (MPD)

Morphophysiological dormancy is another dormancy class that is common in some Australian plant families. Many species from the Apiaceae, Dilleniaceae, Haemodoraceae, Stylidiaceae and Ranunculaceae have seeds with morphophysiological dormancy (Baskin and Baskin 2014). Like embryos found in seeds with morphological dormancy (see section 7.5.4 below), a defining characteristic of seeds with MPD is that they have underdeveloped embryos at the time of **dispersal** (Figure 7.8a). However, seeds with MPD additionally have a physiological constraint to embryo growth – as for seeds with physiological dormancy (Section 7.5.2). Therefore, seeds with morphophysiological dormancy have both morphological (section 7.5.4) and physiological dormancy. For germination to occur the embryos must grow inside the seed to a critical size, and PD of the embryo must be alleviated (Figure 7.8b).

This requires:

- Exposure to appropriate conditions to alleviate embryo dormancy, for example, temperature changes such as after-ripening or stratification.
- Suitable conditions to allow embryo maturation and growth inside the seed; conditions are likely to be similar to those supporting germination.

Morphophysiological dormancy can be particularly complex and there are nine levels of sub-classification of MPD. In some species, seed embryo growth and dormancy break occur under the same environmental conditions, but in other species different conditions are needed for each process (Baskin and Baskin 2014).

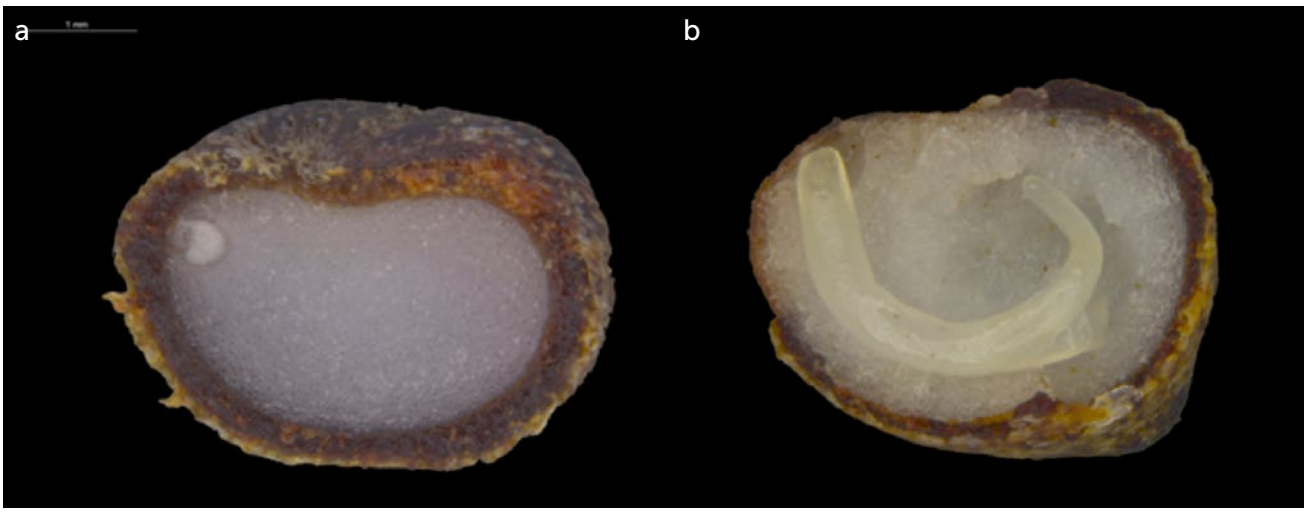

Figure 7.8: **(a)** Closeup example of the internal structure of *Marianthus erubescens* seeds (Pittosporaceae) which possess MPD. Seeds have underdeveloped embryos that grow within the seeds for a period of time prior to radicle emergence and germination. Prior to embryo growth the embryo is tiny (< 300 µm – left image – top left corner of the seed visible as a small bean-like structure); **(b)** After several weeks growth the embryo is significantly larger (>2 mm long) with clearly defined cotyledons now visible. (Images: Arielle Fontaine)

7.5.4 Morphological dormancy (MD)

Morphological dormancy refers to seeds that are permeable to water, but that have an embryo that is not fully developed at the time of seed dispersal. The embryo is commonly tiny compared to the rest of the surrounding endosperm and the size of the seed. The embryo may be differentiated (i.e., cotyledon(s) and a radicle can be distinguished) but underdeveloped, or undifferentiated. This form of dormancy is rare, being found in only ~80 plant families worldwide (Baskin and Baskin 2014). An Australian example of MD (Figure 7.9) can be found in the **endemic** genus *Burchardia* (Colchicaceae), which occurs across SE and SW Australia, with 5 of 6 species endemic to the SW (Kildisheva *et al*. 2020). For these seeds to germinate, the embryo requires time to mature and grow within the seed following imbibition to a critical size at which germination (radicle emergence) is possible. This generally occurs within a month of incubation under appropriate conditions, with the embryo growing several times its original length prior to radicle emergence.

Figure 7.9: Close-up example of the internal structure of *Burchardia congesta* seed (Colchicaceae) which has MD. Seeds of *B. congesta* have a small undifferentiated embryo <1 mm in length compared to the remaining seed. (Image: Arielle Fontaine; This figure is a derivative of Figure 3 in Kildisheva *et al*. (2020) and is used under CC BY 4.0.)

7.5.5 Combinational dormancy (PY + PD)

Seeds with combinational dormancy have a seed or fruit coat that is impervious to water and thus have physical dormancy, as well as a physiologically dormant embryo (Baskin and Baskin 2014). Some species within the Fabaceae, Malvaceae, Rhamnaceae and Sapindaceae are known to have combinational dormancy.

In these species:

- The seed coat must be scarified to allow water uptake.
- The embryo must then be cued to provide sufficient growth potential to overcome the mechanical restraint of the surrounding tissues, either through after-ripening or stratification.

7.6 Approaches to overcoming dormancy in Australian species

Dormancy classification is an extremely useful first step to eliciting germination of a viable seed for which little or no specific information is available. Treatments can then be designed based on those known to be effective for that dormancy class. Seeds differ in the complexity of treatments required for dormancy loss and the rate at which this occurs. Germination experiments may vary from a few days to weeks or months in duration, even up to one or two years or more in extreme cases (Turner 2013; Commander *et al*. 2017). In the case of taxa that have deeply dormant seeds, it is often useful to develop a greater understanding of ecological aspects of dormancy loss/induction **in situ** to pinpoint key environmental factors (i.e., specific moisture and temperature regimes) regulating dormancy status. Laboratory experiments can be based on environmental data such as soil temperatures and the time of year when most rainfall occurs. Careful experimental design, documentation and patience is necessary. Dormancy can sometimes be alleviated by more than one factor, with seeds requiring exposure to multiple cues to break dormancy and maximise germination (e.g., warm stratification + smoke treatment).

7.6.1 Alleviating physical dormancy

To determine whether a species has physical dormancy, an imbibition test (Section 7.3.2, Box 7.4) is conducted. If imbibition testing confirms the presence of physical seed dormancy, there are several options to removing the barrier to water uptake. For small numbers of seed, scarification is possible via manual nicking of the seed coat, gently rubbing seeds on an abrasive paper, or through using a mechanical scarifier (Kildishieva *et al*. 2018). Hot water treatment (80–95 °C for 30 seconds to 5 minutes) is also highly effective for breaking dormancy in many species (though not all) (Box 7.7), as is exposure to concentrated acid (98 % v/v H_2SO_4, use caution and appropriate Personal Protective Equipment (PPE)) for several minutes to many hours, followed by rinsing in water and washing in a weak sodium bicarbonate solution (100 mM $NaHCO_3$) to neutralise any remaining acid (Turner and Dixon 2009). Dry heat treatment is also an option (see Case Study 7.1 as an example using dry heat treatments). For species which have combinational dormancy (i.e., PY + PD), seeds must firstly be made water permeable then require some additional treatment such as stratification or dry after-ripening to overcome non-deep PD (Turner *et al*. 2006a).

Box 7.7: Hot water treatment to break physical dormancy

The easiest treatment to use, hot water ruptures a small region in the seed coat known as the water gap, allowing water entry (see comparative Figure 7.10a untreated seeds and Figure 7.10b treated seeds). Hot water treatment is only applicable to species with physical (or combinational) dormancy though not all species with physical seed dormancy respond well to this treatment, so care is needed. No other dormancy type appears to be overcome with hot water treatment and when used inappropriately it can easily kill seeds. For species with combinational dormancy, both the physical and physiological component must be overcome before germination is possible. However, for species where the physiological component appears to be non-deep, typically a short period of dry after-ripening (Section 7.6.2.1) or stratification (Section 7.6.2.2) will suffice.

Method: A water temperature greater than 90 °C is used, although cooler temperatures (>60 °C) can also be effective for more sensitive species (e.g., *Senna* spp.). Actively boiling water should be avoided (i.e., immersing seeds into boiling water on a stove), as it is easy to damage the seeds. A simple method is to boil a kettle and then let the water cool for a few minutes before immersing the seeds for between 30 seconds and 5 minutes, depending on the species, the seed size, and the relative hardness of the seed coat.

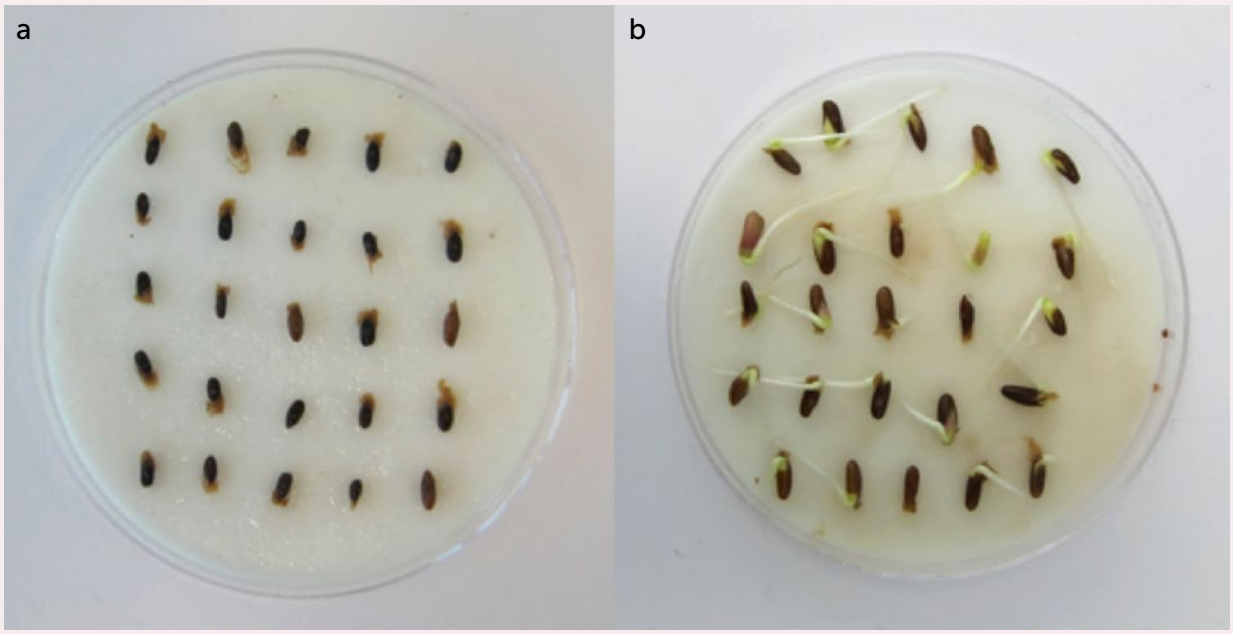

Figure 7.10: Alleviating PY in *Acacia grasbyi*: **(a)** untreated seeds of *Acacia grasbyi* after four days incubation at 20 °C; **(b)** after seeds were pretreated in hot water for 1 minute to rupture the hard seed coat prior to incubation then incubated at 20 °C four days. (Images: Shane Turner)

Case Study 7.1: Physical dormancy in Tasmanian *Pomaderris* (Rhamnaceae) collections: Heat shock and mould

James A. Wood

The presence of Physical Dormancy (PY) in seeds of Rhamnaceae is widely recognised (Hanley and Lamont 2000; Turner *et al.* 2005; Ooi *et al.* 2014). An efficient technique to alleviate PY in large quantities of seeds arose as part of a larger study of dormancy and germination between *Pomaderris* species across different Tasmanian **provenances**. Dry heat shock treatment was selected for investigation as hot water treatments were considered cumbersome when processing large quantities of small seeds in a short amount of time.

Initial investigations were conducted on *Pomaderris pilifera* (Wood 2020). Heat shock (HS) treatments were applied with a fan-assisted laboratory oven and two pans filled with 1.5 cm dry sand (Figure 7.11a). Seed samples were placed in a single layer within folded aluminium foil envelopes and placed on the heated sand (Figure 7.11b). A second tray placed on top sandwiched the envelopes between two bodies of heated sand (Figure 7.11c) and envelopes retrieved after the allotted HS time (Figure 7.11d). Three temperatures (90, 105, 120 °C) and four times (1, 2, 5, 10 mins) were trialed. A control treatment and a manual scarification trial were conducted to gauge the effectiveness of the HS treatments. Trials consisted of three x 40 seeds sown on 9 cm Petri dishes of 1 % agar. Remaining seeds were cut tested at the end of the test. Treatments and results are presented in Table 7.3.

Figure 7.11: Sand pan heat shock application.

Table 7.3: Heat shock trial results.

Treatments	mean %	s.e.	t50 (d)
15 °C (control)	0.8	0.8	14.0
Chip-> 15 °C	30.8	5.5	22.5
HS (90 °C/1m)-> 15 °C	34.7	1.4	30.3
HS (90 °C/2m)-> 15 °C	41.5	2.3	25.7
HS (90 °C/5m)-> 15 °C	62.7	7.5	27.7
HS (90 °C/10m)-> 15 °C	78.2	5.4	29.4
HS (105 °C/1m)-> 15 °C	84.6	3.9	26.3
HS (105 °C/2m)-> 15 °C	89.2	4.4	25.7
HS (105 °C/4m)-> 15 °C	80.7	4.3	25.7
HS (105 °C/10m)-> 15 °C	88.3	4.1	25.7
HS (120 °C/1m)-> 15 °C	87.3	0.1	24.7
HS (120 °C/4m)-> 15 °C	89.2	5.8	24.1
HS (120 °C/10m)-> 15 °C	94.1	3.1	24.6

The control test confirmed the presence of PY in the *Pomaderris* collection. Surprisingly the HS trials proved to be far more effective than the manual scarification trial, partly due to seeds succumbing to mould. Short durations at 90 °C proved to be ineffective at alleviating PY, as most seeds were not imbibed. Treatments at 105–120 °C were effective at alleviating PY with little difference in final germination or germination rate. Decreasing mould on seeds and **elaiosomes** were observed as treatments became hotter and longer. At 120 °C for 10 minutes, almost no mould growth was observed.

Further testing was conducted with *Pomaderris elliptica* to assess the upper tolerance for HS treatments, with temperatures of 100, 120, 140 and 160 °C applied for 5, 10 and 15 minute intervals. The results confirmed that 120 °C for 10 minutes is within a safe optimal range for breaking physical dormancy with good results at 5, 10 and 15 minutes. 140 °C for 5 minutes killed nearly all seeds and durations and temperatures above that were lethal. These findings are in line with other reports of HS treatments in Rhamnaceae and Fabaceae.

Routine testing of *Pomaderris* collections within the TSCC identified a small number of collections (9 out of 55) succumbing to mould very rapidly, thus reducing total germination. Slow imbibition of seeds over water (after PY alleviation but before sowing on agar) or sowing seeds on compost instead of agar reduced this problem, which may be a result of imbibition shock.

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Wood JA (2020) Dealing with physical dormancy in Tasmanian Pomaderris (Rhamnaceae) collections: Heat shock, seed size and mould issues. *Australasian Plant Conservation* **29** (3), 20-21.

7.6.2 Alleviating physiological and morphophysiological dormancy

There are a variety of methods for promoting germination of seeds with physiological (PD) or morphophysiological (MPD) dormancy. Physiological dormancy may be alleviated via dry after-ripening, stratification at warm or cool temperatures, or combinations of these treatments, including cycles of wetting and drying. The use of germination promoting compounds (i.e., GA_3, smoke, KAR_1 or **ethylene**) in combination with these treatments may also be necessary following the dormancy alleviating treatment. For seeds with PD or MPD, an understanding of the time of year and environment into which they are shed is vital to selecting treatments. For example, seeds from many species from the Mediterranean and arid climatic zones that are shed into dry soil during the warm to hot temperatures of late spring and summer often require weeks or months of after-ripening (i.e., warm dry storage). Grasses (Poaceae) and daisies (Asteraceae) are particularly responsive to this type of treatment.

Seeds may also respond to warm stratification; conditions analogous to those experienced during the first rains of autumn in many parts of southern Australia (Turner *et al*. 2006b). Such species often have a low to cool temperature preference (10 to 20 °C) for germination that aligns with temperatures experienced during winter. In contrast, many species originating in cooler temperate climates such as parts of Tasmania and alpine regions in New South Wales and Victoria respond to cold stratification, as the seeds over-winter in the snow following shedding in autumn and subsequently germinate the following spring or even summer as the temperature warms the soil (Sommerville *et al*. 2013). Some of these species require warmer temperatures (>15 °C) to initiate germination to minimise exposure to late frosts (Hoyle *et al*. 2015).

7.6.2.1 After-ripening

A dry after-ripening requirement for dormancy loss appears to be common in the Australian flora, with species of Apiaceae, Asteraceae, Brassicaceae, Haemodoraceae, Poaceae, Solanaceae and Stylidiaceae documented as responding to this treatment (Merritt *et al*. 2007; Hoyle *et al*. 2008; Turner *et al*. 2009; Commander *et al*. 2009; Erickson *et al*. 2016). Seeds that after-ripen do so more rapidly at warmer temperatures and are usually placed at a constant temperature between 20–35 °C to facilitate dormancy loss. Constant temperatures can be achieved using an incubator or laboratory oven, which experimentally is the preferred way to apply an after-ripening treatment. During the after-ripening treatment, control of seed **moisture content** (**relative humidity**, RH), is also desirable for efficient and repeatable results, as there is often a narrow range of seed moisture contents, equivalent to those between 10–50 % RH, that are more effective (Hoyle *et al*. 2008; Turner *et al*. 2009; Erickson *et al*. 2016). Factorial experiments testing germination of seeds stored at different water contents and temperatures are useful to identify conditions that will effectively alleviate dormancy in the shortest time. But a good mid-point that should be effective for most species is 25–30 °C and 50 % RH. Generally, lower RH are more effective at higher temperatures (and avoid risks of rapid aging) while slightly higher RH are preferable at lower temperatures, but there is considerable variation in the rate of after-ripening across different species in response to changes in these conditions (Commander *et al.* 2009).

To alter the storage relative humidity, seeds are placed in air-tight plastic boxes such as polycarbonate electrical enclosure boxes (Figure 7.12a) containing a LiCl salt solution to regulate RH and sealed (Figure 7.12b). Altering the concentration of LiCl will vary the RH within the box which will subsequently affect the seed moisture content as the seeds equilibrate to the surrounding environment (Hay *et al.* 2008). Seeds can either be left in the box permanently (recommended to check regularly as conditions within the box may change over time) or

removed once equilibrated and hermetically sealed in laminated foil bags and stored at the same temperature. Seed moisture content (MC) can be determined gravimetrically (gH_2O/g dry weight) to confirm the seed MC that was present while seeds were after-ripening. Alternatively, the relative humidity (RH) and temperature of air in the sealed container can be monitored with the use of a small data logger such as a T-Tec[49]. An alternative approach to adjust the storage relative humidity is to use a programable incubator which can generate different relative humidity environments as needed. The incubator is set to the desired temperature and relative humidity and the seeds are placed inside in a porous breathable container such as paper envelopes, or cloth bags.

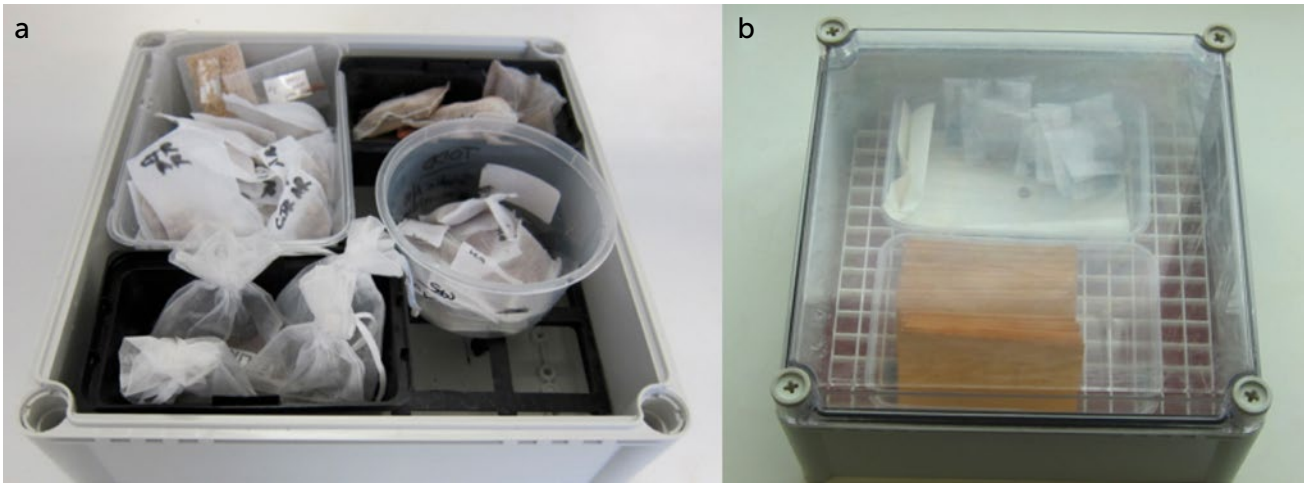

Figure 7.12: **(a)** Commercially available, sealable electrical enclosure boxes are ideal for aging and after-ripening seeds at a specific relative humidity. Seeds are stored over a saturated salt solution which precisely adjusts seed moisture content as seeds equilibrate to the surrounding relative humidity, which can be set anywhere from <10 % to 100 % (Hay *et al.* 2008). Seed moisture content directly affects seed aging and dormancy status over time; **(b)** Lid attached and unit is sealed, ready for use. (Images: Shane Turner)

For some situations (e.g., community seed banks or native plant nurseries), a more basic approach to after-ripening may be all that is needed. A simplified approach to after-ripening is to store seeds on an indoor bench at room temperature (c. 23 °C) for several weeks or months, or to store seeds inside a dry shed (preferably with some form of air conditioning during summer). At regular intervals seeds can then be tested for germination as needed, with and without germination promoting compounds, as in some cases these may be needed following after-ripening (Merritt *et al.* 2007).

On occasions, particularly for species of arid regions, higher temperatures of 40–45 °C can be used to accelerate the after-ripening process, although care must be taken to prevent rapid seed aging and seeds must be at a low water content, equivalent to c. 10 % RH (Commander *et al.* 2009). For some species, accelerated after-ripening can be achieved through exposure to short pulses (e.g., 10–180 mins) of very high temperatures (e.g., 80–120 °C). This treatment has been proved to be effective in various species of Cyperaceae, Haemodoraceae, Poaceae, Restionaceae, Rutaceae and Stylidiaceae (Tieu *et al*. 2001a; Merritt *et al*. 2007; Turner *et al*. 2009; Turner 2013; Mackenzie *et al*. 2016; Cross *et al*. 2017).

49 https://www.t-tec.com.au

7.6.2.2 Stratification

Stratification refers to the moist incubation of seeds at warm (≥ 20 °C) or cool (1–10 °C) temperatures, prior to incubation in the light at an optimal germination temperature (e.g., 15 to 20 °C), and is a technique known to alleviate physiological and morphophysiological dormancy in a broad range of species (Baskin and Baskin 2014). Many studies have demonstrated the applicability of stratification to a diverse assortment of Australian taxa, including several previously considered to produce 'deeply dormant' seeds. Several south-west Western Australian species, including those of Asparagaceae, Iridaceae, Byblidaceae, Elaeocarpaceae, Zygophyllaceae, Dilleniaceae, and Pittosporaceae respond to a brief period (6 weeks) of warm (> 25 °C) stratification (Turner *et al*. 2006b; Merritt *et al*. 2007;Tuckett *et al*. 2010; Hidayati *et al*. 2012; Cross *et al*. 2013). Cool stratification (at 5–10 °C) has been shown to be effective for some species of *Eucalyptus*, and *Allocasuarina*, *Wollemia nobilis* and many alpine species (Beardsell and Mullett 1984; Moncur *et al*. 1997; Offord and Meagher 2001; Sommerville *et al*. 2013).

One approach to testing stratification is to use a 'move along' experimental design (Baskin and Baskin 2003) (Table 7.4). Seeds are placed at a range of constant or alternating temperature regimes indicative of the different seasons (i.e., spring, summer, autumn, and winter) from where the focal species is naturally found (Table 7.5) (Turner *et al*. 2006b; Hidayati *et al*. 2012). For problematic or data-deficient species, alternating temperature regimes may be preferable to constant temperatures, as these are likely to be more reflective of natural temperatures and have been shown to be more effective in promoting germination in several species (Cochrane *et al*. 2011). In a 'move-along' experiment, seeds incubated on a moist substrate are moved step wise every 4–8 weeks from cooler to warmer (e.g., winter, spring, summer, autumn, winter…) or warmer to cooler (e.g., summer, autumn, winter…) temperatures (Baskin and Baskin 2003; Kildisheva *et al*. 2020). The experiments can be run concurrently to test if seeds respond to warm and/or cold stratification as seeds move across different seasons. If seeds respond to stratification, then increased germination should be observed after seeds are moved to a higher or lower temperature (relative to the control seeds incubated at one temperature regime for the entire duration of the experiment). It may take two or more cycles to alleviate dormancy in some species (Baskin and Baskin 2003). Additionally, seeds may be either incubated under alternating light/dark conditions (e.g., 12 hr light/12 hr dark) or constant dark conditions during the move-along experiment, as both factors have been shown to interact and either promote or suppress germination (Tuckett *et al*. 2010). Unless prior knowledge is available on the response of the study seeds to different light conditions, it is recommended to start with a 12 hr/12 hr diurnal light/dark regime.

A simpler approach to stratification is to incubate imbibed seeds at a single stratification temperature either ~5 °C (cold stratification) or ~25 °C (warm stratification) for 4–12 weeks, and then move the seeds to a second temperature appropriate to support germination (e.g., 15 to 20 °C), with control seeds left permanently at each temperature for comparative purposes (Tuckett *et al*. 2010; Dalziell *et al*. 2018).

The assistance of volunteers with the time-consuming work of germination testing, including the move-along approach, has been helpful in conservation seed banks including the Tasmanian Seed Conservation Centre (see Case Study 7.2 for further information on how germination testing is performed at the Tasmanian Seed Conservation Centre).

Table 7.4: **'Move along' experimental approach.** Proposed experimental approach for determining whether seeds respond to cold or warm stratification (modified from Baskin and Baskin 2003; 2004b). Seasons are used here as a proxy for specific temperatures which will vary depending on the location from where the seeds were originally collected. NOTE: In most cases, the same temperature regime can be used for both spring and autumn but there may be instances where separate regimes are required.

Incubation time (week number)	Temperature regimes indicative of different seasons from where the species is naturally found				
	Control treatments (constant temperature regimes)			'Move along' treatments	
0	Winter	Spring/Autumn	Summer	Winter	Summer
6	Winter	Spring/Autumn	Summer	Spring/Autumn	Autumn/Spring
12	Winter	Spring/Autumn	Summer	Summer	Winter
18	Winter	Spring/Autumn	Summer	Autumn/Spring	Spring/Autumn
24	Winter	Spring/Autumn	Summer	Winter	Summer
30	Winter	Spring/Autumn	Summer	Spring/Autumn	Autumn/Spring
36	Winter	Spring/Autumn	Summer	Summer	Winter
42	Winter	Spring/Autumn	Summer	Autumn/Spring	Spring/Autumn
48	Winter	Spring/Autumn	Summer	Winter	Summer

Table 7.5: A sample of indicative alternating seasonal temperature regimes obtained from the Bureau of Meteorology[50] for Australian capital cities that could be adopted for assessing the germination of species located in proximity to these urban centres.

Capital City (North to South)	Season			
	Summer (Jan)	Autumn (April)	Winter (July)	Spring (October)
Darwin	32/25 °C	33/24 °C	31/19 °C	33/25 °C
Brisbane	29/21 °C	26/17 °C	20/10 °C	26/16 °C
Perth	31/18 °C	26/14 °C	18/8 °C	23/12 °C
Sydney	26/19 °C	22/15 °C	16/8 °C	22/14 °C
Adelaide	30/17 °C	23/12 °C	15/8 °C	22/12 °C
Melbourne	26/14 °C	20/11 °C	14/6 °C	20/10 °C
Hobart	23/12 °C	18/9 °C	12/4 °C	17/8 °C

50 www.bom.gov.au

Case Study 7.2: Germination testing at the Tasmanian Seed Conservation Centre

James A. Wood

The germination testing program of the Tasmanian Seed Conservation Centre (TSCC) began in August 2006, with one staff member. Starting with two incubators, the program now uses 12 incubators and most testing is performed by a team of eight volunteers, with the same staff member orchestrating collections and selecting test conditions. Germination testing requires regular and consistent volunteer involvement. Applicants are briefed prior to induction about the level of commitment required.

Tests are conducted on 9 cm petri dishes of 1 % agar (plain water agar or agar with 0.01 M KNO_3 or a 4 % dilution of SmokeMaster Regen 2000) (Figure 7.13). Test plates are labelled with an **accession** number, date started, test code letter/s, replicate number and test conditions (Figure 7.14 and 7.15). Testing aims to achieve >75 % germination from the viable seed. Weekly germination scoring is a major time commitment, considering that tests can take as little as five weeks but generally take about 20 weeks and sometimes, over two years! Test durations are a consequence of the dormancy types and level expressed by wild seed. Non dormant, conditionally dormant and physically dormant seeds can generally be germinated very quickly given appropriate conditions. Other dormancy types can impose a series of blocks that need to be overcome successively.

It's not unfair to describe germination scoring as long periods of boredom punctuated with brief moments of excitement and this is explained in the process of volunteer introduction. While volunteer programs often have a group participation element that facilitates social engagement, the germination program does not necessarily have that framework, so it is important to explicitly talk about this aspect to properly set expectations.

Wild seed testing is particularly challenging with a vast range of plant families producing seeds with different internal morphologies and successfully germinating in a variety of ways. Volunteers rely on staff input for cut testing, finishing/transfer of tests and feedback on results, which means that staff time in supervision is significant.

Learning from the volunteer program at TSCC:

- Minimal distraction is preferable, to avoid momentary lapses in concentration (e.g., miscounting of seedlings or missing a transfer date).
- Volunteers are supported to select a manageable workload and not to take on too much.
- Volunteers are advised to select a mixture of collections; some that are expected to germinate relatively quickly (<20 weeks) and some that will require lengthy, complex move-along trials (1–2+ years).
- In 2019, the TSCC moved to a bicolour coding system for the incubator regimes (Figure 7.15 and Table 7.6) to replace writing the test temperature onto the plate. This system was adopted to help volunteers quickly spot plates going into the wrong incubator. Colour coded labels are attached so the code is visible for all plates in a stack. For move-along experiments, the next regime label is placed on top of the previous label on the day of transfer.

Many volunteers at TSCC have been with the seed bank between 5–10 years; they are self-motivated and find their work fascinating and rewarding (Figure 7.16). Staff don't shy away from sharing their frustration or excitement about test results and it's great to see that reflected by volunteers. Although the primary goal of wild seed banks is the **ex situ conservation** of

seed-bearing plants, the germination testing we conduct is equally as important. The RTBG began sharing germination data with the public in 2008 via the TSCC Germination Database on the RTBG website[51].

Table 7.6: Bicolour coding for incubator regimes at the TSCC.

Temperate Regime	Colour Code	Photo-period
00 °C		10/14
08/02 °C		10/14
05 °C		10/14
12/00 °C		10/14
10 °C		10/14
17/05 °C		10/14
15 °C		10/14
22/10 °C		10/14
20 °C		10/14
27/15 °C		10/14
25 °C		10/14
32/20 °C		10/14
30 °C		10/14
35/23 °C		14/10

Figure 7.13: Germination test set-up equipment: petri dishes of agar, test sheets, sowing grid, seed boats and fine bamboo dibber.

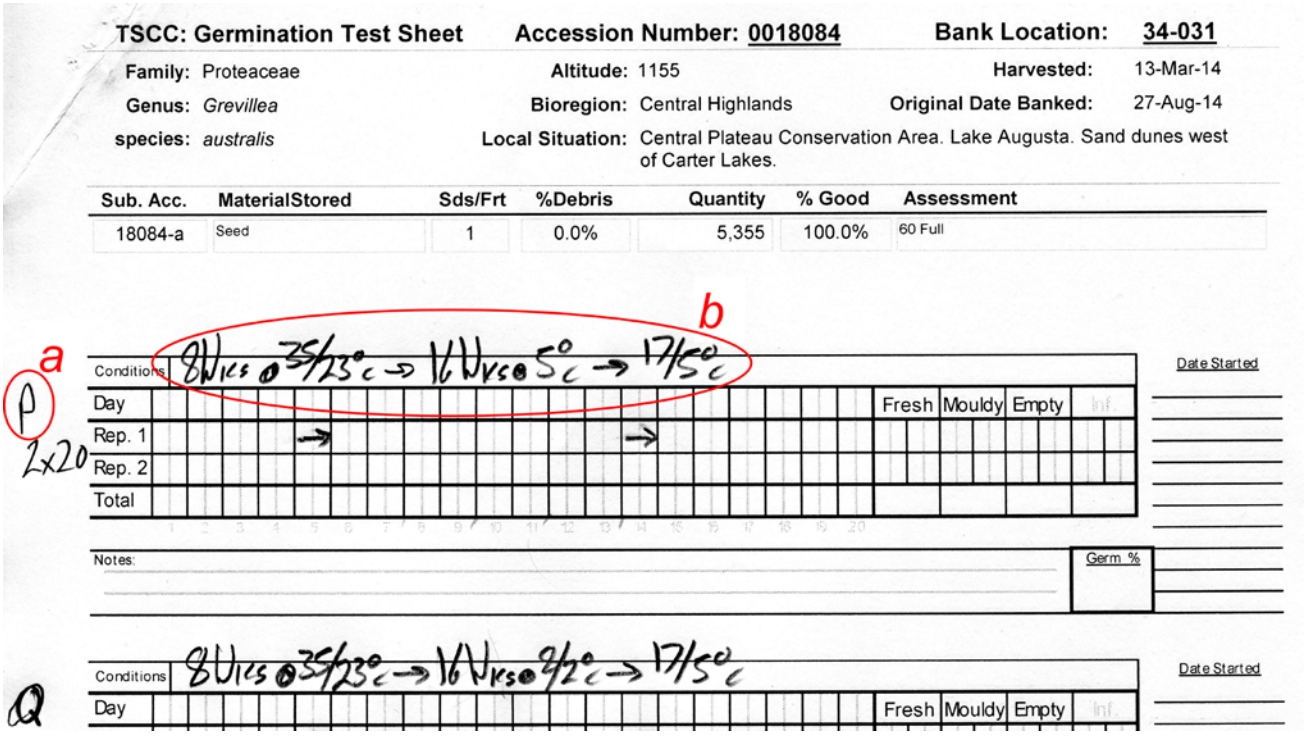

Figure 7.14: Typical TSCC test sheet: **(a)** test plate code, **(b)** test treatments.

51 https://gardens.rtbg.tas.gov.au/conservation/tsccgerminationdatabase/

Figure 7.15: Sown and labelled 6cm Petri dishes.

Figure 7.16: Seed bank volunteers at the TSCC.

Further reading

Wood J (2020) Germination testing at the Tasmanian Seed Conservation Centre. *Australasian Plant Conservation* **29** (2), 15-18.

7.6.2.3 Wetting and drying cycles

Another approach that combines the concepts of after-ripening and stratification is the use of alternating wetting and drying cycles (Baker *et al*. 2005; Hoyle *et al*. 2008). In these experiments, dry seeds are incubated at warm temperatures (i.e., equivalent to after-ripening) punctuated every week or two with short periods of hydration (e.g., imbibed for 1 to 2 days and then re-dried). This mimics the natural hydration/dehydration cycles that seeds experience in the soil seedbank, analogous to summer thunderstorms or sporadic early autumn rainfall. There is evidence that the rate of dormancy loss is increased in some species using this approach (Baker *et al*. 2005; Hoyle *et al*. 2008; Tuckett *et al*. 2010; Lewandrowski *et al*. 2018) and problematic species responding well, including *Persoonia longifolia* and *Hibbertia* spp. (Hidayati *et al*. 2012; Chia *et al*. 2016;). See Case Study 7.3 on the processing and germination of Australian *Persoonia* species.

Case Study 7.3: Processing and germination of Australian *Persoonia* seeds in the lab

Nathan J. Emery

Persoonia (Proteaceae) are spreading to decumbent shrubs or small trees that occur in 64 of the 87 Australian bioregions, but are absent in the arid **ecosystems** of central Australia (Emery and Offord 2018). Known as 'Geebungs' in the east and 'Snottygobbles' in the west, *Persoonia* species attract strong interest in the horticulture industry for their striking light green foliage and small, tubular yellow flowers. The genus is also a key inclusion in the **restoration** industry with nine species listed as threatened, endangered or critically endangered (eight of these species occur in New South Wales). *Persoonia* have complex dormancy mechanisms, and poor propagation success has historically limited their use in these industries (Emery and Offord 2018).

Laboratory processing

A *Persoonia* fruit is a fleshy drupe comprising of a leathery exocarp and fleshy mesocarp layer that cover a woody, but water-permeable endocarp that mechanically restricts the embryo within from germinating (Figure 7.17). In the laboratory, retaining the endocarp typically results in no germination, but removing at least 50 % of the endocarp significantly increases germination above 50 % (Chia *et al.* 2016). At the Australian PlantBank, the endocarp is most often removed using a scalpel, bench-mounted vice or Dremel® power tool. Care is required to not damage the seed when removing the endocarp. Treating the extracted seeds with a mild beach solution (ca. 2 %) for 15–30 minutes further improves germination by minimising fungal contamination. Several eastern Australia *Persoonia* species display complex germination responses to diurnal incubation temperatures that likely reflect their distribution, life-history traits and habitat (Emery and Offord 2019; Catelotti *et al.* 2020). Specifically, optimal day temperatures for germination of these species tend to range from approximately 15 °C to 25 °C, but the seeds may also require a cold or warm stratification pre-treatment to increase germination success (refer to section 5.4.2. Physiological Dormancy). Time to first germination is slow and species-specific, often requiring at least two weeks from commencing the incubation treatment to radicle emergence.

Figure 7.17: Cross section of a *Persoonia hirsuta* drupe in the field showing the green-white outer fleshy layers surrounding the woody endocarp and the white seed within. (Image: Nathan Emery)

The endocarp

While the endocarp is water permeable, its hardness is species-specific, requiring between 27–70 kg of force to crack when dispersed from the maternal plant (Table 7.7; Norman and Koch 2008). Under natural conditions in the field, the endocarp slowly breaks down over time, and recent research has demonstrated that significant wet-dry climate cycles can accelerate endocarp weakening (refer to section 5.6.2.3 Wetting and drying cycles; and Chia *et al.* 2016). Similarly, other disturbances such as mechanical soil movement, fire and animal consumption of fruits are likely to also weaken the endocarp, but these factors and their interactive effects have not been rigorously tested. Even with these elements the endocarp may still require 12 to 36 months post-dispersal to suitably weaken and allow the seed to germinate through the woody shell.

Table 7.7: Mean (± SE) pyrene weight and strength of six eastern Australia *Persoonia* species. Twenty pyrenes of each species were weighed and cracked within one month of collection. Endocarps were cracked using an Enpaix Digital Force Gauge. The peak force required to crack the endocarp was recorded, and 100N of force roughly equates to 10 kg.

Species	Pyrene dry weight (g)	Pyrene strength (N)
P. asperula	0.085 ± 0.002	421.3 ± 23.5
P. hirsuta	0.126 ± 0.002	274.0 ± 10.9
P. levis	0.188 ± 0.010	461.0 ± 20.7
P. pauciflora	0.151 ± 0.002	472.1 ± 8.1
P. silvatica	0.165 ± 0.002	493.6 ± 26.1
P. stradbrokensis	0.172 ± 0.012	607.3 ± 24.8

References

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Chia KA, Sadler R, Turner SR, Baskin CC (2016) Identification of the seasonal conditions required for dormancy break of *Persoonia longifolia* (Proteaceae), a species with a woody indehiscent endocarp. *Annals of Botany* **118**, 331-346.

Emery NJ, Offord CA (2018) Managing *Persoonia* (Proteaceae) species in the landscape through a better understanding of their seed biology and ecology. *Cunninghamia* **18**, 89-107.

Emery NJ, Offord CA (2019) Environmental factors influencing fruit production and seed biology of the critically endangered *Persoonia pauciflora* (Proteaceae). *Folia Geobotanica* **54**, 99-113.

Norman MA, Koch JM (2008) The effect of in situ seed burial on dormancy break in three woody-fruited species (Ericaceae and Proteaceae) endemic to Western Australia. *Australian Journal of Botany* **56**, 493-500.

7.6.2.4 Precision nicking

Precision nicking of the seed coat (and, in some cases, a portion of the endosperm) is effective for a wide range of dormancy types (see Case Study 7.4 on techniques used in precision nicking). For seeds with physiological or morphophysiological dormancy, precision nicking can remove the mechanical restriction imposed on embryo growth by the surrounding tissues (Baskin and Baskin 2014; Kildisheva *et al*. 2020). The location and depth of nicking is species specific, though generally the seed coat needs to be breached and the nicking undertaken at, or near, the radicle end of the seed (Cross *et al*. 2017). Care must be taken not to damage the embryo, particularly the radicle, if the seeds are to be nicked close to the radicle end. This technique is also highly effective for species with physical (or combinational) dormancy as it overcomes water impermeability, though care must be taken as many seeds with PY have very tough seed coats that are difficult to cut (Turner and Dixon 2009).

Case Study 7.4: Dormancy breaking techniques for genus *Acronychia*

Ganesha S. Liyanage, Catherine A. Offord and Karen D. Sommerville

Genus *Acronychia* (Family Rutaceae) contains the endangered species *Acronychia littoralis* and species that are key components of endangered rainforest communities. The persistence of these in the wild is threatened with further habitat loss. Immediate implementation of ex situ conservation strategies, such as seed banking, is therefore important. However, slow and poor germination due to physiological dormancy is common for the genus, as it is for many species from the Rutaceae family (Martyn *et al*. 2009), and negatively impacts the germination testing that is needed for viability assessments and restoration activities.

We tested the germination of three *Acronychia* species – *A. imperforata*, *A. oblongifolia* and *A. laevis* – in response to a series of treatments to identify an effective technique for breaking dormancy (Liyanage *et al.* 2020). Mature fruits were collected from 3–4 well established adult plants from The Australian Botanic Garden, Mount Annan. Four dormancy breaking treatments were performed on healthy-looking seeds of each species: (i) incubation of intact seeds on agar incorporating Gibberellic Acid (GA_3); (ii) full seed coat removal (de-coating);

(iii) scarification near the radicle emergence point (scarification-emergence point) (Figure 7.18); (iv) scarification opposite the radicle emergence point (scarification-back); (v) intact seeds were placed on 0.8 % agar as an untreated control. Due to the small size of the seeds (2–4 mm), all scarification and de-coating treatments were performed under a stereo microscope.

Seeds from each treatment were incubated at alternating temperatures of 25/10 °C with a 12/12 hour light/dark cycle. The GA_3 treatment (i) was not tested for *Acronychia laevis* due to limited seed numbers.

An imbibition test (refer to 7.3.2 and Box 7.4 for further information) was also performed and confirmed that the seed coat is permeable in all three study species (Figure 7.19).

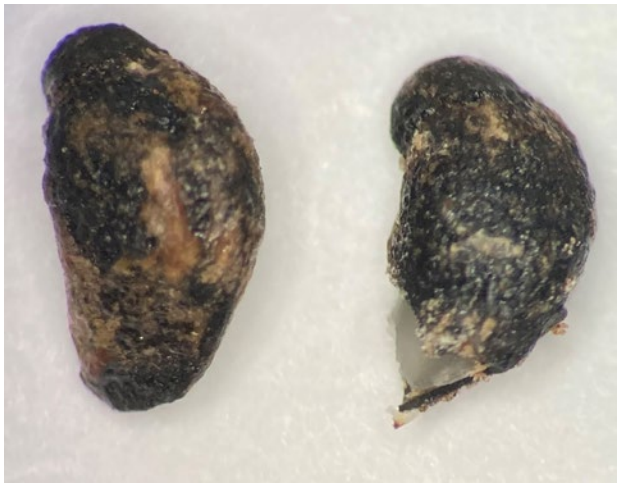

Figure 7.18: Precision nicking of *Acronychia* (Image: G.S. Liyanage)

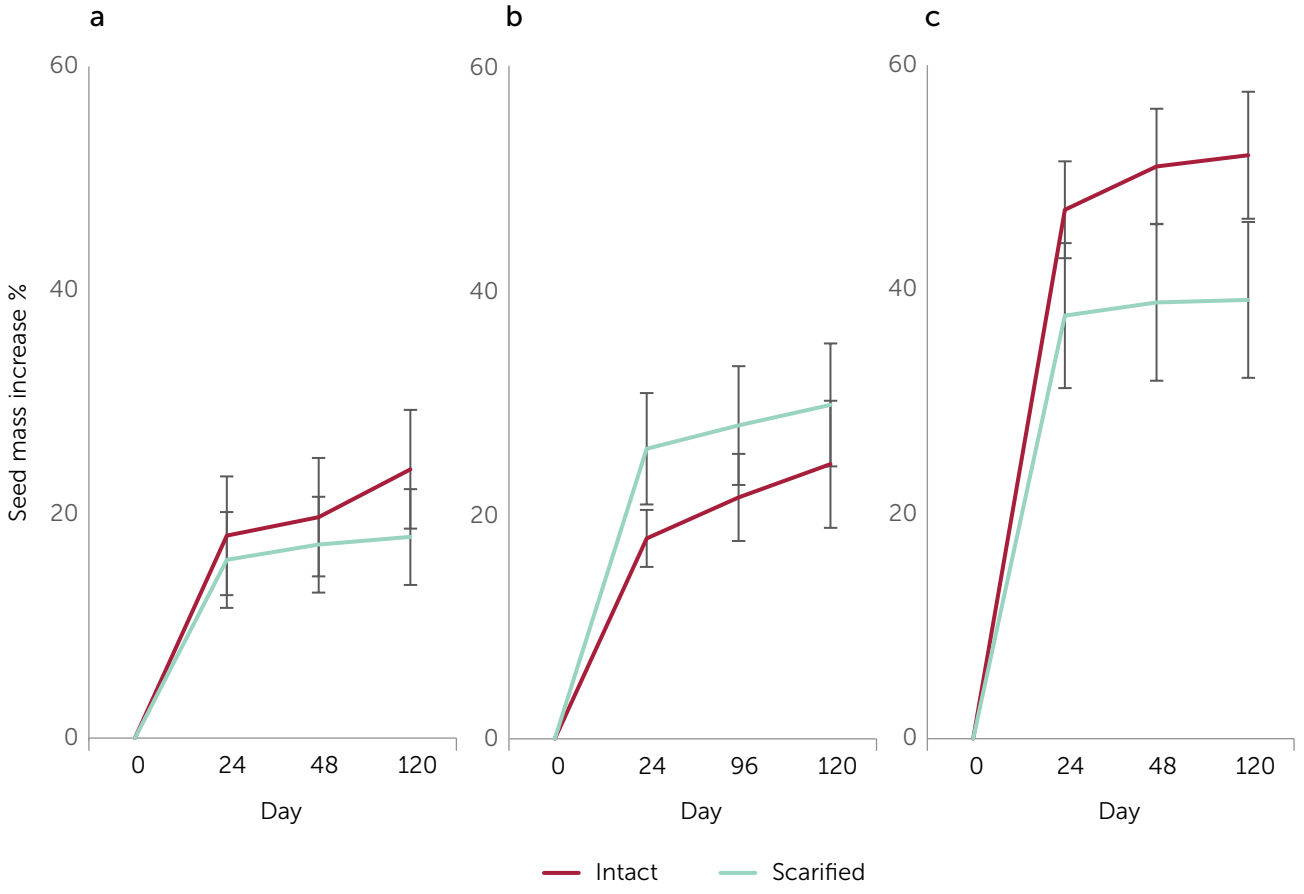

Figure 7.19: Cumulative mean seed mass increase (± s.e.) for intact and scarified seeds of **(a)** *Acronychia imperforata*, **(b)** *Acronychia laevis* and **(c)** *Acronychia oblongifolia* during an imbibition test. This figure was originally published as Figure 2 in Liyanage *et al*. (2020) and is used under CC BY-NC 4.0.

The responses of all three species to the dormancy-breaking treatments were similar, with highest germination observed when the seed coat was scarified near the radicle emergence point (Figure 7.20). Higher germination was also achieved in the 'de-coated' treatment, but this was lower than that achieved by the 'scarification-emergence point' treatment, possibly due to radicle damage during the seed coat removal process. The 'scarification-back' treatment resulted in very low germination for all three species, indicating the importance of clearing the radicle emergence point to promote germination.

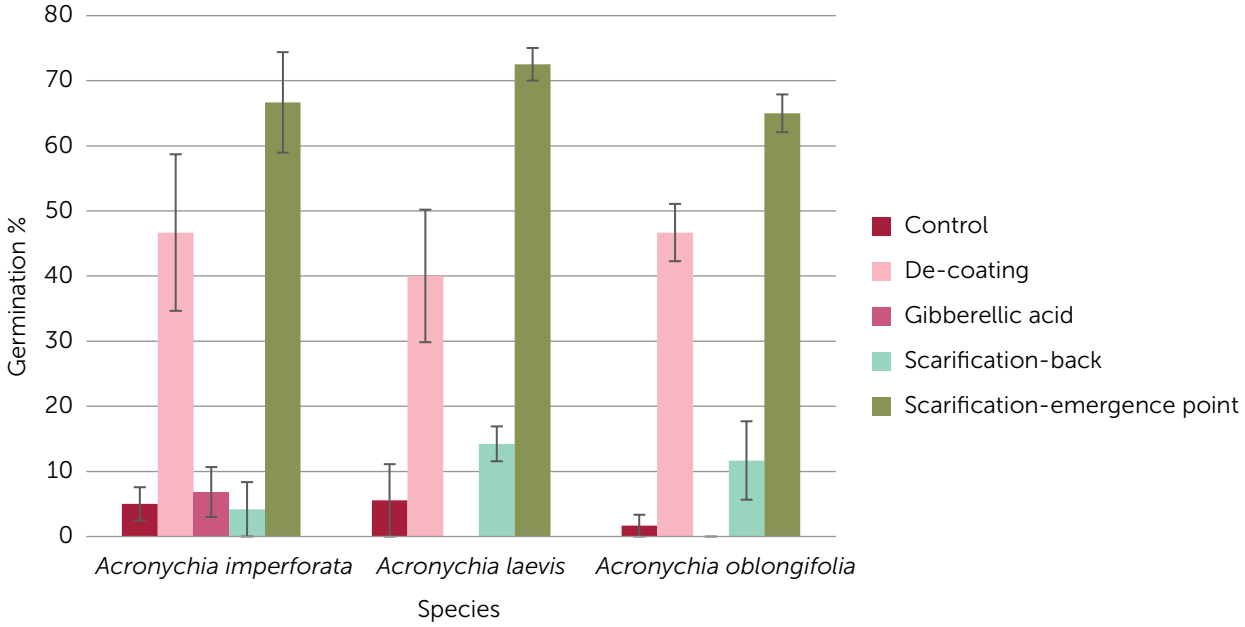

Figure 7.20: Mean germination percentages of three *Acronychia* species in response to different dormancy breaking treatments. This figure was originally published as Figure 1 in Liyanage *et al*. (2020) and is used under CC BY-NC 4.0.

The results indicate that the seed coat near the radicle emergence point acts as a mechanical barrier constraining embryo emergence. The removal of the seed coat near the radicle emergence point (precision nicking) is proposed as a useful technique for breaking dormancy in genus *Acronychia* and other related Rutaceae species with non-deep physiological dormancy.

References

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Martyn AJ, Seed LU, Ooi MKJ, Offord CA (2009). Seed fill, viability and germination of NSW species in the family Rutaceae. *Cunninghamia* **11**, 203-212.

7.6.2.5 Use of germination promoting compounds

Many chemical compounds promote seed germination. Commonly reported agents include smoke (crude smoke products such as plant tissue-derived aerosol smoke or **smoke water**, or synthetically-derived **Karrikin KAR1** – Flematti *et al*. 2005), the plant growth regulators gibberellic acid and ethylene, and nitrate compounds (Kildisheva *et al*. 2020). Smoke products are particularly important germination stimulants in Australian species. Smoke and KAR_1 has been found to have a stimulatory effect on the germination of at least 95 genera of Australian plants (Dixon *et al*. 1995; Merritt and Dixon 2003; Merritt *et al*. 2007). Chemical growth stimulants such as potassium nitrate (KNO_3), and gibberellic acid (GA_3), are commonly used to alleviate physiological dormancy (Kildisheva *et al*. 2020). See Case Study 7.5 for the application of the plant growth regulator gibberellic acid to aid germination in an Australian native crop species.

Case Study 7.5: Germination of Australian native crop wild relative *Sorghum* species

Sally L. Norton

The genus *Sorghum* belongs to the grass family Poaceae and includes 26 recognised species that vary significantly in plant **morphology**, **genetic diversity**, and geographic distribution. Commonly known as the agricultural crop Sorghum, or Milo, the single cultivated species is grown throughout the world as a cereal grain for human and animal food, as well as pasture for animal fodder. The majority of the genus are wild relatives, with 17 species native, and 14 endemic to Australia (Figure 7.21a).

Seed dormancy and seed shattering have been lost during Sorghum domestication, and as such, the cultivated Sorghum species is relatively simple to germinate through sowing directly into soil with a minimum soil temperature of around 15 °C, and an optimal temperature around 23 °C. The germination and establishment of the crop wild relative (CWR) *Sorghum* are inherently more difficult in terms of temperature, moisture and daylength requirements, with strong seed dormancy in most species, and reports of after-ripening required prior to germination for a few. The differences in germination requirements are largely due to the varying environmental conditions in their natural habitats, and due to seed morphology, with the wild species caryopsis tightly bound by the palea and lemma (Figure 7.21b).

Sorghum CWR species obtain optimal germination when the climatic, diurnal and soil conditions of their natural habitat are mimicked. Almost all Australian CWR sorghum occur naturally in tropical/subtropical environments, with only one species in the temperate to cold temperate zones of southern NSW and Victoria. As such, all the Australian species require stratified temperature regimes to germinate. Strong seed dormancy exists in most CWR species both as physical dormancy and as innate physiological dormancy. The dormancy can be overcome by excising the embryo and through the application of growth stimulants. Without these specialised treatments, the CWR *Sorghum* species will fail to germinate, or germinate very poorly.

The protocol developed to maximise germination of the Australian *Sorghum* species involves excising the caryopsis (under a stereomicroscope) to remove the physical constraint, surface sterilisation in 1 % available chlorine with weak surfactant for around 15 minutes, with seeds then germinated between filter papers in an incubator using a diurnal temperature regime of 25 °C (16 hours) and 35 °C (8 hours) and 14 hour daylength. Physiological dormancy can be overcome

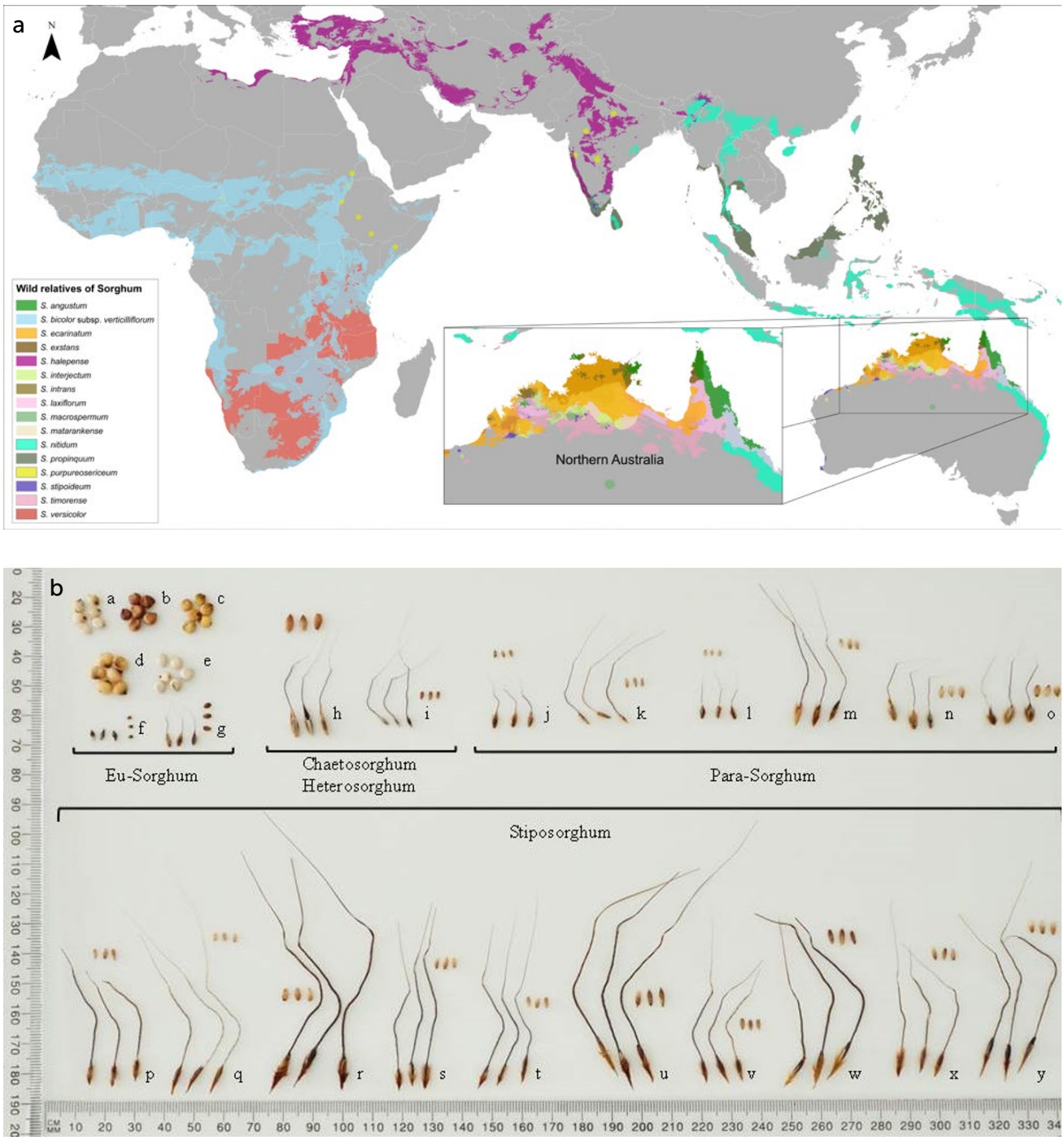

Figure 7.21: **(a)** Distribution of wild sorghum species globally, showing Australia as the origin for a majority of tertiary gene pool species. Figure shows natural distribution and does not include cultivated non-native distributions of the weedy species. **(b)** Morphological variation in Sorghum seed showing the wide range of diversity in the seed structures and caryopsis size. Letters on figure denote species, with a–e: S. *bicolor* AGG 322649, 322618, 322620, 322666 and 322611 caryopses, respectively. Then the seed and caryopsis of: f: *S. propinquum* 302546; g: *S. halepense* 300167; h: *S. macrospermum* 322277; i: *S. laxiflorum* 302503; j: *S. leiocladum* 300170; k: *S. matarankense* 302521; l: *S. nitidum* 302539; m: *S. timorense* 302660; n: *S. purpureosericeum* 321133; o: *S. versicolor* 321126; p: *S. amplum* 302623; q: *S. angustum* 302604; r: *S. brachypodum* 302480; s: *S. bulbosum* 302646; t: *S. ecarinatum*; u: *S. exstans* 302577; v: *S. interjectum* 302563; w: *S. intrans* 302390; x: *S. plumosum* 302489; y: *S. stipoideum* 302644. (Images: Shapter *et al*. (2018) 'The domestication, spread and uses of sorghum as a crop', in W. Rooney (Ed.), Achieving sustainable cultivation of sorghum. Volume 2: Sorghum utilisation around the world. (Burleigh Dodds Science Publishing: Cambridge, UK). Reproduced with permission from Burleigh Dodds Science Publishing, www.bdspublishing.com)

by the application of Gibberellic Acid (GA_3) as either a pre-soak priming treatment (3 mM GA_3, 15 hrs at room temperature) prior to placing onto filter papers, or as a 1 mM GA_3 wetting agent as the first water treatment during germination on filter papers (Kew SID; Cowan *et al*. 2020). The filter papers must be kept moist with de-ionised water during the germination process, but not too wet as this will result in seed rot and/or fungal contamination. Using this approach, germination results for the native *Sorghum* species is quite high (Figure 7.22), with most seeds that are capable of germinating ready to transplant to a loose draining light soil within 5–10 days depending on the species.

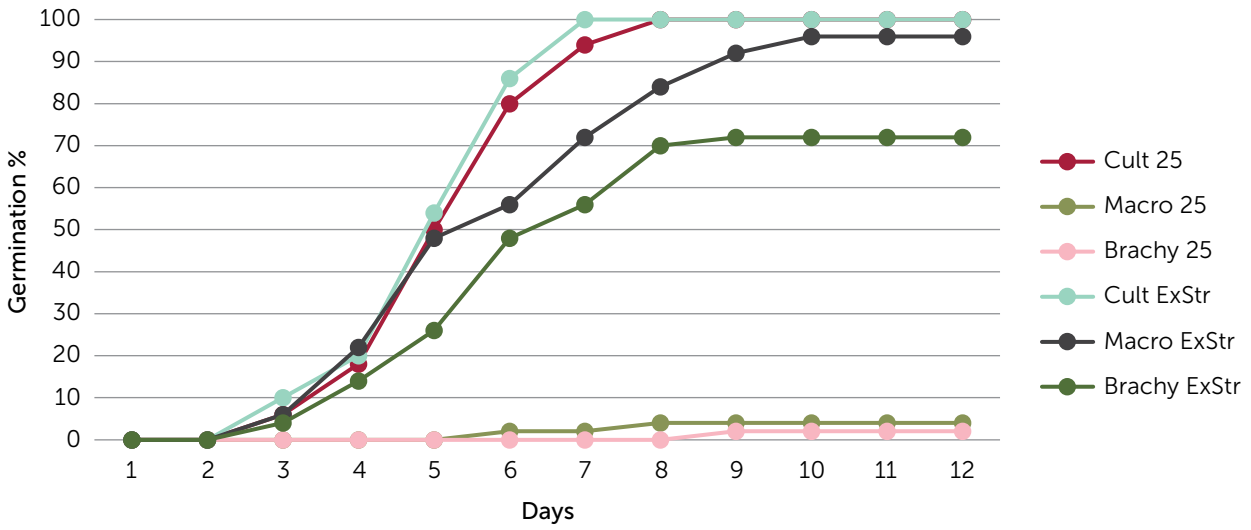

Figure 7.22: Germination frequency for cultivated and wild sorghum under different germination protocols. Where Cult: Cultivated species *S. bicolor*; Macro: wild *S. macrospermum*; Brachy: wild *S. brachypodum*; 25: constant 25 °C temperature, no seed treatments; ExStr: excised caryopsis, striated temperature 25 °C–35 °C, 1 mM GA_3 applied.

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Kew SID database: https://data.kew.org/sid/SidServlet?Clade=&Order=&Family=&APG=off&Genus=Sorghum&Species=&StorBehav=0

7.6.3 Seed burial experiments

For many native species, seed dormancy is not easily overcome and is a considerable obstacle to ex situ germination. In situ, many species spend several seasons or years in the soil seedbank before dormancy is lost (Tieu *et al*. 2001b; Baker *et al*. 2005; Ooi *et al*. 2007; Turner 2013; Chia *et al*. 2016). It should also be recognised that within any population of seeds, individual seeds may lose dormancy at different rates. This is a strategy to increase the chances of seedling recruitment as seeds become germinable at different times, but one that can create difficulties in the laboratory or nursery when synchronous germination within a short period of time is desirable (Ooi *et al*. 2007; Chia *et al*. 2016). Particularly challenging species occur in many genera of Cyperaceae, Dilleniaceae, Ericaceae and Restionaceae (Ooi *et al*. 2007; Hidayati *et al*. 2012; Turner 2013). Species in these families require a complex sequence of cues for dormancy break and germination stimulation including the application of smoke at specific times (Merritt *et al*. 2007).

In such cases, soil burial experiments, which involve studies of germination ecology in the field and/or controlled greenhouse conditions, can assist in identifying the cues seeds require to break dormancy. In particular, these experiments can aid identification of seasonal changes in moisture and temperature that are needed for seeds to germinate. In these types of experiments, seeds are buried and checked regularly to determine viability decline over time and when germination occurs in response to changing soil conditions. Temperatures and rainfall are monitored at the same time, and germination stimulants can also be used at certain times to promote germination of naturally cued seeds (Hidayati *et al*. 2019). See Box 7.8 for more details on undertaking seed burial experiments and Case Study 7.6 for an example of how this approach has helped in understanding the ecology of a critically endangered species.

Box 7.8: Seed burial protocol

First assess initial seed viability and germination of freshly collected seeds (both are critical to establish an accurate control to compare with future observations). Then under standard laboratory conditions:

1. A known number of seeds (e.g., 100–300 filled seeds) for each of the desired number of retrieval times are sealed in porous bags (e.g., 0.75 µm nylon mesh) containing a small amount of washed river sand or field soil.
2. These bags are buried at a depth of 1–2 cm in an environment representative of the natural habitat of the species and ideally at a time that coincides with natural seed shed.
3. A temperature and soil moisture data logger can be buried at the same time to allow for analyses and identification of critical temperatures and periods of moisture availability for dormancy break and germination.
4. Seed bags are retrieved at regular intervals (at least seasonally) and the seeds re-assessed for seed viability, embryo growth and germination (with and without germination promoting compounds), using the same standard approaches at each retrieval time.
5. Once germination has occurred, temperature and rainfall and/or soil moisture data can be used to assess the environmental cues the seeds received from the point of dispersal to the point of dormancy break and germination.
6. These factors can then be incorporated into future ex situ germination treatments.

Valuable insights into the germination requirements of many species with different classes of seed dormancy have been attained using variations on this general approach. For more information, see Tieu *et al*. 2001b; Baker *et al*. 2005; Ooi *et al*. 2007; Turner 2013; Downes *et al*. 2015; Chia *et al*. 2016; Liyanage and Ooi 2017; Hidayati *et al*. 2019.

Case Study 7.6: Ecological insights provided through long-term burial and retrieval of seeds from the Critically Endangered *Symonanthus bancroftii* (Solanaceae)

Shane R. Turner and Eric Bunn

All evidence to date suggested that Bailey's Symonanthus has always been exceedingly rare, having only been collected a handful of times since it was first discovered in 1892. By the late 1990's after not being seen for over fifty years *Symonanthus bancroftii* was considered **extinct**. Then in 1997 a single male plant was found in a disturbed area near the small Western Australian wheatbelt town of Ardath. After intensive searching another plant (female) was found in 1998 with both successfully established in tissue culture. These two original **genotypes** were used for producing several hundred plants for **translocation** to several sites near where the original collections were made (Figure 7.23). Around eighty plants were successfully established in situ with plants eventually producing over 10,000 viable seeds. A subsample of these seeds was subsequently used to undertake a series of dormancy and germination experiments to understand more about the general seed biology and ecology of this species.

Freshly collected seeds were used to implement a large-scale seed burial experiment that was situated adjacent to the translocated plants. Four replicate sites were used with 48 bags of 100 seeds carefully counted out, sealed in highly porous nylon bags then buried in situ for future retrieval and assessment. As the experiment was designed to potentially run for >20 years bags were placed into fine grade porous stainless-steel mesh bags for added durability (Figure 7.23). Seed viability as well as germination capacity were initially assessed with high viability but very low germination recorded for fresh seeds – seeds were considered to have physiological dormancy. Bags with seeds were buried ~ 1cm below the soil surface in November 2009 with four bags retrieved from each burial site every autumn and spring for the first three seasons then again four years later in autumn with more retrievals planned. Upon retrieval the fate of all seeds was determined by carefully sorting out the contents of each bag under a microscope with all intact seeds then assessed for **seed fill** with those that were filled subsequently assessed for germination capacity (15 °C on either water agar alone or with KAR_1). Results so far strongly suggest that seeds of *S. bancroftii* appear to be long lived (Table 7.8) in the soil seed bank with over 80 % of seeds still filled and viable after six years burial in situ. As well, seeds even after six years burial had a very strong response to KAR_1 germinating to over 80 % (Figure 7.23). Seeds were also found to exhibit very marked dormancy cycling becoming relatively non-dormant at the beginning of the wet season and then appearing to re-enter dormancy again with the onset of spring. A discovery in 2017 of a large (>200 plants) **extant** post-fire population in a reserve in the same region verifies that fire plays an integral role in seedling recruitment and confirms that seeds of *S. bancroftii* can indeed persist in the soil seed bank for considerable periods of time (at least several decades) between major fire events. Based on all available evidence it appears that this species is an **obligate seeder** and post-fire opportunist, with a relatively short lifespan (~15–25 yrs.). Several more seed retrievals are planned for this species with enough samples remaining to keep this experiment active for many years to come.

Table 7.8: Seed fill and germination results from seeds of *Symonanthus bancroftii* retrieved and assessed regularly as part of a long-term seed burial experiment.

	BURIAL DURATION							
	0 months (11-11-09)	**5 months** (16-4-10)	**12 months** (4-11-10)	**17 months** (12-4-11)	**24 months** (8-11-11)	**28 months** (29-3-12)	**36 months** (16-10-12)	**76 months** (14-3-16)
	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn
% Seed fill	94.3 ± 2.5 %	88.8 ± 2.1 %	90.3 ± 1.4 %	89.3 ± 2.8 %	80.8 ± 3.0 %	84.7 ± 2.2 %	89.0 ± 2.0 %	88.5 ± 3.3 %
Laboratory germination								
Water	0.0 ± 0.0 %	0.0 ± 0.0 %	0.5 ± 0.5 %	0.6 ± 0.6 %	0.0 ± 0.0 %	3.1 ± 1.7 %	0.9 ± 0.9 %	2.9 ± 1.6 %
1 µm KAR_1	0.0 ± 0.0 %	16.7 ± 7.6 %	20.7 ± 11.3 %	81.1 ± 14.4 %	14.7 ± 2.7 %	79.0 ± 7.7 %	2.5 ± 0.8 %	86.4 ± 5.1 %

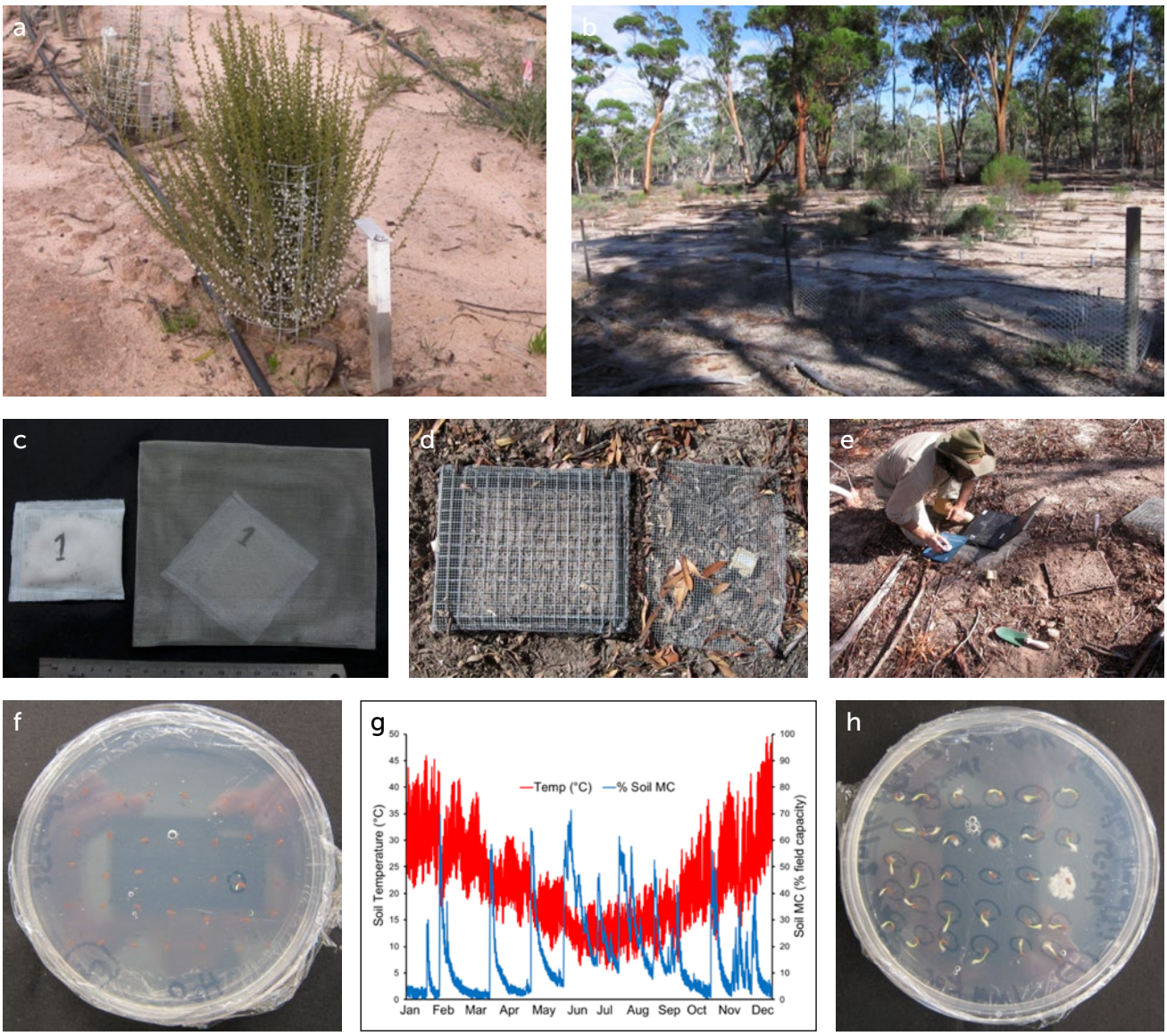

Figure 7.23: (L-R): **(a)** Mature *Symonanthus bancroftii* plant as part of a translocation (Image: B. Dixon); **(b)** Fenced translocation site in the West Australian wheatbelt (Image: S. Turner); **(c)** Nylon seed burial bag + seeds and sand and bag inside stainless steel mesh bag (Image: S. Turner); **(d)** Caged in situ burial site with adjacent Tiny Tag logger (Image: S. Turner); **(e)** Downloading soil temperature and moisture data from buried logger (Image: B. Dixon); **(f)** Laboratory germination (<5 %) of *Symonanthus bancroftii* seeds on water agar after 6 years in situ burial (Image: S. Turner); **(g)** Soil temperature and moisture adjacent to buried seeds recorded over one year (Image: S. Turner); and **(h)** Laboratory germination (>80 %) of *Symonanthus bancroftii* seeds on KAR_1 agar after 6 years in situ burial (Image: S. Turner).

7.7 Smoke for seed germination

Smoke is a primary cue responsible for the flush of seedling emergence following fire. Seeds of a wide range of species from across the globe are stimulated to germinate by smoke. Germination promoting chemicals in smoke that have been identified include the karrikins, which are the predominant group, and the cyanohydrin, **glyceronitrile** (Chiwocha *et al*. 2009; Flematti *et al*. 2011). Smoke and the synthetic karrikins not only promote germination but can also increase the speed and uniformity of germination and enhance seedling growth and tolerance to environmental stress (Kulkarni *et al*. 2011).

In seeds of some species, the germination response to smoke is inherent, and smoke will elicit high germination of a seed batch regardless of time since collection or storage history. But for many species the sensitivity of seeds to smoke is influenced by seed dormancy status. Seeds with PD or MPD commonly require exposure to short durations of elevated temperatures (e.g., 10–180 mins at c. 80–120 °C), or weeks or months of after-ripening, stratification, or wet/dry cycling treatments, with seed sensitivity to smoke increasing progressively as dormancy is lost (Merritt *et al*. 2007). Such patterns have been observed, for example, in species of Asphodelaceae, Asteraceae, Brassicaceae, Haemodoraceae, Poaceae, and Stylidiaceae (Turner *et al*. 2009; Long *et al*. 2011; Downes *et al*. 2014; Erickson *et al*. 2016). Seed burial experiments also show how there can be complex interactions between smoke sensitivity and the seasonal cycling of seed dormancy as influenced by soil temperature and moisture. Examples include species of Apiaceae, Ericaceae, Dilleniaceae, and Gyrostemonaceae (Baker *et al*. 2005; Ooi *et al*. 2006; Hidayati *et al.* 2012) where seeds germinate in response to smoke cues when retrieved from soil in the growing season (e.g., autumn/winter in temperate Australia), but remain dormant and do not respond to smoke if retrieved outside of these times (e.g., spring/summer; see Case Study 7.6).

Seeds may have an obligate requirement for smoke, or for some species a proportion of the seed batch may germinate without smoke exposure and smoke acts to increase the extent of germination (Merritt *et al*. 2007; Collette and Ooi 2017). The requirement for smoke may also be lessened over time and seeds may increasingly germinate without smoke, indicative of a widening of environmental conditions suitable for germination as dormancy is lost (Hidayati *et al*. 2012, 2019). Variation in sensitivity to smoke can be seen in different batches of seeds of the same species collected from separate locations, or from the same location in different years; congruent with variation in the depth of dormancy amongst the seed population as influenced by genetic factors and the maternal environment during seed maturation (Stevens *et al*. 2007; Gorecki *et al*. 2012). The practical implication regarding this plasticity in smoke-response is that for seed batches of many species, their treatment with smoke may not necessarily elicit a high level of germination; the seed batch may require appropriate additional dormancy breaking pre-treatment(s) to initiate or maximise the response to smoke (Merritt et al. 2007).

7.7.1 Preparation and use of smoke

Smoke water is produced by burning plant material in a steel drum of c. 200 L. Most dried non-woody plant material is suitable for burning. Hay or straw can be used as a cheap source of bulk material, mixed with some leaf material from native plants (Box 7.9). The smoke generated is drawn through a 20–30 L plastic drum filled with water via use of a vacuum. A second, empty drum can be connected in series to the water-filled drum, with the vacuum attached to the empty drum to prevent water from entering the vacuum. Drawing smoke through the water for 60 minutes produces a concentrated product that is suitable for use at dilutions of 1:10, 1:100, or even 1:1,000, depending on the species (Box 7.10). For most species, a 1:10 solution is effective.

Seeds can be soaked directly in the diluted smoke water for up to 24–48 hours and then re-dried prior to sowing, or, if germination testing in Petri dishes, then a diluted solution can be used to irrigate the Petri dishes. Note, prolonged exposure times or concentrations of crude smoke water can be detrimental to germination and pre-soaking seeds is a preferable option. Other ways to apply smoke include the use of commercially available perlite- or vermiculite-impregnated smoke, with seeds sown into these carriers and placed in soil punnets or Petri dishes.

As an alternative to smoke water, dry seeds can be aerosol smoked prior to sowing for up to 60 minutes, with the smoke in this case pumped into a plastic tent or small shed (Box 7.11). This technique works well if there is a need to smoke large quantities of seeds for field or nursery sowing. Aerosol smoke can also be applied directly to soil trays with sown seeds for nursery propagation, to trays containing topsoil seed banks sampled from the field, or directly to the soil whilst in the field.

Box 7.9: Equipment required for generating smoke

- 200 L steel drum with a removable and sealable lid.
- Air pump and voltage regulator to control fan speed for drum inlet pipe (with accompanying car battery if aerosol smoking field plots).
- Pipe(s) to carry smoke to water filled drum (or to a plastic tent or shed for the application of aerosol smoke).
- A vacuum capable of drawing sufficient air and smoke through plastic drums. (for the production of smoke water only).
- A 20-30 L plastic drum filled with water through which smoke is drawn, with a second empty plastic drum connected in series (for the production of smoke water only).
- Hay or straw bales, or dry leaves and other plant material, for burning and producing smoke.
- Builders plastic and wooden stakes for building tents if smoking field plots.
- Appropriate personal protective equipment (PPE) and fire extinguisher.

Box 7.10: How to make smoke water

Seek permission to use this method, and undertake a risk assessment considering potential health impacts, appropriate protective equipment, and the potential environmental impacts of starting a fire. Fires should only be lit when and where they are legally allowed, and the authorities notified ahead of time, in case the public alert them to a potential emergency if smoke is observed (Commander 2021).

1. Position a 200 L steel drum in a well-ventilated outdoor area.
2. Attach an inlet pipe to the drum wall near the base to pump air into the drum.
3. Attach an outlet cooling pipe of 1 m in length near the top of the drum wall for the smoke to exit the drum.
4. Attach the cooling pipe to a smaller 20-30 L plastic drum via a flexible hose to direct smoke into the drum. Position the end of the flexible hose below the water line.
5. Attach a second flexible hose to the water drum above the water line to allow excess air/ smoke to exit the drum. A second empty drum can be connected to the first drum in series to prevent water from entering the vacuum.
6. Attach a vacuum to the flexible hose to draw the smoke through the water drum.
7. Place plant material in the drum, ignite and seal the drum with the drum lid. Turn on both the inlet air pump fan and vacuum.
8. Adjust the air pump and vacuum to ensure smoke production in the steel drum and movement through the water drum.
9. Burn plant material for approximately 60 min (see Figure 7.24). Extra plant material can be added if required at 15–20 min intervals to maintain smoke generation. *Do not open the lid of the steel drum whilst burning without first turning off the air pump to prevent flames flaring.

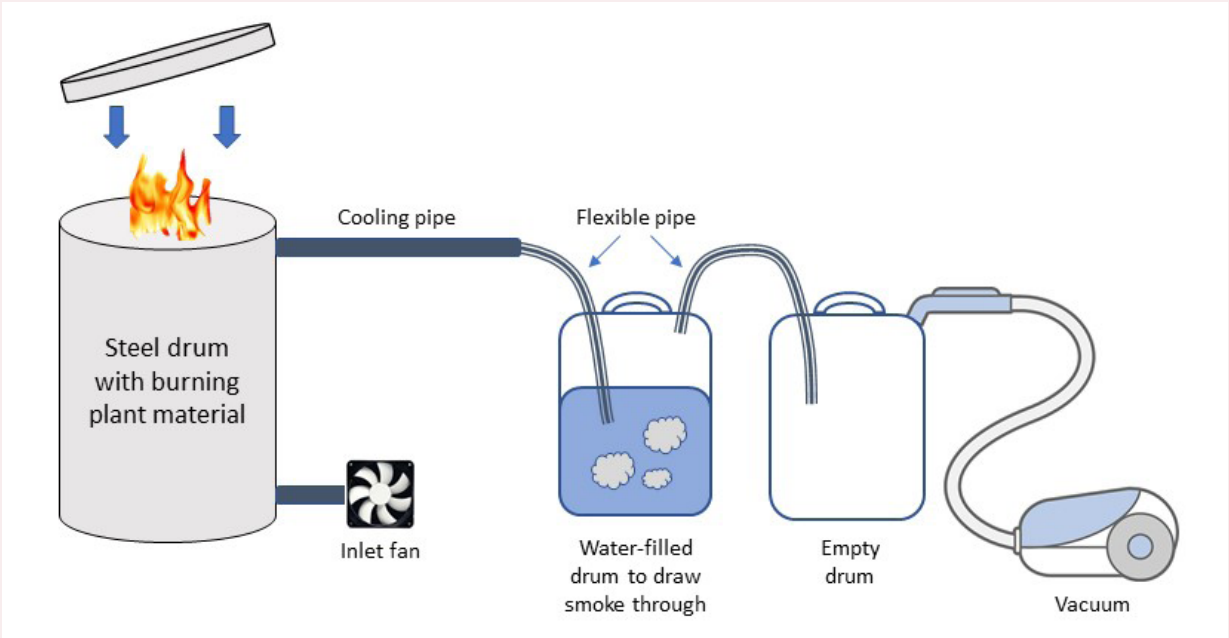

Figure 7.24: Smoke water is prepared through burning plant material and drawing smoke through a water filled drum. If necessary, a second, empty drum can be connected in series to the water-filled drum, with the vacuum attached to this empty drum, to prevent water entering the vacuum. (Image David Merritt)

Box 7.11: How to apply aerosol smoke to dry seeds or soil

1. Assemble smoking equipment in a similar manner as for making smoke water but dispense with the water drum and vacuum.
2. Attach to the outlet cooling pipe 1–3 flexible hoses for feeding into a plastic tent, shed, or replicate plastic tents in the field.
3. If aerosol smoking field plots, set up replicate plastic tents of c. 5 m^2 and seal the sides by heaping soil. Slide the flexible hose under an edge of the plastic near the centre of the plot.
4. Ignite and burn plant material for 60 min as per for smoke water generation, but pump smoke through the flexible hoses into the tent(s) or shed through use of the fan only, powered by a car battery.

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Chapter 8
The role of the plant nursery in ex situ conservation

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This chapter is a revision and significant expansion of the vegetative propagation sections of the previous edition's chapter by Anne Cochrane, Andrew D. Crawford and Catherine A. Offord, 2nd edition, pp. 35-62. Copyright 2009, Australian Network for Plant Conservation Inc. (ANPC).

8.1 Introduction

The primary role of a nursery is to propagate and grow-on plants using **seeds** or vegetative methods (via cuttings, division, grafting or plant **tissue culture**) for various purposes. Some species may also be held in a nursery for extended periods of time as an ex situ potted/ containerised collection where other storage options are not easily available, or as an adjunct form of **conservation** run in parallel with other techniques for **germplasm** storage (see Chapter 2). A major advantage of nursery collections is that germplasm can be kept in an active living state in one, or preferably more locations, as a metacollection (Griffith *et al*. 2020), spreading the risk of loss and making material available for vital research as well as an ongoing source of material for propagation purposes.

Plants may be grown in the nursery for:

- Living collection, display and education (Chapter 11)
- **Translocation** (Chapter 15; Commander *et al*. 2018 Chapter 6, especially 6.4)
- Stock plants as the core of an ex situ collection
- Seed production (Chapter 11 and Gibson-Roy *et al*. 2021)
- Field genebanking (FAO 2014; Chapter 11)
- Research (students/ staff both internal and external to an organisation) (Chapter 15)
- Regeneration of collections (Chapter 15)
- Distribution of germplasm to other conservation organisations
- Capacity building and horticultural development.

Nursery propagation involves good planning and staff training, as well as the development of strong partnerships within and across organisations. The importance of partnerships and working across diverse organisations to achieve threatened species conservation is highlighted in Case Studies 8.1 and 8.2. Specialist staff, experienced in conservation horticulture, are invaluable when working with threatened species. Training to value the importance of accurate scientific record keeping and good hygiene is essential and should be updated regularly as part of staff professional development. Staff continuity for long term projects is preferable as their observations and insights into plant propagation gained over time will be immensely useful. Even with thorough documentation of records and events, having access to a specialist's experience in growing and observing a threatened species over a long period of time is very valuable and difficult to replace.

Growing threatened species in a nursery is often highly intensive and complex. It requires a very detailed level of data recording and specialised knowledge and skills, as many native **taxa** have often not previously been propagated or cultivated. Much can be learnt about the biology of threatened species in a nursery and recording of success and failure of propagation and growing techniques can be greatly beneficial to their management ex situ and **in situ**. If contracting-out propagation or setting up partnerships, it is important to use a reputable, experienced grower such as a botanic garden or specialist nursery with a strong conservation and/or **restoration** focus and ability to work with the often extended timelines required when working with threatened species. If using specialised plant production nurseries, it is important to establish binding contracts that uphold the conditions of Permits to Collect. These conditions which may prohibit the release of plant material for commercialisation, as well as outlining hygiene standards required.

Many threatened species have quite particular requirements, based on their biology, and these need to be considered on a case-by-case basis with some likely to be challenging to maintain for long periods under horticultural conditions.

The following are examples of plant groups that require specialised nursery care and further considerations that may also include laboratory and living collections management:

- Orchids – terrestrial, epiphytic (Chapter 12);
- Plants that have essential specific **mycorrhizal** or symbiotic associations (Chapter 12);
- Plants from extreme environments (Perrins 2020);
- **Parasitic** / **hemiparasitic** species (Chapter 13);
- Cryptogams e.g., algae, lichens, mosses and ferns (for ferns see Chapter 13);
- Carnivorous plants which may have highly specific soil (acidic, nutrient poor soils) and watering requirements (water-logged substrates) as well as unusual growing conditions (ie. high light and high humidity) (see Chapter 13);
- Plants impacted by special **threats** such as disease diseases like Myrtle Rust and Phytophthora Dieback. There may be a need for risk mitigation and management strategies dealing with highly susceptible species (Stanley and Bodley 2020, Viler and Offord 2020, see also Case Study 8.1).

It is particularly important to maintain the highest levels of nursery hygiene when working with threatened species. The phytosanitary conditions expected are dealt with in Commander *et al*. 2018 (Translocation Guidelines 3rd Ed. section 6.4.3), and Turner *et al*. (2021), which are in line with the minimum standards and best nursery practice expected under the Nursery Industry Accreditation Scheme Australia Best Management Practice Guidelines (Greenlife Industry Australia 2019). Plant propagation techniques used in the nursery are varied and include both

seed-based and vegetative techniques. These must be appropriate for the focal species, the time frame of the project or collection, and the intended end use of the germplasm, including consideration of the required genetic composition of the germplasm (see Case Study 8.3).

It is recommended that maternal seed lines are used when propagating threatened species rather than bulked seed lots (see Chapter 3, particularly Box 3.2 'Why not pool **maternal lines**?'). Whether from vegetative material, maternal seed lines or bulked seed lots, the genetic identity of each plant should be known and traceable with a unique identifier from the time of collection and maintained for the life of the plant, even when returned to the wild (Chapter 3). Generally speaking, this requirement for tracing of individual plants is a major point of difference between how threatened species are managed in a nursery (in these guidelines) and how more common plants are propagated and grown in a nursery environment for restoration purposes, where plants are labelled by seed lot rather than by **genotype** or mother plant (Turner *et al*. 2021). While it is important that propagation material be sourced from appropriate **provenance** for restoration, the fate of individual genotypes is rarely closely monitored or documented in **ecological restoration**, unless for specific experimental purposes (e.g., Broadhurst 2012; Krauss *et al*. 2013).

Record keeping and planning are therefore of utmost importance in a nursery situation (Chapter 15). As with all aspects of germplasm conservation of threatened species, careful planning of nursery-based activities and identification of key steps is required. Key activities, such as obtaining permits, collection, propagation, labelling, data recording, potting-on, fertilising, pruning, and pest and disease control, must be carried out in a timely fashion because such plants may be in a nursery for months, years and even decades on occasion. Above all, watering and disease control are critical. Failures in watering for example, could lead to catastrophic loss of important germplasm. Diseases introduced or not controlled in the nursery can lead to loss of germplasm, and possible introduction of disease to other nursery-held plants and wild sites if accidentally introduced with translocated plants. In this regard, maintenance of potted plants in a nursery should be considered relatively high risk and resource intensive. Nevertheless, it may be the only option for some species and extremely rare species or unique genotypes, like the sterile (non-seed producing) *Hakea aenigma* and *H. pulvinifera.* For further consideration of techniques, refer to Commander *et al*. (2018) Section 3.5 of the Translocation Guidelines and Turner *et al*. (2021).

This chapter provides guidance on:

1. Planning and partnerships
2. Optimising plant growth
3. Nursery hygiene (pest, disease and weed control)
4. Propagation techniques
5. Nursery record keeping

8.2 Planning and partnerships

While seed banks, tissue banks and horticulturally-managed living collections can conserve representative **genetic diversity** of a species over time, it is more often than not, the fate of such material to pass through a plant nursery at some stage during the plant production cycle.

Planning is critical to successful **ex situ conservation** with a nursery component and must remain focussed on the desired end uses of the collection. Considerations should include:

- When and where will collections be used? This should be part of the planning prior to undertaking field collecting (Chapters 2 and 4).
- Are the nursery resources suitable for the short to long term needs of the collections, ie. infrastructure, equipment, size, access, biosecurity and quarantine for sensitive collections?
- Has the expertise of available personnel been considered? The participation of professional horticulturists and database experts, experienced in propagation, plant and data management, both within and across organisations should be ensured.
- How and when will plants be hardened off/acclimatised for translocation or other planting? This will be determined by the nursery resources and climate as well as the plant's needs.
- Material handling, storage and transport logistics are also important to consider.

Consideration of the climatic conditions in which a species naturally occurs and the environments in which it will be propagated and grown-on are important factors in success (refer to Turner *et al*. (2021) for hardening-off and Commander *et al*. (2018) Translocation Guidelines Ch 4 for source and recipient **populations**). Where possible, grow the species in a similar climatic zone to where it naturally occurs and will be translocated. This will benefit plant growth and may reduce transport/travel costs and carbon emissions. Climate is also a consideration when finding suitable locations for ex situ conservation of species that will be adversely affected by threats such as climate change (see Case Study 8.2), or disease (e.g., species affected by Myrtle Rust may benefit from a drier and cooler climate where the disease can be better managed).

Examples of planning and partnerships for threatened species conservation are found in Case Studies 8.1, 8.2 and 8.3.

Case Study 8.1: The importance of partnerships for securing threatened species

Stig Pederson

When we engage in conservation work, we often do so with great enthusiasm. We want to make a difference; we want to preserve the relevant species or ecological community and to help secure its future. Often, we come up with methods we see as 'best fits' to meet our goals. However, we may neglect to acknowledge the threats that external factors might have on what we set out to achieve. For example, the impact of fires, pests and diseases, and loss of specialist knowledge due to staff turnover. This becomes relevant and can have serious impacts if a threatened species or community is confined to only a few localities, or indeed a single location, or when working in isolation.

What to do about it? Put simply, we need to spread the load and need to come up with an insurance policy. One such policy is to form partnerships that work together on common goals to ensure that threats don't become catastrophes (Pederson 2021). Such an approach was put into practice when three botanic gardens agreed to work together on the Myrtle Rust-affected Scrub Turpentine (*Rhodamnia rubescens*) (Figure 8.1).

Figure 8.1: Scrub Turpentine (*Rhodamnia rubescens*) in flower. (Image: Julie Percival)

Myrtle Rust (*Austropuccinia psidii*), first detected in Australia in 2010, is a fungal disease that affects plants in the Myrtaceae family, with a range of consequences from deformed foliage through to death of individual plants. One affected species, distributed from Batemans Bay on the NSW South Coast through to Bundaberg in Qld, is the species *Rhodamnia rubescens*. Once common, it is now rapidly dying from the impacts of Myrtle Rust and is one of four species that in NSW has been declared as being Critically Endangered.

To address the rapid loss of *Rhodamnia rubescens* plants, the Booderee Botanic Garden (BBG), the Eurobodalla Regional Botanic Garden (ERBG) and the Australian National Botanic Gardens (ANBG) formed a partnership in 2019, with the aim to establish ex situ collections in all three botanic gardens. Based on several observations in the wild, where affected plants struggled to produce **fruit**, it seemed highly unlikely that any fruiting material would ever be found. As a result, it was decided to forge ahead using cuttings as the only viable propagation method.

Once the partnership was established, it quickly swung into action. No fewer than ten sites, spread throughout the Jervis Bay and Batemans Bay regions of New South Wales, were visited in the winter of 2019, and cuttings taken from all sites. The material was evenly split between ERBG and BBG, and several dozen plants were subsequently grown at both gardens. Plant identification was maintained through careful data management, with use of common **accession** numbers and references linked to collections enabling the tracking of **clones** during and after field work collection events. This method had previously been used successfully on non-seed conservation projects and ensured that diversity within the holdings were known and able to be tracked.

And then disaster struck. The Currowan fire, one of many 2019–20 Black Summer fires, affected large areas of the NSW South Coast and burned most of the sites where cuttings were collected only six months earlier. Not only that, the fire overran the ERBG and killed all but three *Rhodamnia rubescens* nursery plants propagated the previous winter. Thanks to the partnership that had earlier been formed, not all was lost. Due to the foresight of distributing propagation material between the partners, BBG still held genetic material representing many genotypes from the locality affected by fire. The ex situ plants that currently flourish at BBG may now be the only genetic source representing the southern extent of the species.

The impacts caused by the Currowan fire brought into focus the importance of working in partnership for conservation of threatened species. As another fire season approaches, the BBG will hand over a number of plants to the ANBG, and in time to the ERBG when they have recovered. The next step is to propagate from existing nursery stock to build up clone numbers with the aim to distribute to botanic gardens beyond the current partnership, as part of a metacollection strategy (Chapter 11, section 11.10). Ultimately, the hope is that the valuable work performed by all partners will lead to re-establishing the genetic material into the wild. This should further increase the species' chance of survival.

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Case Study 8.2: Establishing a metacollection of threatened species from Far North Queensland's cloud forests

Warren Worboys and John Arnott

The tops of the high peaks of Far North Queensland are the cloud forest **habitat** for a broad range of **endemic** flora and fauna, including **relicts** of **Gondwanan** species of flowering plants, conifers, ferns, mosses and other non-flowering taxa, including species that have only just been discovered during this project (Figure 8.2 and 8.3).

Modelling by the Australian Tropical Herbarium (ATH) (Costion *et al*. 2015) has predicted that climate change has the potential to have significant impacts on the clouds which normally enshroud these peaks, with the cloud base rising above the peaks, thus considerably reducing total moisture inputs. Rainfall on some peaks can exceed 12 000 mm annually (Bureau of Meteorology 2020) while cloud moisture condensing on foliage and dripping on to the soil, a process called 'cloud stripping', can contribute an additional 50 % to the water reaching the forest floor (D. Crayn, Australian Tropical Herbarium, pers. comm.). The cloud base rise is predicted to result in drying of the cloud forests and habitat loss.

A grant from The Ian Potter Foundation has enabled the ATH to collaborate with the Australian National Botanic Gardens, Canberra (ANBG); Australian Rhododendron Society, Victoria Branch; Cairns Botanic Gardens; Mossman Botanic Gardens; Royal Botanic Gardens and Domain Trust, Sydney (RBGS); Royal Botanic Gardens Victoria (RBGV), Traditional Landholders, and Wet Tropics Management Authority, Qld., funding a project involving a series of plant collecting expeditions to these peaks.

This project will enable project partners to secure the future of Australia's climate-threatened tropical mountaintop plants by building a multi-strategy ex situ conservation reserve to 'backup' at-risk wild populations and support research, display and education. The novel research on seed banking strategies, genetic diversity, and plant tolerance of extreme climates will ensure that the reserve collections, distributed across multiple botanic gardens and seed banks in a metacollection (IUCN SSC Conservation 2014-64; Griffith *et al*. 2019) along Australia's east coast, are genetically and physiologically diverse, and climatically matched to wild habitat.

The ATH and JCU shortlisted 70 species of cloud forest endemic flora most under threat then undertook intensive research to develop a field trip strategy to enable collection of herbarium **vouchers** and genetic samples (ATH), seed for long term storage (ANBG and RBGS) and asexual propagation material (ANBG and RBGV). Genetically diverse propagation material is being collected and the provenances of the material is being accurately recorded to ensure the diversity is tracked.

This ground-breaking collaborative approach is enabling the varied skills of participants to be utilised to help establish ex situ nursery collections which will then be distributed to appropriate botanical institutes through an expression of interest and site evaluation process for in-ground cultivation.

Most of these plants have never previously been grown in cultivation and novel methods of propagation are being adopted and a diversity of standard practices are being experimented with to ascertain the best way to grow the species. While some taxa have grown quite readily from cuttings, zero survival rates from others has made it necessary to revisit some sites to collect more material in different condition. For example, *Lenbrassia australiana* (Figure 8.4), is in Gesneriaceae, a family whose members are typically easy to grow from soft to medium hardness shoot tip cuttings, but the first collections didn't survive. On a follow-up visit, hardwood stem cuttings were taken, and these have resulted in a good strike rate.

Once established in the ground, these plantings will form a genetically diverse conservation metacollection housed at a number of sites providing security, a resource for re-introduction to their natural habitat, and material for future display, scientific research and education.

Figure 8.2: Boulder field on Mt Bartle Frere, the only known habitat of *Eucryphia wilkiei*, Cunoniaceae (Image: Warren Worboys)

Figure 8.3: Clouds move in on the Mt Bartle Frere cloud forest (Image: Warren Worboys)

Figure 8.4: *Lenbrassia australiana* var. *australiana*, Gesneriaceae (Image: Donna Davis)

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Case Study 8.3: Using clonal genetic structures to inform and develop an ex situ conservation strategy for *Grevillea renwickiana*

Edward J. McAuliffe

Grevillea renwickiana F.Muell. (Nerriga Grevillea) is a critically endangered prostrate root-suckering shrub (Makinson 2002, Figures 8.5 and 8.6). It is endemic to New South Wales, confined to the Braidwood-Nerriga area where it grows on sandy soils in open dry sclerophyll forest. Flowering occurs during summer months, but appears to be irregular and the flowers are putatively sterile.

In 2011, members of the Australian National Botanic Gardens' (ANBG) Living Collections staff located a population of Nerriga Grevillea on private property east of Nerriga.

Identifying an opportunity to include the new population in ongoing genetic studies, and recognising potential for developing a scientifically backed ex situ conservation plan for the species, we discussed a sampling strategy with Elizabeth James (Royal Botanic Gardens Victoria, Melbourne) and Keith McDougall (NSW Department of Planning Industry and Environment). Genetic analyses of the species had identified eight genetically distinct individuals that were generally restricted to single populations (James and McDougall 2014). We modified our standard collection method of taking a herbarium specimen and cuttings from a single plant or area to match the methodology used by James and McDougall (2014), enabling comparisons to be made with previous work.

Samples were collected from three accessible locations over a total distance of 2.5 km, maintaining a minimum distance of 10 m between sampled plants. Two herbarium specimens were taken, one from either end of the collection transect. This resulted in 16 new collections (accessions), which were each given a unique field number, locality description and geocode. For each new collection, samples consisting of four or five leaves were placed into silica gel for genetic analysis and a small number of cuttings were collected and tracked using the same field collection number as the corresponding genetic sample. The cuttings struck well and multiple plants from the 16 collections were later grown and maintained as individual accessions in the ANBG Nursery.

A genetic analysis of the 16 samples taken from the newly located population identified two genetic individuals, so, the ANBG condensed the 16 accessions into two separate accessions, retaining their genetic identity. This reduced the complexity of short-term management of the lineages and dramatically increased the feasibility of retaining the long-term scientific integrity of the ex situ collection.

In designing an ex situ management plan for this species, its typical growth habit, characterised by its rhizomatous spread, must be considered. The plant's ability to sucker from rootstock and shed old stems, and to spread with new above-ground stems has the potential to complicate or undermine lineage tracking in cultivation. In the case of *Grevillea renwickiana*, the ANBG deliberately planted a single clonal lineage in a section of the garden (Figure 8.5) and separated the plants from other clonal lineages with a physical barrier such as a road to minimise the possibility of confusing lineages. When appropriate records are maintained, long-term management of clonal lineages is achievable at a relatively low cost and resource effort if the cultivation requirements of a species are straightforward. The lifespan of individual plants, care in labelling and maintenance of planting records, as well as data management are important considerations in maintaining living collections (McAuliffe 2020).

One of the key objectives of an ex situ collection is to facilitate in situ conservation options. Good conservation outcomes can be achieved through working collaboratively with the scientific and natural resource management communities. This is especially the case with sterile species, such as *Grevillea renwickiana*, where seed banking is not an option and knowledge of genetic structure can have a large impact on collection management decisions and improve the quality of the ex situ conservation collections. ANBG now holds 20 % of the known diversity of the species and has the capacity to hold and maintain the other eight lineages identified in the earlier study. It is important to recognise that the security of both ex situ collections and conservation of wild populations of the species will be best achieved if the known diversity of *Grevillea renwickiana* is held in more than one botanical institution. This may be possible through the collaboration of the NSW South-East Bioregion Working Group, a conservation-focused collaboration between the ANBG, the Australian Botanic Garden Mount Annan, Wollongong Botanic Gardens, Booderee Botanic Gardens, Eurobodalla Regional Botanic Gardens and Illawarra Grevillea Park.

Early and open communication between government agencies and conservation practitioners, including private landholders, was in this instance, a key factor that influenced the ANBG's approach. By considering and contributing to the latest genetic analysis of *Grevillea renwickiana*, the ANBG has been able to apply the most appropriate collection management practices and horticultural knowledge to its ex situ holdings of the species.

Figure 8.5: One of the newly identified **genomes** of *Grevillea renwickiana* growing in the Australian National Botanic Gardens. (Image: J. McAuliffe)

Figure 8.6: *Grevillea renwickiana* sterile flower from Corang B site. (Image: E. James)

References

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8.3 Optimising plant growth

8.3.1 Potting media

Living plants can be cultivated in various media, including potting mixes and soil in pots, large or small, or in garden beds. Special attention should be paid to ensuring the appropriate medium, fertiliser and watering regimes, and the reader is referred to Handreck (2001) for consideration of growing conditions for Australian species.

Consider the components, nutrients, pasteurisation, and timely use of potting media. Avoid situations where the medium becomes hydrophobic (repels water) and ensure appropriate moisture retention or drainage. Water soluble surfactants can be used at potting and 3 monthly intervals (particularly important during the warmer months of the year) to improve water permeation into the pot medium. Storing potting media for extended periods, generally longer than 3 to 6 months may result in pathogens infecting the mix and detrimentally affecting the potted plants. Depending on storage conditions, potting medium may degrade over time, particularly if stored under high temperatures and with significant amounts of moisture present. Potting media may become unsuitable for use due to nutrients breaking down (particularly added synthetic fertilisers), and the presence of microbes that may change soil attributes such as pH, oxygen availability (soil may become anoxic), and the production of toxic compounds.

8.3.2 Plant size

For translocation, consider the size of the plants to be used (standard forestry pots are ideal for most species) and the amount of potting media being transferred to the wild/natural environment. This reduces production, transport, planting and aftercare costs and requirements. Larger containerised plants require more watering to establish when planted and may also be root bound so establish poorly when planted out. For collections that need to be maintained in the nursery for long periods ex situ, larger pots might be used, or consider repropagation to renew the collection.

8.3.3 Potting up

Aim to optimise root health through appropriate pot or container choice. Consider size, type (e.g., forestry tube vs standard), the use of air pruning, and anti-spiral pots (root trainer pots for the growing of tree species) all help enable the establishment of good root structure and assist in the success of translocation hygiene and establishment. After potting on or planting out, most pots may be sterilised using chemicals such as sodium hypochlorite (bleach) or benzalkonium chloride solutions, or steam, and the pots may then be re-used multiple times.

Avoid using a pot that is too large as this can encourage rot as the plant is not capable of using the moisture and fertiliser in the mix. This is especially important at the first potting on stage. In most cases, a 50 mm forestry tube is sufficient and generally preferred for both nurseries and translocation. However, some taxa will grow better in smaller or larger standard tubes depending on their growth rate and natural habit. Potting on or planting out should ideally be undertaken when roots are growing actively (see Figure 8.7).

Figure 8.7: Root system of a forest tube-potted plant which has been in the tube too long (left) with roots becoming amber coloured and less active, compared to active, white coloured roots of the plant (right) which indicates an ideal condition for planting. NB Vertical lines of roots are from the 'root trainer' sides of the tubes. (Image: Warren Worboys)

8.3.4 Fertiliser regime

Many native species are sensitive to high levels of phosphorous (particularly Proteaceae), and a number of species are also sensitive to high levels of ammonium nitrate. Hence, attention should be paid to the phosphorous and nitrogen components and what percentage is present when selecting a controlled release fertiliser. As there is not much data available on individual species, a good strategy is to halve the suggested application rates and monitor plant health and growth as indicators of sensitivity. Symptoms of excess nutrients can include poor growth rate, brown or burnt leaf margins and plant death. Some slow release fertilisers are marked with the number of days over which the fertiliser is released, e.g., 30 days or 90 days. If using slow release fertiliser formulated for native species, it can be beneficial at the outset to blend 33 % x 30 day pellets and 66 % x 90 day pellets, providing a smaller burst of nutrient. As the 30 day fertiliser is depleting, the plant is growing and utilising the nutrients, and the 90 day fertiliser is starting to peak in nutrient release to sustain the growth. However, do not fertilise plants just before planting in the ground unless there will be a good level of plant monitoring and watering, as new growth induced by the fertiliser will be softer and subject to desiccation and pathogen attack.

8.3.5 Watering

The watering regime is very important at every stage and must be flexible to allow for weather changes. Consistent monitoring of soil moisture levels is important to ensure that plants receive adequate water to support growth whilst not adversely affecting root structure. During the hardening-off stage, less water can be applied, but this needs to be carefully monitored to avoid drought. Capillary mats are useful for avoiding overhead watering of pot-grown species that don't tolerate high humidity, but extra care is required to avoid waterborne pathogens. Drip irrigation may be suitable for more advanced (larger pot size) longer-term potted collections.

To facilitate a balanced watering regime, nursery stock can be 'hydrozoned' by placing plants with similar watering requirements and pot sizes together. Hydrozoning is essential in nurseries with automated irrigation systems to ensure appropriate water application.

8.3.6 Records and program planting

Good records provide information on how long it takes to propagate a species (from seed or cuttings) and to grow it on to a size suitable for planting out or long-term maintenance (Section 8.6 and Chapter 15). These records can be used to ensure that batches of plants are produced to within a week or two of when they are required for planting out, assisting in scheduled project planting timelines.

8.4 Nursery hygiene (pest, disease and weed control)

Pest and disease control are essential in any nursery and includes animals (e.g., birds, insects), weeds and diseases caused by micro-organisms (e.g., fungi, bacteria, virus). Any of these could badly affect the operation of the nursery and plant production. If plants are being produced for restoration, there is a possibility of carrying the problem into the recipient site.

Integrated pest management (IPM) is highly valuable in any nursery. IPM uses a range of cultural practices to manage or control infection that can include:

- Ensuring plant material is clean, or treated for pests, when it arrives in the nursery.
- Quarantine and monitoring of incoming plant material for a period of time.
- Using pasteurised potting media.
- Using new or sterilised pots.
- Regularly sterilising tools and work areas.
- Keeping potted plants, tools and equipment off bare soil or areas subject to foot traffic.
- Ensuring there are no weeds in plant pots or in the growing area.
- Timely use of chemical and/or biological controls to manage insect and disease infections. Consider using predatory insects to control pest species instead of chemical sprays.
- Capillary mats may rapidly spread disease if good hygiene is not maintained.
- Fences and cages, glasshouses and tunnel houses, may need to be used to exclude larger animals, like birds.
- Optimising watering, light levels, air flow and fertilising regimes and the application of appropriate growing conditions to ensure good plant health.
- Regular inspection and monitoring of all nursery stock to ensure plants are in optimum health, including carefully removing plants from pots to inspect for soil infections such as Root Mealybug or root rot.

Early preventative action can avoid more extreme actions later on (such as chemicals sprays over large areas).

8.5 Propagation techniques

Before choosing a technique, consult with the literature and/or propagation experts on what is known about the propagation of the target species or, if information on that species is not available, find information on a related species of the same genus, family or **clade**. The first consideration is what material to collect (Chapter 4 and Turner *et al*. 2021), such as seeds or vegetative material and, in some cases, both may be possible and worth trying. Then, it's where and when to collect material, taking into consideration flowering and fruiting times or seasonal growth of vegetative material (Chapter 4). Always consider whether appropriate material is already available ex situ in a seed bank, botanic garden, commercial or community nursery or from specialist amateur growers (e.g., Australian Native Plant Society [ANPSA] study groups[52]), before committing to wild harvesting which may further threaten a species or population.

To maximise genetic diversity, propagation from seeds is preferable and, in most cases, also much cheaper compared to either vegetative propagation or tissue culture. For threatened species, seeds collected from different plants in the wild or from field genebanks and subsequently propagated should be labelled and kept separately. This ensures there are representatives from each parent plant (maternal lines) and that the resulting plants are not overly biased towards particular genotypes or populations (see Chapter 3 Box 3.2 'Why not pool maternal lines?').

Where seed is unavailable, of poor quality or cannot be germinated, or the genotype of the individual needs to be replicated, vegetative propagation through cuttings, division, grafting or tissue culture may be appropriate. Generally, an ex situ population established using vegetatively propagated material should represent as many genetically individual plants as possible, being mindful of the constraints imposed by the plant's interaction with the cultivation environment as well as any physical and resource limitations, such as sufficient staff and space to maintain large numbers of plants. While the techniques for vegetative propagation are not treated as a separate chapter in these guidelines (cf. seed **germination** and tissue culture) they are valid and widely used. In some cases, vegetative propagation is recommended over seed germination and tissue culture. Examples include propagation by cuttings of *Boronia*, *Hibbertia* and *Myoporum* species, or division of some Restionaceae (rushes) and Cyperaceae species (sedges).

Many references detail the principles and practices of different types of propagation, e.g., Davies *et al*. (2017) or Beyl and Trigiano (2015) for detailed general principles, Toogood (2019) is excellent for the beginner or low budget grower with step by step illustrations that may be applied to most plant species. Also see Bowes (1999) for conservation collections, or Stewart (2012) and Turner *et al*. (2021).

8.5.1 Seed propagation

If considering propagation by seeds, ask the following:

- Are suitable seeds available in a seed bank or is collection from the wild required (Chapters 2 and 3)?
- If collecting from the wild, find out what is known about the timing of flowering and fruiting and if there may be seed quality or germination issues (Chapters 4, 5, 7).
- What type of fruits and seeds do they produce (Chapter 4)? Are seeds/fruits released upon maturity or are they retained upon the parent plant for a length of time following maturation i.e. **serotinous** species such as many Banksias (Chapter 7)?

52 http://anpsa.org.au/study.html

- What type of seed storage is suitable? Can seeds be dried and stored until used, or do they need to be kept moist? (Chapter 6)
- What is known about seed propagation of the species, especially the likely viability of seeds and presence of **dormancy** or other germination requirements (Chapters 5 and 7)?
- If suitable seeds are not available, can the species be propagated vegetatively or consider a **hybrid** approach whereby any available seeds may be used as the starting material (via **embryo culture** or **in vitro** germination) for the initiation of different clonal lines via plant tissue culture (Chapter 9).

Factors that limit the use of seeds for propagation include:

- A species may not produce seeds or seeds are not **viable** ie. not filled (See images in Case Study 8.4), dead or are predated (Chapter 5).
- Seeds of some species have complex dormancy barriers that are yet to be understood thus cannot be easily germinated (Chapter 7).
- Sporadic and erratic seeding, making collection at appropriate times difficult or constrained by resources and accessibility issues such as restricted access due to the threat of bushfires or the wet season (Chapter 4).
- Seed production could be low; thus, it may not be possible to collect without risk to the regeneration capacity of the donating population (Chapter 4).

Information from seed germination experiments in the laboratory or the nursery can be used to make best use of what is often small amounts of seeds available for threatened species (Chapter 5–7). This is particularly important for seeds that demonstrate dormancy, in which case, find out the dormancy breaking treatments most likely to work.

Further considerations for optimal seed germination include:

- What is the best air and substrate temperature?
- Which seed-raising substrate to use?
- What is the best time of year for onward growth of seedlings?

Benefits of using seeds:

- May be the only option available for some species.
- Seedling-grown plants often display juvenile characteristics, which can include vigour and better ability to establish in the wild.
- This juvenile vigour can be used to advantage for next generation tissue culture, if required.
- Root systems of seed derived plants are often better structured (with a dominant/leading tap root) and more vigorous than cutting derived plants.
- Potentially high **genetic variability**.
- **Orthodox** seeds can be stored for long periods, with minimal maintenance required.
- Many seeds germinate easily when provided with ideal conditions.
- Cheaper than other forms of plant propagation.

Risks of using seeds:

- Plants propagated from seed may take longer to reach sexual maturity than vegetatively propagated plants, especially many woody species. For example, seedling grown *Telopea speciosissima* (Waratahs) take more than four years to flower/seed, in comparison cutting grown plants may take less than 18 months if propagated from adult phase material (see also Viler and Offord 2020).
- Many species do not produce seeds or produce low numbers of poor-quality seeds.
- **Non-orthodox** seeds can only be stored for short periods (days to several weeks) before they lose viability (Chapter 6).
- Greater than 40 % of threatened species have some class of dormancy and thus require some form of dormancy-alleviating treatment which in many cases is not well understood at present (Chapters 6 and 7, Offord *et al*. 2004).

8.5.2 Vegetative propagation methods

Over the millennia, humans have developed and refined vegetative propagation methods as a resource for their own benefit, in particular to produce food. For example, banana plants are produced by the separation and transplantation of banana suckers from the parent plant and ginger by dividing clumps of **rhizomes**. Potatoes are cut up so that each portion with an 'eye' may be planted to produce a new plant with many new **tubers**.

In recent centuries, vegetative propagation has been refined: layering and cutting-grown food, medicinal and culinary plants have been produced, and with gentrification of society, expanded to grow plants of aesthetic value, growing many plant clones for their beauty or fragrance rather than their food value. These years of experimenting with vegetative propagation have provided the conservationist with an invaluable knowledge resource for propagating and curating plants of threatened species.

The various forms of vegetative propagation all rely on the natural ability of the plant, or the plant part, to regenerate itself using unspecialised cells that may be found in different plant organs such as shoots, rhizomes, roots, and leaves to adapt and form new tissues as required for the regeneration of a new plant ie. to form roots, shoots or leaves. This ability is known as '**totipotency**' and its utilisation is potentially a very powerful way to produce large numbers of plants without the need for seeds (Davies *et al*. 2017). Nearly all plants have this capacity to some degree. The way plants regenerate vegetatively varies widely and likely relates to other life history traits of the species such as: the capacity of the species to re-sprout after fire compared with those that are killed; the capacity to naturally spread via underground rhizomes; or, through the production of new shoots directly from roots.

A plant that has been propagated vegetatively and is identified as being genetically unique is often called a 'clone' e.g., Clone '1', Clone '2'.....Clone 'X'. Clonal progeny are 'carbon copies' of the parent, essentially genetically identical to both the donor plant as well as other plants vegetatively sourced from the same donor plant. It is often helpful to determine how many cloned individuals to propagate and maintain in the nursery through genetic sampling of the source population prior to collection of material – see Case Study 8.3.

Benefits of vegetative propagation:

- Genetic sampling of individuals within a population may identify specific genotypes and their inter-relatedness or kinship, potentially improving the chances for natural increases in population numbers when translocated using an appropriate genetic mix (Chapter 3 and see Case Study 8.3, McAuliffe 2020).
- Can be used when insufficient germinable seeds are available.
- If species recovery is time sensitive, due to clearing, wildfire or drought for example, vegetative propagation may be the only short-term option if seeds are unable to be collected (but not generally a recommended course of action for threatened species).
- If whole plants are being removed for transplant, additionally taking cuttings may increase the chances of success and may be an easier/more practical way to preserve threatened genotypes in some situations.
- Some species may be easier and/or quicker to strike from cuttings than to germinate seed (especially deeply dormant seeds which might take months or years to germinate).
- Advanced plants can be rapidly obtained which can be used for further vegetative propagation or even seed production (via a managed seed production area) more quickly than if relying on seed-grown plants.
- Vegetative propagation – particularly cutting propagation – can often result in more uniform growth rates amongst resulting plants.
- With the right infrastructure and equipment, cuttings can be produced in large numbers for many species at any time of the year as required.
- Parental plant vigour may be enhanced, and the production of new shoots or flowers may be stimulated with the removal (pruning) of cutting material.
- Often vegetative propagation success is greatly increased once material sourced in the wild is growing in cultivation under optimised conditions.

Risks of vegetative propagation:

- Potential narrowing of genetic diversity in ex situ and translocated populations.
- Damage could occur to plants in the original population resulting in direct and indirect loss of plants through overharvesting of material, introduction of disease or weeds through the use of contaminated equipment, and plant wounding which could result in infection from opportunistic pests and diseases, as well as soil disturbance.
- Loss of plants during establishment from cuttings or transplants due to horticultural knowledge gaps or lack of care. It is reasonable to expect, however, that not all cuttings of threatened species will strike and a conversion rate of healthy cuttings to established plants might be low, typically 30–75 % (noting that the full range is 0–100 %).
- For many species, the seasonal timing of material collection and propagation plays a major factor in the success rates.
- Maintaining large genetically diverse ex situ collections is resource intensive, often only funded for a short period of time and requires specialist horticultural and record keeping protocols.
- Cutting-grown plants may not establish as well as seedlings in translocations, due to various factors including differences in plant habit, stage of juvenility and poor root system development and structure.
- There are often greatly varying results between genotypes in terms of growth, rooting success, and susceptibility to pests and diseases.

- Plant material generally will only last for a short period after cutting from the plant, so needs to be kept cool and moist, by wrapping in moistened newspaper, placed in a plastic bag, in a cool box or refrigerated at 3–5 °C, and processed as quickly as possible, ideally within a few days.
- Failure to accurately track clonal lineages, especially over multiple generations. This should be considered in timeframe planning, particularly for difficult or slow-growing species.

8.5.2.1 Cutting propagation

Propagation from cuttings is the most common form of vegetative propagation in many plant nurseries. It involves 'striking' roots onto 'cuttings' (generally, shoots taken from the stems or branches of the plant).

Many species, like some *Boronia, Eremophila, Hibbertia* and *Myoporum,* that are challenging to grow from seed, may propagate easily from cuttings, while others, like *Persoonia* and *Leucopogon*, may be very difficult to propagate vegetatively, so other forms of propagation may need to be considered. Some species propagate from vegetative cuttings best in spring/summer while others will do significantly better if the material is collected in autumn/winter. Most species will propagate more readily from shoots taken from near the base of the plant, such as basal suckers, rather than from older woody stems high up on the plant; this is especially so for large shrubs and trees. However, some species with distinct juvenile and adult growth stages may pass on the juvenility to cutting progeny that are produced.

If nothing is known about the target species, find out what is known about vegetative propagation of related species (either the same genus or family) or other co-occurring species with a similar growth form. It is also important to understand what happened in previous propagation attempts, regardless of whether they were successful, particularly the approaches used, the type of material trialled, and the range of conditions and treatments assessed (see also Turner *et al*. 2021). The type of cutting made is important. Cuttings may not strike if too woody or too soft (Box 8.1) and it is best to seek expert advice on the best material which may vary, depending on the species, and time of year.

Preparation of cuttings

Once cuttings are cleaned (often with mild bleach solution, e.g., 1 % sodium hypochlorite), lower leaves and stems are removed. It is natural for plants to endeavour to reproduce to survive and thus may put their energy into flower and fruit production rather than new shoot or root growth. This takes essential energy and moisture from cuttings therefore, it is important to remove any flowers, flower buds and/or fruit from cuttings while they are being prepared. Different types of cuttings can be made, depending on the species and the material available (Box 8.1).

Root stimulating treatments

In most cases, the bases of the cuttings are dipped into powder or solution containing a root-stimulating plant growth regulator (PGR – see Box 8.2), such as IBA (indole-3-butyric acid), although some species will produce roots without a PGR stimulus. With most species, the root primordia – the initial specialised cells which form the roots – are not capable of expanding through the bark of stems and will develop roots through the base of the cutting. Therefore, only the base of a cutting needs to be dipped into the growth regulator solution. Often, **callus** tissue (parenchyma cell proliferation from cut tissue in response to wounding) will develop at the base of a cutting (Davies *et al*. 2017). The callus seals the wound and is capable of absorbing sufficient

moisture to keep a cutting alive for months (and even years with some conifer species), however the callus is often thick enough to prevent the root primordia from extending and forming roots. It then becomes necessary to trim off at least some of the callus, back to the original stem base, to allow the roots to develop. In some taxa, such as many herbaceous Asteraceae, the root primordia can expand through the stem's epidermal layer.

Growing conditions for cuttings

The bases of the cuttings are dibbled (inserted) carefully into plugs (see Case Study 8.5) or punnets containing propagation mix, using an appropriately sized skewer or chop stick the same thickness as the cutting. The propagation substrate should have a high air-filled porosity (>20 %), as well as having good water holding capacity (Australian Standards, Potting Mixes AS 3743-2003, 2003; Handreck 2001). Generally, cuttings propagated in a heated environment should only be inserted into the cutting mix to a depth where they are held firmly upright. The deeper the base of the cuttings the more bark is in contact with the moisture retentive cutting substrate, increasing the opportunity for rotting.

The cuttings in punnets or plugs are then placed into a propagation environment with high humidity (>70 % **relative humidity**) out of direct sunlight (generally 50–70 % natural sunlight), such as in a controlled environment greenhouse, where they remain until 'struck' i.e., they develop new roots and may have begun to grow new shoots or leaves. Understorey species may be sensitive to excessive light and require extra shade in propagation. Plants from arid environments or with fine hairs tend to be prone to rotting and will likely respond better with dry fog or with covers in mist houses to stop foliage becoming too wet.

For many species, 'bottom heat' from specialised equipment such as a heat mat or built in bench heater, is applied during propagation to provide warm soil temperatures to induce root growth. Cuttings from alpine and cold environments, however, may have better success without traditional bottom heat. As a guide, the bottom heat temperature should generally be 5° to 7 °C above the annual mean average temperature of the natural habitat of the plant (e.g., 20 to 25 °C in temperate Australia). The temperature may need to be higher for some tropical species, or lower for some alpine species.

When to pot-on

Some species will start producing roots on cuttings (Figure 8.8) within two weeks and are ready for potting within five weeks e.g., *Scaevola* and *Dampiera.* Other species may take 12 to even 24 months to form roots, e.g., some conifer and rainforest species which are able to remain alive by absorbing moisture through their base and callus tissue.

Sometimes there is temptation to 'test' if cuttings are starting to develop roots by tugging the cuttings out of the propagation substrate. This should either be done very gently or avoided altogether as it may easily result in damage to delicate new roots, causing fungal infection, rotting of the roots and death of the cuttings. A simpler way to determine whether roots have formed is to look for roots growing out of the bottom of the pot, punnet or plug (Figure 8.14) which for many easy-to-strike species are readily visible several weeks after propagation.

Hygiene during cutting propagation

All tools used to prepare the cuttings should be razor sharp to minimise plant tissue damage via crushing or tearing that may occur with the use of blunt implements, which increases infection opportunities. All tools, dibblers and workbench tops should be cleaned down with 70 % alcohol solution or other sterilising agent between different batches of plants, to avoid possible cross-contamination.

Figure 8.8: New root development on recently struck cuttings of *Androcalva perlaria* (Malvaceae) which is a critically endangered species from south west Western Australia. (Image: S. Turner).

Box 8.1: Types of cuttings

Stem cuttings

Stem cuttings can be categorised into a number of different types:

- Softwood – generally the softer new-seasons growth taken from the tips of the main stem or side shoots. They are usually prepared by cutting 30 to 100 mm from the top of the stem just below a node. The 3 to 6-month-old stems are often green and flexible but not too soft and must be firm enough that they cannot be easily crushed between the thumb and fore-finger. This type of cutting generally requires very high humidity, >80 %, to avoid wilting. These cuttings are highly susceptible to fungal infection.

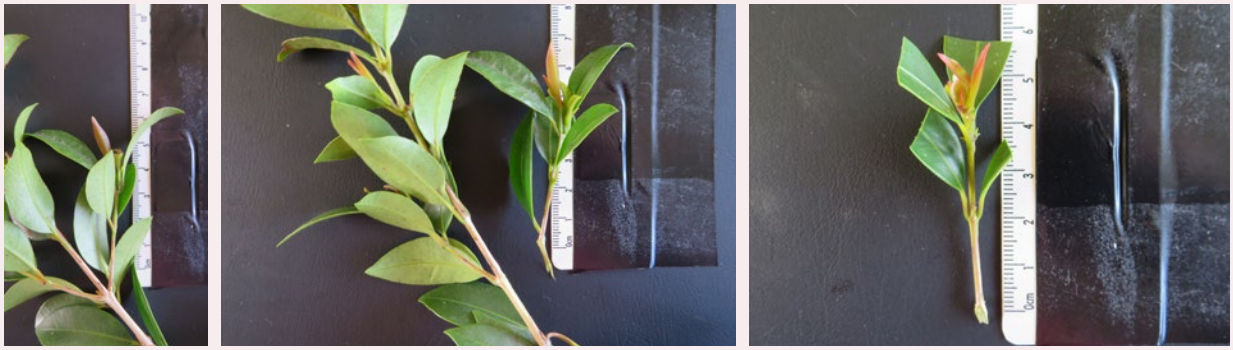
(L) Field collected stem of *Correa* prior to taking cuttings. (R) A softwood cutting has been removed from the shoot end and leaves reduced, ready to insert. NB Colour change of stem in lower 15 mm. (Images: Warren Worboys)

- Semi-hardwood (semi-ripe wood) – taken from firmer shoots of the main stem or side branches and may be from the tips or further down the stem. They are usually the hardened tissue from the current season's growth but may be up to 15 months old. They are generally prepared by cutting the stem into 70 to 100 mm lengths. The stems may be green and/or starting to colour grey or brown. The stems can usually be held between the thumb on one side and forefinger and middle finger on the other side and the stem will snap like a match when pressure is applied. These cuttings are generally less susceptible to wilting and fungal infection. This is the most common type of cutting method used.

(L) Field collected stem of *Grevillea* prior to taking cuttings. (R) A semi-hardwood cutting has been prepared and leaves reduced in length. NB Long internodes necessitate long cuttings. (Imagea: Warren Worboys)

- Hardwood – taken from older mature stems, ideally vigorous single season stems generally 18 months or more in age, where the bark is immature, and the wood is very firm. Commonly used for deciduous non-native plants. Some easily struck evergreen native plants with short internodes (e.g., *Leptospermum* and *Micromyrtus* species) may be grown from hardwood shoots that are two or three years old. Some woody herbs, like *Senecio*, and the shrubs *Melastoma* and *Lenbrassia,* can also be grown successfully from this type of propagation material.
- Mallet – the generally firm, semi-hardwood sten is cut with sharp secateurs approximately 8 mm above and 20 mm below a healthy leaf with a good bud in the axil of the petiole. Depending on its size, the leaf may need to be trimmed, then the base of the 'mallet' is inserted into the cutting mix to the base of the petiole. This method can produce many cuttings from a single stem section but is not appropriate for many species. In general, species from the families Araceae and Araliaceae will grow readily from mallet cuttings.
- Heel cuttings – are generally made from semi-hardwood side-shoot material that has been carefully pulled downwards from the main stem resulting in a 'heel' at the base which is a small amount of tissue from the main stem. This heel area contains a higher concentration

of unspecialised cells still present from before the bud sprouted to form the side shoot, and these cells specialise to form new roots. The firmer wood helps reduce the chances of the base of the cutting rotting. Often, a strip of bark is peeled off with the heel when pulling off the side shoots, and this should be trimmed off with a sharp knife or snips before being inserted in the cutting mix as the dying bark may cause rotting of the cutting base.



(L) Field collected stem of *Syzygium* prior to taking cuttings. (Centre) The stripped off heel cutting prior to preparation. Note the long tail of bark attached to the heel, at base of cutting. (R) The finished semi-hardwood cutting with trimmed leaves and heel. (Images: Warren Worboys)

NB: The length of cuttings mentioned above are of a general nature. Internodal length and species will considerably influence cutting length. For example, *Ewartia nubigena* may be grown from cuttings 8 to 10 mm long whereas *Tasmannia* sp. Mt Bellenden Ker may have internodal spaces of up to 200 mm between closely whorled leaves, necessitating quite long cuttings.

Root cuttings

Not a common method of propagation for Australian plant species but sometimes the only method available. For example, *Hakea aenigma*, a rare species from Kangaroo Island which is apparently a single clone, is completely infertile and is propagated from 100 to 200 mm long sections of the root, ideally with young shoots already sprouting, dug up from around the parent plant. This method has the potential to kill the parent plant if not carried out with care and should only be used as a last resort.

Rhizome cuttings

A method of propagating rhizomatous and stoloniferous herbaceous plants, whose above ground stems may be short lived and unsuitable for traditional cuttings. Examples include most species in Zingiberaceae (gingers) and stoloniferous grasses like *Hemarthria uncinata* (Mat Grass). Rhizomes or stolons are cut into sections, generally 70 to 100 mm long, depending on the source material, and individual pieces are laid flat in trays (see Case Study 8.4). This material generally has few or no roots and is therefore treated as cuttings, not as divisions which have roots already established.

Leaf cuttings

Individual leaves with the whole of the petiole attached may be removed from the parent plant as close as possible to the base of the petiole and inserted in the cutting mix. The leaves will develop roots then a shoot will grow. This method is successful with some herbaceous taxa including some *Brachyscome* species (Schaumann *et al*. 1987).

8.5.2.2 Considerations for collecting cuttings from the wild for propagation success

Refer to Chapter 4 for general information on collecting vegetative material.

- Plan to get material back to the nursery as soon as possible, as propagation delays may lead to a reduction in propagation success.
- After propagation material is removed from the parent plant, natural degradation of the tissue commences, in the process forming **ethylene** which can lead to further plant tissue damage. If the material is being stored for more than two days, the material should be aired and given a fine mist spray with water to help dissipate the ethylene and help keep the material turgid and reduce degradation.
- Whilst collecting and transporting, place the collected propagation material in ethylene absorbing plastic bags or roll it up in slightly moistened newspaper, which will also absorb ethylene. If remaining in the bags or wrapped in newspaper for more than 24 hours, the cuttings should be exposed to release any ethylene which may be accumulating and if the foliage is dry, sprayed with a fine mist of water.
- If in the field for long periods of time, keep material cool and moist, preferably in a cooler box.
- Generally, cutting and grafting material is collected by pruning off the end shoots of side branches. These shoots normally have a number of secondary branches which may be used to make additional cuttings once returned to the nursery.
- Remove excess leaf, flower, fruit and woody stem material to minimise the moisture loss from the propagation material.
- Always leave some excess stem material on the cuttings so that the final preparation in the nursery results in a fresh cut immediately before the cutting is dipped in growth regulator and/or dibbled into the cutting mix.
- ALWAYS inspect material for pests and diseases and avoid collecting infected material where possible. Isolate infected material from other collected material and ensure that nursery staff are informed of the infection prior to the material being exposed in the nursery.

Case Study 8.4: Rhizome cuttings of the herbaceous perennial *Xerochrysum palustre* (Flann) R.J.Bayer

Warren Worboys

Xerochrysum palustre (Swamp Everlasting) is a rhizomatous perennial recorded from mainly lowland areas of southern Victoria and eastern Tasmania, with some montane occurrences in NSW and eastern Victoria that may represent a distinct species (N. Walsh, pers. comm.). It is generally confined to wet situations such as permanent lowland swamps, winter wetlands, and stream margins (Wilson 2017), usually on black cracking clay soils but it is rare due to habitat depletion. It is classified under the EPBC Act 1999 as vulnerable (VU) and vulnerable (v) in Victoria and is listed in the *Flora and Fauna Guarantee Act* 1988 (Stajsic 2018).

This species does produce seed but **seed fill** and viability (see Chapter 5) may be extremely low in some populations (Sunner 2020, see Figure 8.9) necessitating vegetative propagation from some provenances.

Because stems die back annually, they are not a reliable source for traditional cuttings. An alternative propagation method is to use rhizome cuttings. It should be noted that rhizome cuttings vary from rhizome division because they generally do not have sufficient established roots to support the child plant, whereas divisions have sufficient roots for the plant to be immediately self-sufficient.

Figure 8.9: (a) Mostly filled seed of *Xerochrysum palustre* with a few unfilled seed at right. **(b)** Unfilled seed of *X. palustre*, note withered and sunken characteristics. (Images: N. Sunner)

Plants can be dug up in late winter/early spring when the new leafy shoots are 100 to 200 mm above the soil surface, thus defining the outer edges of each plant. Washing the soil from the root system can generally be done at the site, which will expose suitable rhizome cutting material which may be removed and allow the replanting of the unused portions of the rhizomes. Rhizomes will vary in colour from pale new growth sections (with the young stems developing), through light brown of one-year-old sections, dark brown older sections and blackening sections which are starting to die off (Figure 8.10). Old stem bases and dark brown and black rhizome sections are far less likely to strike as rhizome cuttings and should be reintroduced to the hole where the material was dug up.

Once taken back to the nursery, leafless sections of rhizome 70 to 100 mm long (Figure 8.11) may be embedded flat on standard cutting propagation mix in community trays, spaced approximately 50 mm apart and covered with approximately 5 mm of cutting mix.

Rhizome sections 70 to 100 mm long with developing leafy stems may be potted into individual tubes of cutting mix or in deeper community trays, however they will need to be inserted sufficiently for the shoots to stay upright. Leafy rhizomes may have developed sufficient new roots to pot them directly into a 50/50 mix of cutting mix and potting mix.

Figure 8.10: Typical portion of *X. palustre* rhizome clump, washed of soil, August 2020. A – New seasons growth. B – Dead shoots from previous year. C – Older, dying, black rhizome section. (Image: Warren Worboys)

All rhizomes pieces should be fully washed and cut with sharp secateurs to minimise chances of infection. The trays or pots of cuttings should ideally be placed on propagation benches with bottom heat in the range of 18 to 21 °C.

Leafless rhizome sections (Figure 8.10) will generally develop sufficient root and shoot growth within 8 weeks to be potted up into standard potting mix. Leafy rhizomes will generally produce roots more quickly.

Allowing the plants to grow for at least one full year in the nursery will facilitate proper establishment, then the plants may be **reintroduced** to the wild or introduced to ex situ cultivation. Ex situ the species will grow well in heavy summer-moist soils in full sun, without requiring the possible annual total inundation they would have experienced in natural conditions, but this inundation does appear to increase spring growth vigour.

If re-introducing to the wild, this is best undertaken in late winter or early spring when the plants are dormant or just beginning to sprout. This allows for the removal of all potting mix and washing the root system to reduce the introduction of non-indigenous biota to the site and the root systems may be settled amongst the muddy soil.

Figure 8.11: Trimmed new shoot with some rhizome and roots and older rhizome section, ready to be inserted as cuttings. (Image: Warren Worboys)

References

Stajsic V (2018) '*Xerochrysum palustre*.' Available at https://vicflora.rbg.vic.gov.au/flora/taxon/2b897163-174b-42a0-94d5-f25fc324977b [Verified 1 August 2020].

Sunner Naomie (2020) Pers. comm. Unpublished data from Masters thesis - Exploring habitat and seed ecology of the vulnerable Swamp Everlasting – *Xerochrysum palustre*

Wilson PG (2017) An examination of the Australian genus *Xerochrysum* (Asteraceae: Gnaphaliae). *Nuytsia* **28**, 33-34.

Case Study 8.5: Using cellular 'plugs' to assist in field collection of cuttings

Warren Worboys

Cellular plugs can be used to improve cutting survival rates if it is necessary to travel into remote locations for a number of days, away from the propagation nursery. Cellular plugs help to maintain cuttings in prime condition rather than being left for days in sealed plastic bags while they wait for processing back in the nursery. The survival of cutting material is highly dependent on their freshness (Section 8.5.2.1) and although some material may last a week or more, plant tissues (leaves, stems, buds) will start to degrade as soon as they are removed from the parent plant and losses will inevitably increase the longer they are left before processing.

Traditionally, cutting material is collected and stored in plastic bags or rolled in moistened newspaper and stored in chilled portable cooler boxes or fridges until returned to the nursery, where it is prepared for propagation (see also Section 8.5.2.2). Propagation plugs may be taken

into the field, even in a hiking pack, allowing for cutting material to be collected, prepared, and inserted in the plugs almost immediately, or at the end of the day. In this way the plant tissue can be kept in a better state prior to nursery processing which may be several days away. It is possible to take 'hormone' root stimulants into the field (Box 8.2) but often these stimulants only speed up the root development; they are not always necessary and, in some cases, as with many Proteaceae, may be detrimental to root development. The significant value of the plugs is that their moisture retention acts like a vase, keeping moisture readily available to the base of the cutting but still allowing some degree of air movement through the plug thus reducing anoxic conditions. The plugs are also rigid, so the bases of the cuttings remain undisturbed when packaging and transporting, unlike standard cutting mixes.

There are a range of cellular plugs available commercially, that are generally available from larger trade horticultural supplies outlet. They are variously made from either rock wool or pure peat to mixtures of propagating substrates which can be formulated to the client's specific requirements. Broadly, the various plugs have similar characteristics and usage traits.

Plugs are generally cuboid or cylindrical and come in a range of sizes from 20 to 50 mm across, designed to suit custom fit plastic or polystyrene trays which may have the capacity of over 200 plugs (Figure 8.12a and 8.13). The plugs are generally purchased in a dry state and are relatively light weight but require soaking in water to fully hydrate them prior to use.

Most plugs have a manufactured hole in the centre into which the cutting is inserted and it is necessary to ensure that the cutting is inserted sufficiently for the base to contact the bottom of the hole, to allow water uptake and to avoid an air pocket. The plugs must be soaked in a container of water for up to 2 hours before use, depending on the type and size.

Figure 8.12: **(a)** Preparing cuttings in peat plugs NB. Stem diameters (approx. 6 to 8 mm) to be inserted in 30 mm plugs. **(b)** Cuttings in peat plugs bundled and bagged ready for transport. (Images: Warren Worboys)

Once the cuttings have been inserted, the plugs may be grouped together in small bundles in a plastic bag, with appropriate labelling (Figure 8.12b). A handy hint is to prepare the cutting in the plugs, cradle the corner of a plastic bag in the palm of the hand and starting in that corner, pack the plugs one at a time side by side, to form a circular bundle which may be held together with a rubber band. Bundles up to approximately 120 mm diameter are usually quite stable to stand upright and to handle and transport. The top portion of the bag can then be unfurled up around the cuttings to act as a cloche or rolled around the cuttings for packaging

and transport. The lower portion of the bag retains water and can help keep the plugs moist.

Roots generally develop from the base of most cuttings thus they are generally in the lower portion of the plug (Figure 8.14). Once the cutting has established roots, there is great benefit in removing the upper section of the plug if there are no roots in it. This allows for potting the cutting with the roots closer to the surface. Leaving the plug whole may result in plant loss due to rotting caused by excess moisture retention around the stem through potting too deep.

Plugs may also be used in the field for collecting very small seedlings. Carefully dig up the seedlings using a knife or similar small blade, wash off soil immediately and keep the seedlings immersed in water until the roots are dibbled into the plug, then water well. Again, this makes water and air readily available to the plant.

Figure 8.13: Typical size of cuttings which may be inserted into 20 mm peat plugs in a custom fit propagating tray. (Image: Warren Worboys)

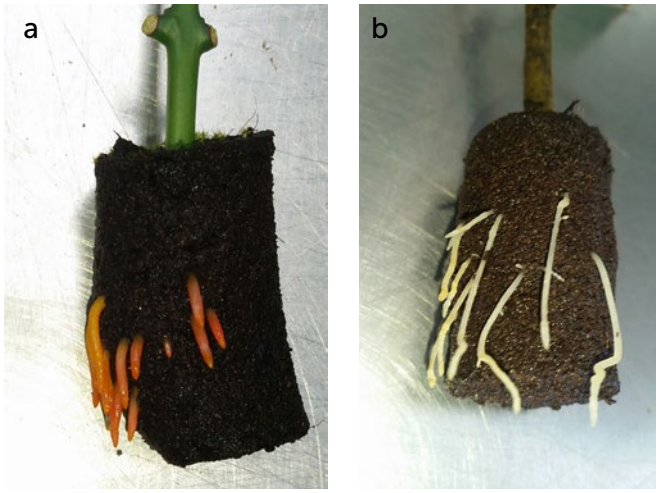


Figure 8.14: (a) Strong root development on *Austrobaileya scandens* six weeks after being inserted in peat plugs in the field. **(b)** Root development and new side shoot on hardwood cutting of *Lenbrassia australiana* var *australiana* twelve weeks after being inserted in peat plugs in the field. (Images: D. Perkins)

8.5.3 Division

To propagate by division, a plant is cut, split or broken up into two or more pieces, each of which is treated as a new plant with established roots, stems and foliage or dormant buds which are ready to shoot. This technique is commonly used for many herbaceous perennials like orchids, (both epiphytic and terrestrial) as well as sub-surface spreading and clump-forming taxa such as *Lomandra*, *Dianella, Gahnia*, and *Juncus* and species of Zingiberaceae, Asteraceae, Liliaceae. In fact, any plant which multiplies itself from rhizomes, bulbs, corms, stolons, tubers, root suckers, tillering or pups (aerial off-set shoots from the main stem, like those of epiphytic orchids) is likely to propagate by division to some degree (see Case Study 8.4).

Depending on the species, division can generally be done at any time throughout the year but the ideal time is when new shoots are just starting to develop, which usually occurs a week or two after new root growth has commenced, thus the plant is in active growth and the divisions will re-establish quickly. It is best avoided in the heat of summer when the new plants are likely to experience transplant shock. As a rule, early morning or overcast days, when temperatures are lower and humidity is high, are most favourable. If the soil is dry and it is possible, the plants should be watered a day or two before division to ensure the entire plant is turgid.

Division can also be an effective method of multiplication in situ with clumps of the target species gently dug up, split onsite into several new plantlets, then each replanted in situ without the need to transport the propagation material back to the nursery. This can be a speedy and cost-effective method of translocation and desirable when there are risks of spreading soil-borne pathogens to other locations.

Many orchid species can be successfully propagated by division. Epiphytic orchids should generally be separated just as the bud for the new pseudobulb (shoot) is starting to swell and new roots are starting to develop at the same time. Terrestrial orchids are best divided when the above-ground parts have died back or are dormant thus are much easier to work with. See Case Study 11.5 and Chapter 12 for more information about orchids.

It is particularly important to consider nursery hygiene and minimise the potential spread of plant diseases and pests during division. During division and transplanting of terrestrial species there will be soil adhering to the plant's roots. This soil can easily carry pests and diseases, especially fungi, which may be detrimental to the propagation process and a potential threat to other nursery stock if introduced to the nursery environment. There is often significant benefit to carry an appropriate substrate such as a 50/50 mix of vermiculite and perlite, moistened, which may be used to protect exposed roots. As soon as possible after it is dug up, wash the soil off the roots of the plant division or transplant and immerse the roots in the substitute substrate, while the roots are still damp from washing. These plants could be placed straight into pots in the field, or carried in plastic bags, which is generally far more practical. This will improve hygiene, helps reduce transplant shock due to root disturbance and may be the only viable option available for maintaining more challenging or fussy species.

To propagate by division in the field, generally smaller manageable parent plants are dug out of the ground, with a sharp-edged spade or trowel to cleanly cut roots. Use the spade to cut a slot all the way around the plant, providing appropriate clearance from the crown of the plant, depending on its type and size. Use the spade to lift the plant out of the ground then shake or tease the soil from around the roots. The amount of root exposure varies considerably between species. Some may be completely washed of soil, whereas other species should have minimal root disturbance and for rare species it is best to err on the side of caution until experience is gained with the individual species.

After excess soil is removed and depending on the plant type, the crown may be divided by pulling apart leafy crowns of tillering plants by hand (e.g., *Celmisia latifolia*). Secateurs can be used for rhizomatous species like Senecio extensus, or a sharp spade for densely crowned species such as Lomandra. Division of side-shooting species like palms, may require the use of an axe or tomahawk. It is generally necessary to reduce the leaf area on the divided plants by cutting back stems or reducing the length of long strappy leaves by as much as 80 % to minimise desiccation (via excessive transpiration) due to root reduction and disturbance. Ensure there are adequate roots attached to the portion to sustain the division when it is replanted or potted up. It is also necessary to ensure that each divided portion has adequate reserves of nutrient and moisture to sustain it during re-establishment and is healthy, with well-formed buds, to produce new leaves and shoots.

8.5.4 Layering

Layering is a propagation method where a new plant develops while still attached to the parent plant. There are two main techniques used: simple (terrestrial or ground) layering and air layering.

8.5.4.1 Simple layering

This propagation technique takes advantage of the natural process in some species, whereby a branch or stem of a plant touches the ground (while still connected to the parent plant) and over time roots gradually develop from the point of contact. In the natural environment, some taxa that are often difficult to grow from cuttings may layer and these layered sections can be successfully separated from the parent (as in many Ericaceae species).

To carry out simple layering in the field, a shallow trench should be scraped into the soil surface, ideally in the humus-rich top layer, and a low growing side branch is pulled down into the trench and firmly pinned into place with U-shaped wire pins 100 to 200 mm long, depending on the soil type. Generally, one to two seasons of tip growth at the end of the shoot is carefully bent upwards and left exposed at the end of the trench. Slight wounding of shoots over 2 mm in diameter, such as slitting the bark with a sharp knife or scalpel at the upward bend, will help speed the root development. It may be necessary to remove some leaves along the stem section which will be buried in the trench. The soil is then pulled back over the stem and firmed into place and watered. The smaller the stem diameter, the shallower the trench will be, generally, a cover layer of soil approximately five times the diameter of the stem is required. Over several months, while still connected to the parent plant, roots will develop adventitiously resulting in the production of a new plant. The buried side branch should be exposed approximately 100 mm from the new plant (or where the bark was slit earlier) to ensure that root development has occurred then the stem may be cut with secateurs to separate it from the parent. After another month the new plant can be gently dug up and potted on.

It is possible to bury pots, with potting mix, into soil and simple layer side shoots directly into the pot, however maintaining moisture in the pot can be problematic. An advantage of pot layering is that a suitable substrate can assist more rapid root development and transplant shock is also reduced, especially if the natural soils (such as soils with high clay or rock content) are not conducive to good air and water movement, both essential for good root development.

8.5.4.2 Air layering

This is a similar technique to simple layering whereby a branch, usually semi-hardwood to younger hardwood (see Box 8.1), is wounded or incised to expose the underlying cambium layer (the region of actively growing undifferentiated cells where new roots are likely to develop) and may also be treated with a growth regulator to stimulate new cell growth (Figure 8.15a). The treated part of the branch is enveloped with moist potting mix (usually peat based) or media such as sphagnum moss which is held in place by wrapping with plastic or aluminium foil (Figure 8.15b, c). Air layering is used where the desired material is unable to come into close contact with the ground and can be particularly effective for climbing and prostrate species.

Figure 8.15: (a) Wollemi Pine bark is wounded to expose the cambium, and a growth regulator is applied. **(b)** The wound is surrounded by sphagnum moss to provide consistent moisture with good aeration and held in place with a plastic container. (Images: Maureen Phelan)

Figure 8.15: **(c)** Wollemi Pine showing where to apply the air layering technique to encourage adventitious root development, with 2-3 nodes above the apparatus. (Image: Maureen Phelan)

8.5.5 Grafting and budding

These techniques use tissue, stem or shoot buds (scion material), taken from a parent plant, which is then attached to a compatible plant with an established root system (stock plant). Grafting uses stem pieces, budding uses a single bud trimmed from a stem.

Grafting is a specialised technique used for maintaining various selections and threatened species including *Eucalyptus, Grevillea, Eremophila* and *Myoporum* (for more examples, see Figures 8.16, 8.17 and 8.19). For example, some rare and threatened *Eremophila* species are grafted on to *Myoporum* rootstock to assist with their survival in higher rainfall ex situ collections which allows them to be planted into display conservation gardens which climatically may be somewhat different from where the species is naturally found. Grafting onto resistant rootstock can help with maintaining species susceptible to disease or soil pests like nematodes.

Figure 8.16: (a) A recent graft union of *Prostanthera lasianthos* var *subcoriacea*, a rare Grampians endemic. **(b)** A *Corymbia ficifolia* selection grafted on to *C. maculata* rootstock. **(c)** The bark difference can be seen in this typical graft union of *Pimelea physodes*. (Images: Warren Worboys)

Closely related species are more likely to be successfully grafted than more distant taxa (Figure 8.16b). Sometimes the rootstock and scion may not be compatible, and it is necessary to have a third, intermediate, scion type which is compatible with the rootstock and the scion which will 'bridge' the material. When all the tissue, bark, cambium, and wood of the scion and rootstock completely unite to form a strong, homogenous stem which functions for the life of the plant, the graft is 'compatible'. An 'incompatible' graft is when the tissue does not properly unite, and even though the plant may grow for several years, it may suddenly break cleanly at the union or the plant may just die due to poor vascular connection (Figure 8.18). When a scion dies for no apparent reason, a cross-section of the graft area may demonstrate poor tissue connection. This type of failure is relatively common and often happens with little warning. Rootstock selection is very important as, if too vigorous, it may require constant maintenance or could even outgrow the scion if left unchecked.

Figure 8.17: The graft union of a select form of *Corymbia maculata* grafted on *C. maculata*. (Image: Warren Worboys)

Figure 8.18: (a) The stump of an incompatible graft of *Corymbia maculata*. **(b)** The trunk of the graft. Note the small amount of ragged tissue near the centre of the trunk – the only point where there was some degree of tissue union. (Images: Warren Worboys)

There are many different methods of grafting and budding and they generally require more advanced skills and knowledge of compatibility and are therefore not dealt with in these Guidelines. It is recommended that specialist advice be obtained if grafting or budding are deemed necessary for the conservation of a particular taxon. ANPSA study groups and specialist growers are often a great source of information.

Advantages of grafting:

- Useful for species with susceptibility to soil borne pathogens and pests e.g., *Phytophthora* or nematodes.
- Overcoming sensitivity to salinity, pH, drought or water logging.
- Enables ex situ collections to be grown in different climatic zones ie. growing Western Australian *Pimelea, Eucalyptus* and *Banksia* species in eastern Australia.
- For species that are difficult to propagate by other vegetative means, ie. cuttings, there may be more success with grafting when seed is not an option (e.g., *Eucalyptus* x *impensa*, a threatened WA species and a naturally occurring hybrid, see Figure 8.19b).
- Can be used to produce more resilient plants that can then be maintained and grown for seed production purposes which otherwise may not be possible.

Disadvantages of grafting:

- Grafting can be labour intensive, expensive and slow with a lot of trial and error often required.
- The process for determining optimum rootstock-scion combinations is often lengthy.
- Specialised infrastructure may be needed, such as, fogging units to maintain optimum conditions for grafting success.
- Long-term incompatibility of the rootstock and scion may not be detected for a number of years.
- Rootstock may become infected to a soil borne pathogen subsequent to grafting.

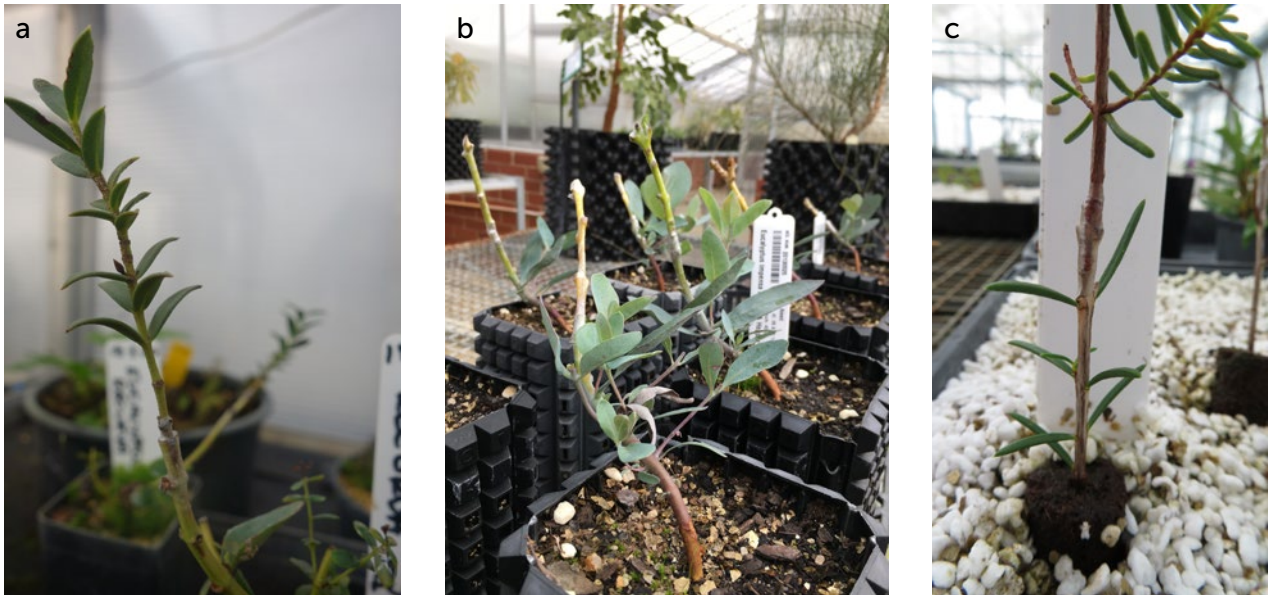


Figure 8.19: **(a)** Grafted *Eucalyptus recurva* at the Australian National Botanic Garden. (Image: Julie Percival) **(b)** 'Mummy graft' of *Eucalyptus* x *impensa* at Kings Park Botanic Garden. (Image: Amanda Shade) **(c)** A cutting graft of *Verticordia straminosa* where the scion material is grafted onto the rooted cutting stock material, *Chamaelaucium uncinatum*. (Image: Warren Worboys)

8.5.6 Tissue culture – micropropagation

Tissue culture is a laboratory-based technique that can be used to propagate some species. The finer technical details of this plant propagation approach are dealt with in detail in Chapter 9. This technique is extremely valuable for propagation of terrestrial orchids and '**exceptional**' (for definition see Chapter 2) or otherwise difficult to grow species. For example, several *Lepidosperma* species and *Gahnia radula*, have been successfully micropropagated for use in restoration when reproduction via conventional seed or division methods have had poor results (Kodym *et al*. 2010, 2012, and 2014; Koch 2007).

In general, the nursery is important in the tissue culture process for the preparation of **explant** material for culture, and for the acclimatisation and growing-on phase. Often, nursery grown plants are used as the starting material (explants) for tissue culture. Nursery grown plants are generally much less likely to be as contaminated with fungi and bacteria than wild plants. This is because they are generally healthier and growing well due to optimised water and fertiliser regimes.

The most common form of tissue culture is '**micropropagation**', a method which is similar to vegetative cutting propagation as it relies on the regeneration ability of the plant to produce new shoots which can then be stimulated to produce roots. Once roots are formed the plantlet can be gently removed from the tissue culture environment and placed into a growing medium (usually potting mix) using approaches and equipment that aligns with more traditional nursery production systems. This 'acclimatisation' phase is a critical step to re-establish tissue culture grown plants in the nursery, as the tissue culture environment is very different in terms of temperature, light, growing substrate and relative humidity. It is often the point at which micropropagation fails (i.e. plants fail to thrive or die) and requires careful monitoring of humidity and light to establish the plantlet. Often, tissue culture plants have poorly formed leaf stomata and epidermis making them more prone to overheating, desiccation and fungal attack until they have acclimated to their new growing conditions which may take several weeks. Once successfully established into the growing medium, plantlets can then be treated in a similar way as other newly established plant in a nursery, being potted-on and hardened-off as needed.

8.5.7 Transplantation of salvage plants

Occasionally it is necessary to dig up whole plants in order to capture the plant material for transplant as part of a translocation (see Commander *et al*. (2018) Translocation Guidelines Chapter 6) or for incorporation into ex situ collections. These plants may be placed in a nursery for horticultural care to overcome transplant shock and to prepare them for replanting at an appropriate time. If the plant salvage process is time sensitive, such as imminent clearing, it may increase the chances of success if cuttings are taken at the same time.

It is particularly important to consider nursery hygiene and minimising the spread of plant diseases and pests during transplantation as soil may be a source of pathogens or weeds.

Box 8.2: Plant growth regulators (PGR) and their role in propagation

A number of chemical plant growth regulators (PGR, commonly known as 'hormones') have been developed for small and large-scale propagation purposes (see also Box 9.2). The types used most in cutting propagation alone or in combination are classed as 'synthetic **auxins**', the most common of which are: IBA (indole-3-butyric acid), IAA (indole-3-acetic acid) and NAA (naphthaleneacetic acid). These PGRs can be used to increase the speed of root initiation and increase the percentage of 'strike' of the cuttings. However, plant species react differently to the different PGRs and concentrations at which they are applied. There are many commercially available products that can be purchased that contain different concentrations of auxins (usually referred to as either low, medium or high concentration on the packaging). With rare species which may not have been propagated before, it is generally safer to divide the batch of cutting material and use zero and/or different types/ concentrations of PGR to ascertain if there are detrimental effects on the cuttings.

Growth regulators are available for application in the following forms:

- *Powder* – the most convenient and readily available through many garden supply outlets. Care must be taken to ensure excess powder is removed from the base of the cutting, as excess powder can hold moisture and cause rotting of the base of the cutting. Generally, this is the most stable product if stored at room temperature.
- *Gel* – helps seal the base of the prepared cutting to keep it moist but it is generally only available from specialist suppliers (e.g., hydroponic shops). Requires refrigeration but remains quite stable for a number of days without refrigeration.
- *Liquid* – convenient to use but less readily available from retail suppliers. It can be prepared fresh in a laboratory. Requires refrigeration for storage and generally the least stable at room temperature.

Comparison of more rapid root development using a powder growth regulator (left) and no treatment (right). (Image: M. Thomson)

The PGR listed above are available at different formulation strengths, and in blends, in the various forms listed and it is usually important not to a use higher strength than required for the cutting type, and for some species (as in many Proteaceae) no PGR might be required. Growth regulators may 'burn' cell tissue and used at too high a concentration can severely damage the cuttings and hence be counter-productive.

Individual operators will have their own style, but generally it is easier to prepare the cutting, then hold the cutting by the upper section between the thumb and the index finger, dip the base of the cutting into the formulation, not too deeply as this can have detrimental effects. If using powder or liquid, it is then possible to gently flick the lower portion with the ring finger or little finger to remove excess formulation, then dibble (insert) the cutting into the cutting mix, all without changing the hold on the cutting.

Whatever type of formulation is being used, for hygiene purposes, it is important to pour out a small portion of the formulation into a small washable container, even a recycled plastic milk carton lid, never the main container lid as this may introduce contaminants into the stock solution. This allows for the disposal of any left-over formulation from the day's work, which will be contaminated with debris and possible pathogens from the cuttings.

8.6 Record keeping in the nursery

Detailed, accurate management of accessions – from collection, through nursery production, and establishment in living collections – is critical in maintaining the scientific and conservation integrity of ex situ collections (Figures 8.20 and 8.21). This is particularly challenging in living collections where multiple generations of plant material may be produced over long periods of time giving room for human error at each step of the chain of management throughout generations of continued production. See Chapter 15 for more details.

It should be noted that maintaining large **clonal collections** for long periods of time is very resource intensive and an institution's capacity to undertake this successfully should be carefully considered and resourced appropriately. Ideally, a central person, such as a threatened species officer or project coordinator, will be available to keep track of what clones are where in both ex situ collections and translocation sites.

Ex situ collections are often duplicated and shared by multiple organisations such as botanic gardens and universities as insurance collections (for example, see Case Studies 8.1, 8.2 and 11.2). These collections are known as meta-collections (Griffith *et al*. 2019). This is a good practice not only for insurance, but for knowledge sharing of cultivation techniques and responses to different treatments and growing conditions. If smaller organisations are to be part of insurance collections, it is best to confirm their capacity to maintain complex records over a long period of time and take measures to reduce the possibility of clone and record mix ups which is a relatively common problem.

Figure 8.20: Labelling of *Hibbertia circinata* (L) including genotype collections at the Australian National Botanic Garden (R). (Images: Julie Percival)

Figure 8.21: Labelling of *Zieria baeuerlenii* permanent holdings collections at the Australian National Botanic Garden. (Images: Julie Percival)

Tips for good record keeping in the nursery include:

- Detailed record keeping and data capture throughout the propagation and production process to highlight successes and failures. When working with threatened species, material can often be limited, so data capture and knowledge sharing is vital to get the best results from often limited and sub optimal material.
- When possible, link propagated plants to herbarium vouchers, citing the unique identifier of the herbarium specimen. This allows both for confirmation of identification if required, as well as giving access to more information about the collection (e.g., habitat, plant size etc.) than a nursery data system might hold.
- Consider the ability to extract data collected over time. Will it be useful? Will it be understandable in 10 to 20 years' time when reviewed by someone not familiar with the species or work?
- Always use permanent markers or waterproof pencils and long-lasting weatherproof labels (Figures 8.20 and 8.21). Will that label still be legible or even still present in a few years' time?
- Have a backup system, such as, an image of pots or containers marked with accession or identifying numbers as well as labels in the pot.

- Where possible, place plants/clones into some sort of logical alphabetical or numerical order so if something goes wrong the location or position of the plants may assist in its re-identification.
- Once **propagules** are potted up, replicate and disperse several labels within the batch of potted plants. Labels are easily displaced by wind, birds or even human misadventure.
- Permanent labelling should be given to each plant as soon as possible.
- Labelling containers with paint pens provides insurance in the case of labels getting knocked out or damaged or batches being mixed up when moving around areas within nurseries and out for translocation.
- For long term collections, mapping both on paper and digitally (Figure 8.22) provides additional insurance if tags/labels disappear or are damaged over time.
- Maintaining paper propagation records as back up to databased records is good practice and can assist in problem solving if there are discrepancies or technical issues. It may seem like a duplication of work but can be very beneficial for threatened species and high value collections where conservation and research results are reliant on accurate records and clonal management.
- It is important to record as much as possible and be consistent with record keeping within each trial/project/species. While this can be time consuming, successes and can often be attributed to multiple factors.
- When recording strike rates, also make note of the quality of root development. This could be a simple grading system (1 = no roots, 2 = weak roots, 3 = large roots) which will be beneficial in determining the best treatments/outcomes of a propagation trial.
- In addition, also record plant growth regulator treatments, propagation and growing media, temperature and humidity conditions, rootstocks used, material type (suckering growth), source (wild or cultivated) and quality, container type and size, bottom heat, capillary mats, additional treatments throughout the propagation period (e.g., callus removal) and reasons for failure if observed (e.g., pest or diseases, systems failures, too wet, too dry). There is just as much to be learnt from failure as from success.

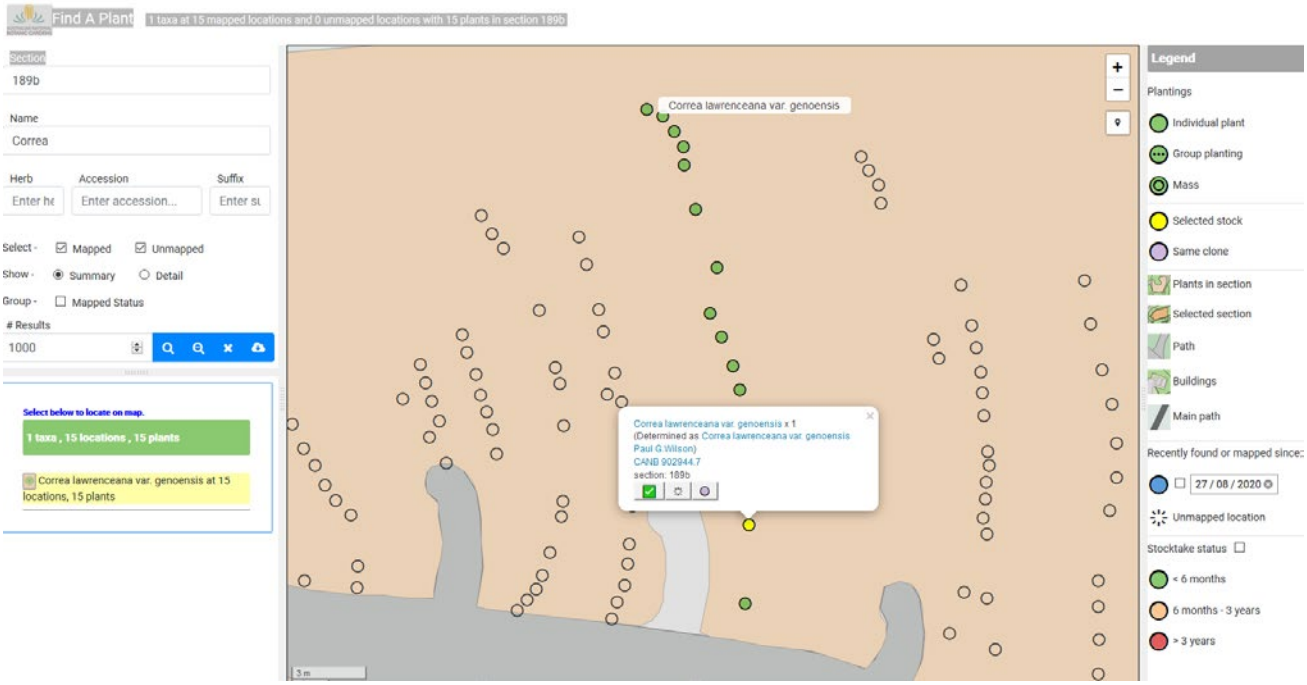


Figure 8.22: Digital mapping of *Correa lawrenceana* var *genoensis* plants at the Australian National Botanic Garden. (Image: ANBG)

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Chapter 9
Tissue culture

Karen D. Sommerville, Eric Bunn, Amanda Rollason, Shane R. Turner

This chapter is a revision of the previous edition chapter by Catherine A. Offord, Eric Bunn, Shane R. Turner, Karen D. Sommerville, John Siemon and Sarah E. Ashmore, 2nd edition, pp. 109-128.

9.1 Introduction

Tissue culture is a term encompassing a variety of techniques for growing plants in artificial media under sterile conditions, in an environment where light and temperature can be controlled to suit the plants' needs. The plants may initially be propagated from a shoot tip, a root, an anther, a petal, a whole **seed**, part of a seed, or even a single cell (Davies *et al*. 2017). The cultured plant parts, known as '**explants**', do not generally have functional roots; the nutrients required for growth are obtained by diffusion from the tissue culture medium which may be a solid gel or a liquid. Plant growth regulators – natural or synthetic compounds that act as hormones – may be added to this culture medium to stimulate different types of growth. For example, **cytokinins** may be added to stimulate shoot multiplication, **gibberellins** to promote shoot elongation, and **auxins** to initiate root formation. Different ratios of these hormones can also stimulate a range of other responses such as **callus** growth (Section 9.4.2) or **somatic embryogenesis** (Section 9.4.4; Ashmore *et al*. 2011; Bunn *et al*. 2011).

Tissue culture is commonly used to mass produce ornamental plants such as African Violets (Stewart 1999), to improve and conserve horticultural crops such as blueberries and avocados (Sharma 2014; Hiti-Bandaralage *et al*. 2017), and to produce plants for research into plant function such as *Arabidopsis thaliana* (Barkla *et al*. 2015). It can also be used to support **ex situ conservation** for species that are highly threatened, do not produce **viable seeds**, or do not produce seeds suitable for banking (Bunn *et al*. 2007). Tissue culture is becoming increasingly important for preserving species heavily impacted by Myrtle Rust (Sommerville *et al*. 2019), and for preserving rainforest species with **desiccation sensitive** seeds (Sommerville *et al*. 2017 and Chapter 6). In this context, tissue culture is generally used in combination with other ex situ conservation techniques such as **cryopreservation** (Chapter 10) and living plant collections (Chapters 8 and 11). Tissue culture can also be useful in its own right, however, for rapidly producing large numbers of plants to support **restoration** projects (Koch 2007).

In this chapter we will outline the benefits and risks of tissue culture, and we discuss the range of techniques utilised in ex situ conservation, the difficulties that may be encountered, and examples of its use.

9.2 Benefits and risks of tissue culture

While highly effective when optimised, plant tissue culture is almost always a last resort for ex situ conservation due to its cost and the need for specialised consumables, equipment, staff and laboratory facilities (Bowes 1999). As a propagation technique, however, it is highly suited to the production of rare species for restoration when seeds are too scarce or too valuable to risk losing via direct sowing. Tissue culture can also be useful for common though problematic species that have poor seed set, poor **germination**, or complex germination and growth requirements such as species of Cyperaceae and terrestrial orchids (Kodym *et al*. 2014; Bustam *et al*. 2014). Consequently, if tissue culture is being considered as part of a conservation or restoration program, it is necessary to have a clear understanding of the potential benefits as well as the potential pitfalls before commencing. Some of the main benefits and risks are outlined in the following section.

9.2.1 Benefits

Tissue culture can be used to replicate a single plant in large numbers. This is useful where **genetic variability** is not required, such as in the propagation of desirable cultivars for horticulture or the production of uniform material for research. As an indication of how effective this can be, Stewart (1999) reported that up to 15 million Kangaroo Paws (*Anigozanthos* spp.) can be produced in 12 months from one starting plant. Many commercially available horticultural species – such as African violets, begonias, dahlias, and most exotic orchids – are produced this way.

Controlled growing conditions provide protection against environmental hazards. Once a plant has been initiated into culture, the sterile and contained environment prevents pest attack and infection by disease-causing organisms. The controlled growing conditions also provide protection from the wind, drought, storms, floods, fires and temperature extremes that can impact plants growing in the field or nursery. Consequently, plants can be maintained in perfect health year-round which may be particularly important for rare or threatened species.

Many plants can be maintained in a small amount of space. The small size of explants means that hundreds of plants can be grown in the same space that might be occupied by a single plant in the field or a container collection in a botanic garden.

9.2.2 Risks

Tissue culture is a highly specialised field that requires specific skills and equipment. Trained personnel are required to perform most tissue culture procedures as the manipulation of plant material must be carried out in sterile conditions. Tissue culture is therefore usually carried out in a laboratory or specialised nursery (though fern and orchid cultures are commonly produced in less specialised environments). Due to the level of training required, and the facilities and equipment needed, the cost of tissue culture is high compared to the production of plants using more traditional approaches (Koch 2007).

Techniques and procedures often need to be optimised for each species. Considerable time and effort can be spent experimenting with different levels of light, nutrients, plant growth regulators, and other growth factors such as vitamins (see, for example, Bunn 2005). Unfortunately, even after considerable research, some species still perform poorly in tissue culture and thus the approach is not applicable to all species.

Maintaining tissue cultures is labour and time intensive. Explants can exhaust available nutrients, or outgrow the size of their container, in a matter of 3–4 weeks for fast growing species or 6–8 weeks for slower growing species, and therefore need to be transferred to fresh media regularly. This process is time consuming so can be expensive in terms of labour.

Maintaining plants in tissue culture for long periods can lead to genetic changes. These changes, known collectively as '**somaclonal variation**', can be the result of spontaneous genetic **mutations** that can be passed on to the next generation, or **epigenetic** changes that generally affect only the current generation (Ashmore 1997) but may also be heritable (Han *et al*. 2018). Somaclonal variation occurs more frequently when cultured cells go through an undifferentiated stage (Sections 9.4.2 and 9.4.3); it is less common when preformed buds are used to establish shoot cultures (Reed *et al*. 2004).

Species may be difficult to transition out of culture and back into the ex vitro environment. This transition is one of the most difficult stages of tissue culture (Barlass and Hutchinson 1996), particularly for many woody species. Some species, for example, fail to grow functional root systems, while others produce fragile shoots (Section 9.5.3) that are susceptible to disease and stress when removed from the tissue culture environment.

Clean cultures can be easily contaminated by microorganisms. Plant cultures can be maintained indefinitely under sterile conditions as long as strict **aseptic** techniques and regular monitoring are adhered to during all subculturing and storage procedures. Failure to do this may lead to accidental introduction of unwanted microorganisms (see Figure 9.3) requiring costly re-sterilisation and rehabilitation of infected cultures or, at worst, resulting in the complete loss of a valuable collection. In some cases, microorganisms that are present inside freshly collected plant tissues but quiescent (inactive) may emerge slowly over time despite careful subculturing.

9.3 When should tissue culture be used?

Tissue culture is more costly than other propagation methods (Pence 2011) so should only be used for ex situ conservation of wild plants when other options are impractical or have proven unsuccessful. This might be the case where a species has poor seed set, deep seed **dormancy**, desiccation sensitive seeds, a poor response to propagation by cuttings, or very few individuals remaining in the wild (Fay 1994).

Tissue culture can also be used in the following situations:

- To multiply specific **genotypes** (e.g., to supply large numbers of naturally clonal species for **revegetation** projects);
- To rescue embryos that would otherwise abort due to incompatibility of **embryo** and **endosperm** (as may happen when two different species are **hybridised**);
- To produce microbe-free vegetative material for long-term storage using cryopreservation (Chapter 10);
- To recover cryopreserved material (Chapter 10);
- To germinate seeds with specific nutritional requirements or requiring the presence of a **symbiont** (e.g., terrestrial orchids with **mycorrhizal** associations; Chapter 12).

Tissue culture has a vast array of other uses in physiological and genetic research, crop improvement, and commercial horticulture. For more information on these topics, see George (1993), Taji and Williams (1996), Bowes (1999), Beyl and Trigiano (2015) and Davies *et al*. (2017).

Rhodamnia rubescens and *Rhodomyrtus psidioides* are two Australian rainforest species severely affected by Myrtle Rust. These species were initiated into tissue culture (Case Study 9.1) as they now produce few seeds in the wild and potted collections grown from cuttings are prone to reinfection with the rust.

Case Study 9.1: Micropropagation of *Rhodamnia rubescens* and *Rhodomyrtus psidioides*

Amanda Rollason and Karen D. Sommerville

Rhodamnia rubescens and *Rhodomyrtus psidioides* are two Australian rainforest species severely affected in the wild by Myrtle Rust, a disease caused by the fungus *Austropuccinia psidii* (Beenken 2017). The two species were both common before the arrival of the pathogen in 2010 but are now listed as Critically Endangered under the NSW *Biodiversity Conservation Act* (2016). The Rainforest Seed Conservation team at The Australian PlantBank began investigating alternative conservation methods for the species in 2014 as one of the impacts of the disease was reduced seed set in the wild (Sommerville *et al*. 2019). Tissue culture was investigated as an option for both direct conservation and a preliminary step to cryopreserving the shoot tips.

Disease-free tip cuttings (Figure 9.1a) were obtained from either a garden specimen (*R. rubescens*) or potted plants (*R. psidioides*). The leaves were removed from each cutting, leaving 2–3 mm of the petiole to protect the nodes during the sterilisation process. Each cutting was then divided into 30–40 mm segments which were washed for 30 minutes by agitation in a solution of 2 % Alconox®, rinsed in water filtered by reverse osmosis (RO water), then surface sterilised by agitation for 30 minutes in 200 ml of 2 % sodium hypochlorite (prepared using White King® household bleach, sterile RO water and ~33 µl Tween® 20). The stem segments were then transferred to a **laminar flow cabinet**, rinsed three times in sterile RO water, and dissected into smaller segments (explants) consisting of a single node with 5–7 mm of stem below the node to enable insertion into the initiation medium. The explants were then inserted into individual test tubes containing ½ strength Murashige and Skoog basal medium (MS, pH 6.0; Murashige and Skoog 1962) with 9 g/L agar and 30 g/L sucrose.

The explants were incubated at 23 ± 2 °C with a 16 h photoperiod and monitored for fungal and bacterial contamination for 6 weeks (Figure 9.1b). Both species were successfully initiated into tissue culture using this protocol, however the number of explants discarded due to contamination was much higher for *R. rubescens* than for *R. psidioides*. Trials to reduce the level of contamination for *R. rubescens* by pre-treating the stem cuttings with 70 % ethanol, Plant Preservative Mixture® or Tilt®250 EC (a systemic fungicide) did not resolve the problem (Sommerville *et al.* 2019).

Uncontaminated explants were transferred to multiplication media consisting of either MS with vitamins, growth regulators and 30 g/L sucrose, or Woody Plant Medium (WPM, pH 5.75; Lloyd and McCown 1980) with growth regulators and 20 g/L sucrose. Both media types contained 1 µmol/L benzylaminopurine (BAP), 0.2 µmol/L indole-3-butyric acid (IBA) and 9 g/L agar. The two species multiplied on both formulations but appeared to perform better, and are now growing vigorously, on the MS-based medium (Figure 9.1c, d).

Root initiation trials were carried out for both species using four levels of IBA (0, 5, 10 and 20 µmol/L; in a base of MS + vitamins, 30 g/L sucrose and 9 g/L agar) with 25 replicates per treatment. The explants were incubated in this medium at 23 ± 2 °C with a 16 h photoperiod for 8 weeks, after which the agar was gently washed off and root development inspected. The plantlets were then transferred to a potting medium consisting of 70 % fine perlite, 20 % vermiculite, 10 % peat and a general fertiliser mix, watered in with 1ml/L Auxinone®, and incubated in a mini-glasshouse for 4 weeks at 25 °C with a 12 h photoperiod. *R. psidioides* performed well in all treatments except at the higher level of IBA (which formed distorted growth); however, this species also produced roots on the multiplication medium described above (Figure 9.1e). *R. rubescens* required higher levels of IBA to produce roots but produced those in much lower numbers than *R. psidioides*. Both species were successfully transferred to pot culture (Figure 9.1f) but further work is required to increase the success rate of transfer for *R. rubescens*. With both species now growing well in tissue culture, experiments to cryopreserve the shoot tips for long-term conservation can commence.

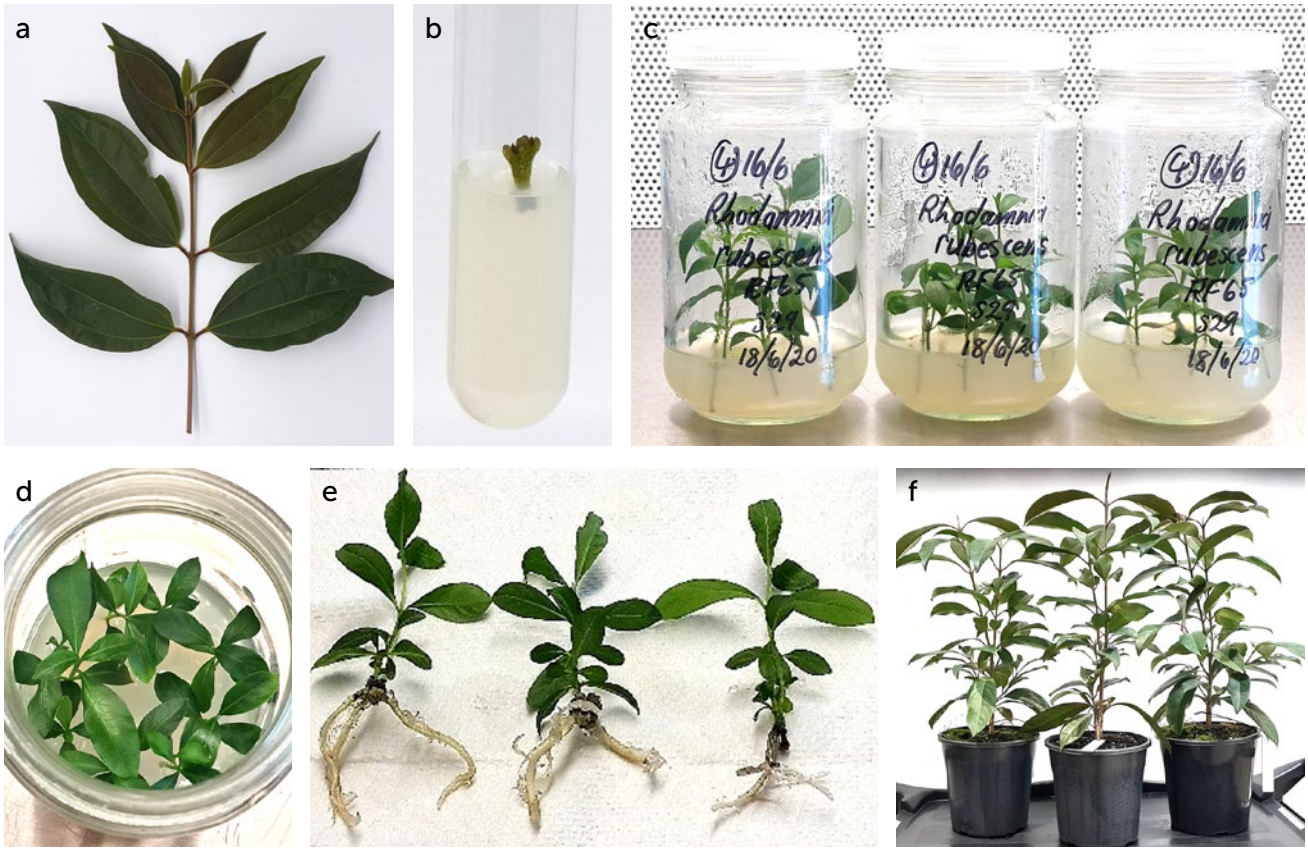


Figure 9.1: L-R **(a)** Stem cutting of *Rhodamnia rubescens* used for culture initiation; **(b)** sterilised *R. rubescens* explant inserted in initiation medium; **(c)** sub-cultures of *R. rubescens*; **(d)** sub-cultures of *R. psidioides*; **(e)** Root development on *R. psidioides* following treatment with IBA; and **(f)** *R. psidioides* successfully transferred from tissue culture to pots. (Images: Amanda Rollason)

9.4 Types of tissue culture

Virtually any type of plant tissue is theoretically suitable for initiating a tissue culture, including pollen, petioles, leaves and roots (Davies *et al*. 2017). However, the most common types of tissue used are vegetative buds or shoots taken from established plants, or **zygotic embryos** taken from seeds. The culture environment determines the type of growth response observed.

The main tissue culture systems used are:

- Micropropagation (including slow-growth storage; Section 9.4.1)
- **Callus culture** (Section 9.4.2)
- **Suspension cell culture** (Section 9.4.3)
- Somatic embryogenesis (Section 9.4.4)

9.4.1 Micropropagation

Micropropagation is the most basic plant tissue culture system. It involves initiating cultures from apical or axillary shoots, stem segments or seeds, encouraging the material to produce multiple shoots, then propagating the plants from miniature cuttings. This technique is commonly used for woody species and is the principal technique utilised for rare native plants at the Australian PlantBank and Kings Park and Botanic Garden (see Case Studies 9.1, 9.2 and 9.3). The four main stages of this technique are initiation, shoot multiplication, root initiation and transfer to potting mix.

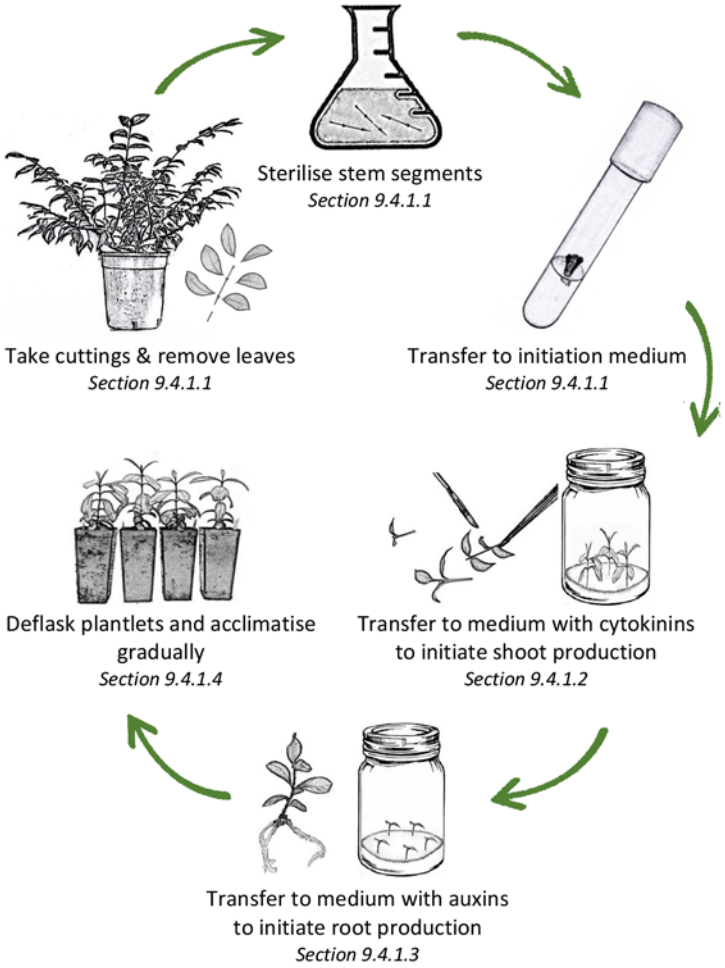


Figure 9.2: Key steps in the micropropagation process. (Images: Amanda Rollason)

9.4.1.1 Initiation of cultures

Healthy, pot-grown plants (or seedlings or seeds) are the best sources of material for initiating tissue cultures when these are available (Figure 9.2), but cultures can also be initiated from material collected from the wild. Seeds and seedlings have an advantage over shoots from adult plants in that they are inherently juvenile and often respond better to culture conditions; however, they make take longer to establish an adult plant ex vitro.

If the source plant is already in a managed ex situ collection, the target material is first made as clean as possible by encouraging healthy new growth so that the microbial load is low. Suitable shoots or stem segments are then selected and cut into sections of about 2–5 cm, each containing a leaf node and an apical or **axillary bud**. The plant sections (explants) are thoroughly washed to remove surface fungi and bacteria, either under running water or in a solution of detergent such as Alconox® or Tween®. Following washing, the explants are placed into a sterilant solution (such as 1–4 % sodium hypochlorite) and agitated gently for 5–30 minutes. A small amount of Tween® 20 or Tween® 80 (e.g., 15–30 µl per 100 ml) is often added to the solution as a wetting agent to improve contact between the sterilant and the plant surface. The type and concentration of the sterilant, and the duration of exposure, are usually determined by preliminary experiments to find the optimal combination providing the cleanest tissue with the least amount of tissue damage. Washing and sterilising are vitally important procedures as any contaminants not removed will rapidly overwhelm the explants once they are inserted in the growing medium; this is one of the main impediments to the successful initiation of plant material into tissue culture.

All subsequent procedures must be performed in a laminar flow cabinet using aseptic techniques to retain the sterility of the explants. Everything that comes into contact with the explants – forceps, scalpel, cutting surface and media – must also be sterile from this point onwards.

After transfer to a laminar flow cabinet, the explants are removed from the sterilant solution and washed several times in sterile distilled water (the shoots may also be reduced in size at this time, then briefly re-sterilised and washed again). The sterilised explants are then placed onto a tissue culture medium that has been heat-sterilised under high pressure (121 °C, 103.4 kPa) for 15 minutes. The medium contains a variety of components that can be modified to suit individual species and specific outcomes but typically consists of:

- A nutrient formulation, which acts as a complete inorganic nutrient source similar to fertiliser (see Box 9.1);
- Vitamins, a few of which are essential for healthy growth;
- Sucrose (or another sugar), which provides energy to the explant;
- Plant growth regulators, which shape the plant's growth response (see Box 9.2).

Figure 9.3: A range of fungal contaminants can appear during the initiation phase of tissue culture. (Image: Amanda Rollason)

The medium may be solidified with a gelling agent (e.g., agar) which binds the various components together and provides a suitable substrate to support the explants. Alternatively, a liquid medium containing the above components may be added to a substrate such as sand or a filter paper bridge. For more information refer to Taji and Williams (1996). The explants are incubated on this medium for 4–6 weeks and monitored for contamination by fungi and bacteria. Contaminated explants are discarded as they appear; clean explants are transferred to a multiplication medium at the end of the monitoring period.

Box 9.1: Nutrient formulations used in tissue culture

The most commonly used nutrient formulations used in tissue culture media are Murashige and Skoog medium (MS; Murashige and Skoog 1962) and Woody Plant Medium (WPM; Lloyd and McCown 1980). These formulations contain the macro and micronutrients needed for plant growth and additional organic components that may be beneficial for some plant species. With the exceptions of agar and sucrose, the concentrations below are in mg/L.

Macronutrients			Micronutrients			Organic compounds		
	MS	WPM		MS	WPM		MS	WPM
NH_4NO_3	1650	400	H_3BO_3	6.2	6.2	Glycine	2.0	2.0
KNO_3	1900	–	$MnSO_4.4H_2O$	22.3	–	Myo-inositol	100	100
K_2SO_4	–	990	$MnSO_4.7H_2O$	–	22.3	Nicotinic acid	0.5	0.5
$CaCl_2.2H_2O$	440	96	$ZnSO_4.4H_2O$	8.6	–	Pyridoxine HCl	0.5	0.5
$Ca(NO_3)_2.4H_2O$	–	556	$ZnSO_4.7H_2O$	–	8.6	Thiamine HCl	0.1	1.0
$MgSO_4.7H_2O$	370	370	KI	0.83	–	Sucrose	30g/L	20g/L
KH_2PO_4	170	170	$Na_2MoO_4.2H_2O$	0.25	0.25	Agar	10g/L	6g/L
Na_2.EDTA	37.3	37.3	$CuSO_4.5H_2O$	0.025	0.25			
$FeSO_4.7H_2O$	27.8	27.8	$CoCl_2.6H_2O$	0.025	–			

9.4.1.2 Shoot multiplication

To encourage the development of multiple shoots, the clean explants are usually placed onto a medium containing a cytokinin (see Box 9.2). Once produced, the new shoots can be carefully removed with a scalpel (under sterile conditions) and placed onto fresh multiplication medium. This process is known as 'subculturing'. After multiplication has commenced, the explants can usually be subcultured every 1–2 months, with the total number of explants increasing with each successive subculture.

A variation in culture conditions that can be used at this stage (and following stages) is **photoautotrophic** culture. This involves growing plantlets on nutrient media without sucrose, in a ventilated flask that allows gas exchange (Nguyen *et al*. 2016). These conditions allow the plantlets to produce their own sugars by photosynthesis and have been reported to reduce contamination, increase the multiplication rate and increase the survival rate on transfer to potting mix (Nguyen *et al*. 2016).

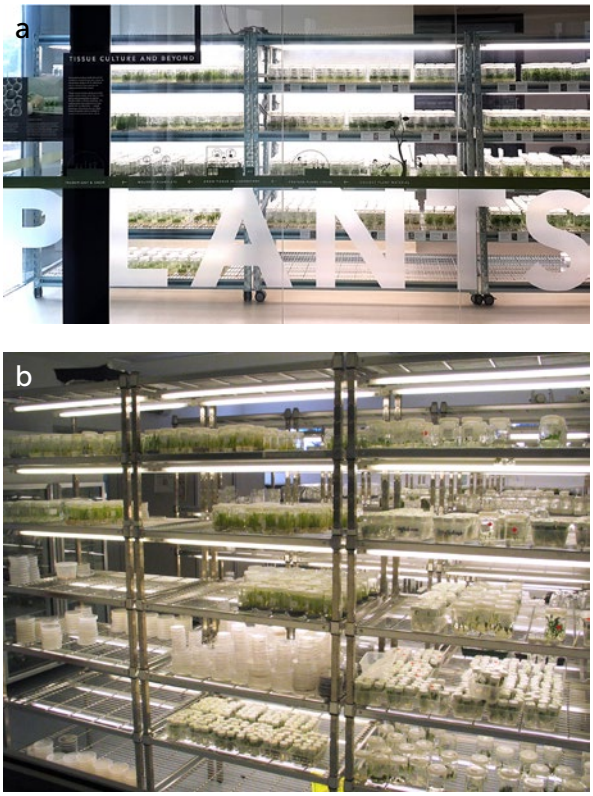


Figure 9.4: A selection of species on multiplication media at: **(a)** the Australian PlantBank (Image: A. Rollason) and **(b)** Kings Park and Botanic Garden. (Image: S. Turner)

9.4.1.3 Root initiation

Once the explants are a sufficient size, they can be moved onto a root induction medium. This involves moving the explants onto fresh medium devoid of plant growth regulators (which can spontaneously stimulate rooting in some species because of their endogenous hormone levels) or, more commonly, onto a medium supplemented with an auxin. Auxins such as IBA or NAA (see Box 9.2) actively stimulate adventitious root production and are key components of various rooting powders and gels used for propagating plants from cuttings (e.g., Yates Clonex Rooting Hormone Gel and Yates Plant Cutting Powder). The auxins may be applied at low concentration over several days to weeks (incorporated into the medium) or may be applied at high concentrations for several minutes or hours (usually as a liquid overlay on the medium or as a concentrated dip). Root initials should appear within a few weeks of treating the explants with auxin. Placing the treated explants on porous agar (solidified agar aerated with a hand-held blender, Barrett Lennard and Dracup 1988), white sand or sterile propagation mix has been found to improve root production for some Australian species (Newell *et al*. 2003).

9.4.1.4 Transfer to potting mix

Once the explants have initiated roots, they can be gently removed from culture (using warm water to remove any adhering agar) and potted into a free-draining propagation mix (e.g., seed raising mix with added perlite). This procedure is often referred to as 'deflasking' or '**exflasking**'. The plantlets should then be placed into a controlled environment with high humidity (> 90 % **relative humidity**), low light (e.g., 70 % shade) and, preferably, controlled temperatures (~15 to 25 °C). A variation on this procedure is to 'direct root' the explants which bypasses the final **in vitro** rooting stage. This involves applying an auxin to the explants (e.g., by dipping them in Clonex Rooting Hormone Gel) before placing them into propagation mix and growing them in a high humidity environment (essentially treating the explants as miniature cuttings).

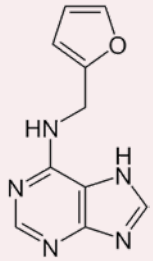
Plantlets transferred to potting mix are initially sensitive to desiccation stress as they tend to have poorly developed waxy cuticles on their leaves and have a limited ability to regulate water loss through stomatal opening and closing. This vulnerability is reduced once the plantlets have had several weeks in the transition environment and have begun to produce new growth. At this stage, they may be moved into a less environmentally controlled structure (e.g., a shadehouse) and may be treated as normal nursery stock propagated from seeds or cuttings.

Figure 9.5: *Syzygium australe* plantlets transferred to potting mix at the Australian PlantBank. (Image: K. Sommerville)

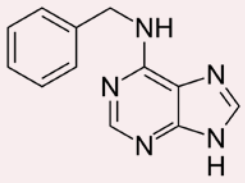
Box 9.2: Plant Growth Regulators

Plant Growth Regulators are natural or synthetic hormones that control the type of growth a plant produces. They are added to tissue culture media in very small amounts, and precise combinations, to stimulate shoot multiplication, shoot elongation, root development, or other types of growth (see Section 9.4).

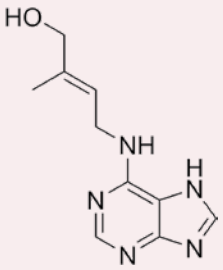
Cytokinins tend to suppress apical dominance and encourage rapid development of axillary shoots. This group includes kinetin, 6-benzylaminopurine (BAP; also known as benzyl adenine or BA) and zeatin.



kinetin

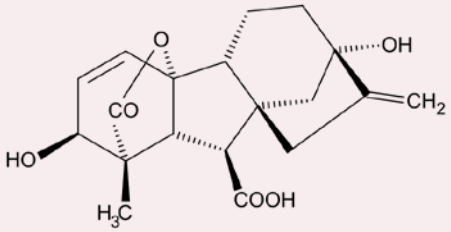


6-benzylaminopurine



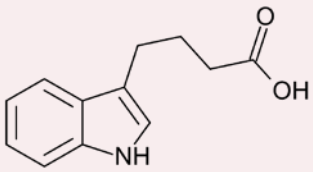
zeatin

Gibberellins, such as gibberellic acid, promote shoot elongation and seed germination.

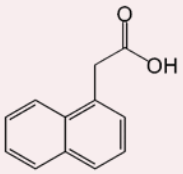


gibberellic acid

Auxins stimulate the development of roots; they can also be used to stimulate the production of callus and somatic embryos. This group includes indole-3-butyric acid (IBA) and 1-napthalene acetic acid (NAA).



indole-3-butyric acid



naphthalene acetic acid

Images: Wikimedia Commons

9.4.1.5 Slow growth storage

Slow growth storage is a method of maintaining plant cultures for long periods without subculturing by altering the chemical composition of the culture medium, and/or lowering the culture temperature to 4–15 °C. This reduces the growth rate of the plants and can extend the subculture period from six weeks to several months or even years. For information on slow growth methods, readers are referred to Engelmann (1991), Ashmore (1997) and Touchell and Dixon (1999). Examples of large collections held in slow growth storage are the global banana and cassava collections held at Bioversity's International Transit Centre (ITC) in Belgium and the International Centre for Tropical Agriculture (CIAT) in Columbia, respectively. Kings Park and Botanic Garden maintain some priority native species in slow growth culture at 12 °C and have found most species able to tolerate storage at this temperature for 9–18 months between subcultures, with some able to tolerate up to two or even three years in storage. There is scope for greater use of slow growth approaches for tissue culture conservation of Australian species, particularly for woody subtropical and tropical **taxa**.

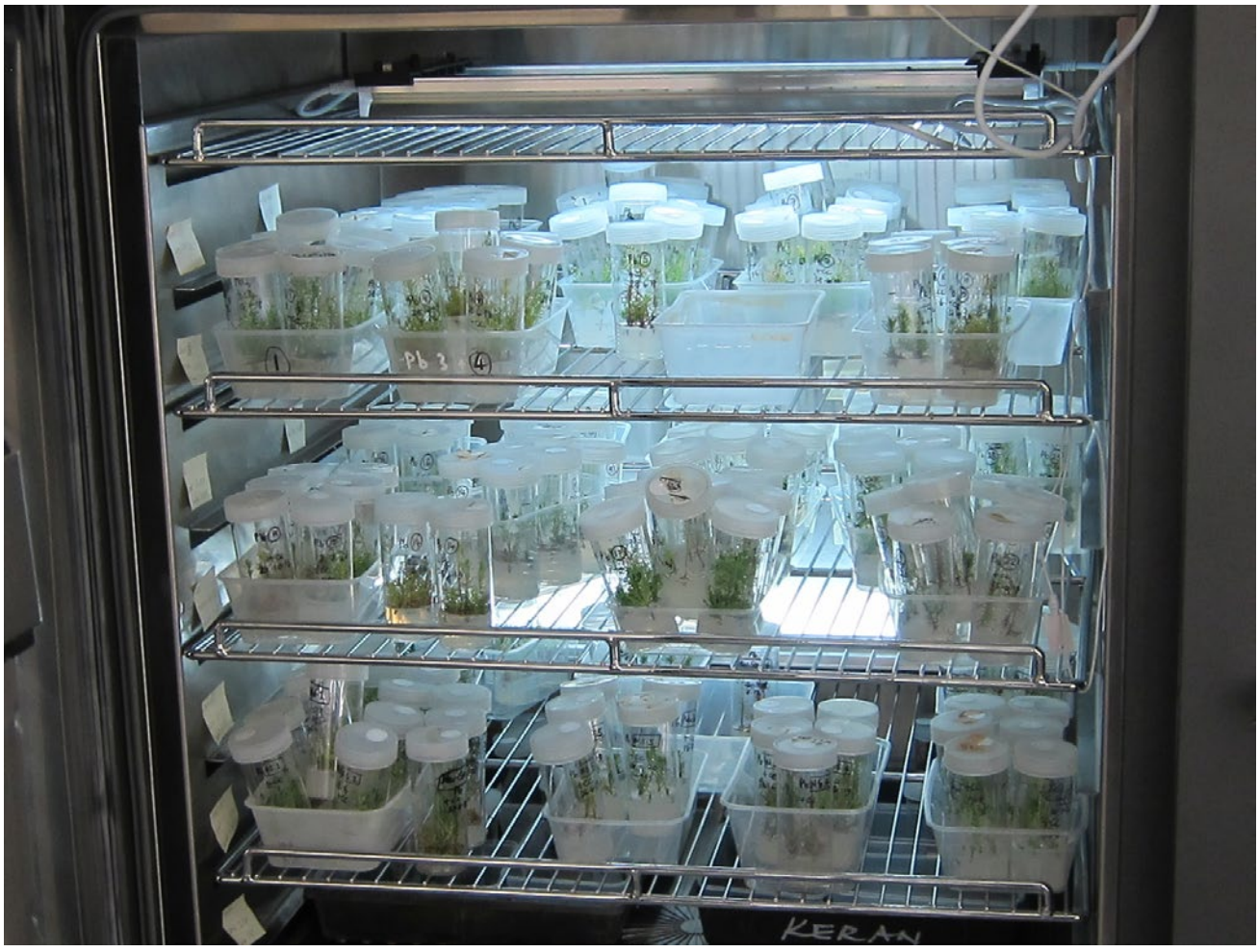


Figure 9.6: Priority species held in slow growth storage at 12 ± 1 °C at Kings Park and Botanic Garden. Slow growth storage is used to reduce the frequency of subculturing from several weeks to > 6 months. (Image: S. Turner)

Table 9.1: Australian native species currently maintained in tissue culture using micropropagation techniques at the Australian PlantBank (PlantBank) or Kings Park and Botanic Garden (KP&BG). Reason for culture: CR, critically endangered; E, endangered; V, vulnerable; DS, desiccation sensitive seed; Int, **intermediate** seed; SL, short-lived seed; DD, deeply dormant seed; H, horticultural potential; P2 or 4, listed as Priority 2 (Poorly-known taxon, known from only one or a few collections) or Priority 4 (Rare, Near-threatened and other taxa in need of monitoring) under Western Australia's *Biodiversity Conservation Act* 2016.

Family	Species	Habit	Reason	Location
Apiaceae	*Actinotus helianthi* cultivars	Shrub	H	PlantBank
Apocynaceae	*Ochrosia moorei*	Tree	E[1,2]	PlantBank
Argophyllaceae	*Corokia whiteana*	Shrub	V[1,2]	PlantBank
Casuarinaceae	*Allocasuarina fibrosa*	Tree	CR[3]	KP&BG
Droseraceae	*Aldrovanda vesiculosa*	Aquatic	P2	KP&BG
Elaeocarpaceae	*Elaeocarpus sedentarius*	Tree	E[1,2], DD	PlantBank
Elaeocarpaceae	*Sloanea australis*	Tree	DS	PlantBank
Elaeocarpaceae	*Sloanea woolsii*	Tree	Likely DS	PlantBank
Elaeocarpaceae	*Tetratheca deltoidea*	Shrub	CR[3]	KP&BG
Ericaceae	*Styphelia longissima*	Shrub	CR[3]	KP&BG
Euphorbiaceae	*Fontainea australis*	Tree	V[1,2], Int	PlantBank
Euphorbiaceae	*Fontainea oraria*	Tree	CR[1,2], Int	PlantBank
Euphorbiaceae	*Ricinocarpos brevis*	Shrub	CR[3]	KP&BG
Fabaceae	*Acacia leptoneura*	Shrub	CR[3]	KP&BG
Fabaceae	*Acacia subflexuosa* subsp. *capillata*	Shrub	CR[3]	KP&BG
Fabaceae	*Ptychosema pusillum*	Herb	CR[3]	KP&BG
Haemodoraceae	*Conostylis dielsii* subsp. *teres*	Herb	CR[3]	KP&BG
Haemodoraceae	*Conostylis micrantha*	Herb	CR[3]	KP&BG
Lamiaceae	*Hemiandra gardneri*	Shrub	CR[3]	KP&BG
Lamiaceae	*Hemiandra rutilans*	Shrub	CR[3]	KP&BG
Lamiaceae	*Hemigenia exilis*	Shrub	P4	KP&BG
Lamiaceae	*Pityrodia scabra* subsp. *scabra*	Shrub	CR[3]	KP&BG
Lauraceae	*Cryptocarya glaucescens*	Tree	Likely DS	PlantBank
Lauraceae	*Cryptocarya microneura*	Tree	Int	PlantBank
Lauraceae	*Cryptocarya rigida*	Tree	DS	PlantBank
Lauraceae	*Neolitsea dealbata*	Tree	DS	PlantBank
Malvaceae	*Androcalva adenothalia*	Shrub	CR[3]	KP&BG
Malvaceae	*Androcalva perlaria*	Shrub	CR[3]	KP&BG
Malvaceae	*Commersonia erythrogyna*	Shrub	CR[3]	KP&BG
Malvaceae	*Lasiopetalum moullean*	Shrub	CR[3]	KP&BG
Monimiaceae	*Wilkea huegeliana*	Shrub/tree	DS	PlantBank
Myrtaceae	*Darwinia masonii*	Shrub	CR[3]	KP&BG
Myrtaceae	*Eucalyptus* × *graniticola*	Mallee	P4	KP&BG
Myrtaceae	*Eucalyptus* × *impensa*	Mallee	CR[3]	KP&BG
Myrtaceae	*Eucalyptus* × *phylacis*	Mallee	CR[3]	KP&BG

Family	Species	Habit	Reason	Location
Myrtaceae	*Eucalyptus argutifolia*	Mallee	CR[3]	KP&BG
Myrtaceae	*Eucalyptus dolorosa*	Mallee	CR[3]	KP&BG
Myrtaceae	*Rhodamnia rubescens*	Tree	CR[2]	PlantBank
Myrtaceae	*Rhodomyrtus psidioides*	Tree	CR[2]	PlantBank
Myrtaceae	*Syzygium anisatum*	Tree	SL	PlantBank
Myrtaceae	*Syzygium australe*	Tree	DS	PlantBank
Myrtaceae	*Syzygium leuhmannii*	Tree	DS	PlantBank
Myrtaceae	*Syzygium moorei*	Tree	V[1,2], DS	PlantBank
Myrtaceae	*Syzygium paniculatum*	Tree	E[1,2], DS	PlantBank
Myrtaceae	*Syzygium pseudofastigiatum*	Tree	DS	PlantBank
Pennantiaceae	*Pennantia cunninghamii*	Tree	DS	PlantBank
Proteaceae	*Banksia ionthocarpa* subsp. *chrysophoenix*	Shrub	CR[3]	KP&BG
Proteaceae	*Banksia montana*	Shrub	CR[3]	KP&BG
Proteaceae	*Banksia serratuloides* subsp. *perissa*	Shrub	CR[3]	KP&BG
Proteaceae	*Conospermum galeatum*	Shrub	CR[3]	KP&BG
Proteaceae	*Grevillea althoferorum* subsp. *althoferorum*	Shrub	CR[3]	KP&BG
Proteaceae	*Grevillea dryandroides* subsp. *dryandroides*	Shrub	CR[3]	KP&BG
Proteaceae	*Grevillea dryandroides* subsp. *hirsuta*	Shrub	CR[3]	KP&BG
Proteaceae	*Grevillea scapigera*	Shrub	CR[3]	KP&BG
Proteaceae	*Hakea aculeata*	Shrub	CR[3]	KP&BG
Proteaceae	*Hicksbeachia pinnatifolia*	Tree	V[1,2], DS	PlantBank
Proteaceae	*Macadamia integrifolia*	Tree	Int	PlantBank
Proteaceae	*Persoonia amaliae*	Shrub	DD	PlantBank
Proteaceae	*Persoonia hirsuta*	Shrub	E[1,2], DD	PlantBank
Proteaceae	*Persoonia nutans*	Shrub	E[1,2], DD	PlantBank
Proteaceae	*Synaphea quartzitica*	Shrub	CR[3]	KP&BG
Proteaceae	*Synaphea stenoloba*	Shrub	CR[3]	KP&BG
Proteaceae	*Telopea speciocissima* cultivars	Shrub	H	PlantBank
Rutaceae	*Drummondita ericoides*	Shrub	CR[3]	KP&BG
Rutaceae	*Glycosmis trifoliata*	Climber	DS	PlantBank
Rutaceae	*Philotheca basistyla*	Shrub	CR[3]	KP&BG
Rutaceae	*Philotheca wonganensis*	Shrub	CR[3]	KP&BG
Sapindaceae	*Planchonella australis*	Tree	DS	PlantBank
Scrophulariaceae	*Eremophila pinnatifida*	Shrub	CR[3]	KP&BG
Scrophulariaceae	*Eremophila virens*	Shrub	CR[3]	KP&BG
Solanaceae	*Grammosolen odgersii* subsp. *occidentalis*	Shrub	CR[3]	KP&BG
Solanaceae	*Symonanthus bancroftii*	Shrub	CR[3]	KP&BG

[1]Commonwealth *Environment Protection and Biodiversity Conservation Act* 1999
[2]NSW *Biodiversity Conservation Act* 2016
[3]WA *Biodiversity Conservation Act* 2016

The following case studies demonstrate the use of micropropagation to conserve endangered species. In the first, Case Study 9.2, the technique was used to conserve *Persoonia hirsuta*, an endangered shrub with poor regeneration in the wild and complex seed dormancy issues. In the second, Case Study 9.3, micropropagation was used to produce multiple **clones** for **translocation** of critically endangered *Eremophila resinosa*.

Case Study 9.2: Using tissue culture to conserve the fire-sensitive *Persoonia hirsuta*

Amanda Rollason and Nathan J. Emery

Persoonia hirsuta (Hairy Geebung) is an Endangered shrub that is restricted to the Greater Sydney region in New South Wales (NSW; Figure 9.7). The species occurs in highly fragmented **populations**, many comprising less than ten individuals or a single isolated individual. Consequently, this **obligate-seeding** species is under significant **threat** from **habitat** loss, **inbreeding**, inappropriate fire regimes and a lack of recruitment success. Highlighting the fragility of *P. hirsuta*, the recent unprecedented fire season in NSW during summer 2019/20 burnt five of the 13 known wild populations. Successful conservation of *Persoonia* species is hampered by complex seed dormancy issues (see Case Study 7.3), and seed production and recruitment events for *P. hirsuta* are rare (Figure 9.8; Emery and Offord 2019). Therefore, tissue culture was investigated as an alternative method for establishing *P. hirsuta* in ex situ conservation (Offord *et al.* 2015).

The material used to initiate *Persoonia hirsuta* into tissue culture was taken from 6-month old seedlings held in the nursery at the Australian Botanic Garden Mount Annan. Cuttings of fresh new growth were removed from the plants and leaves were excised from the cuttings, leaving 2 mm of each petiole for node protection during the sterilising procedure. Each cutting was then divided into segments around 15 mm in length. The segments were washed by agitation for 15 minutes in a solution of 1 % Alconox® and rinsed in water filtered by reverse osmosis (RO water). The pre-washed segments were sterilised by agitation for 15 minutes in 200 ml of 1 % sodium hypochlorite[53] (prepared with White King® household bleach, sterilised RO water and ~33 µl of Tween® 20). The washed and sterilised segments were then transferred to a laminar flow cabinet and rinsed three times in sterile RO water. After rinsing, the segments were dissected into explants around 5–7 mm in length, and the freshly cut basal end of each explant was inserted into half-strength Murashige and Skoog medium (MS; Murashige and Skoog 1962), containing 9 g/L agar and 30 g/L sucrose (pH 6.0), in individual test tubes.

After 4 weeks, 45 % of explants suffered microbial contamination and were discarded. The uncontaminated explants were transferred onto one of two types of media: MS basal medium plus vitamins, growth regulators, 30 g/L sucrose and 9 g/L agar (pH 6.0); or Woody Plant Medium (WPM; McCown & Lloyd 1981) plus growth regulators, 20 g/L sucrose and 9 g/L agar (pH 6.0). In both cases, the growth regulators consisted of 2 µmol/L 6-benzylaminopurine and 0.2 µmol/L indole-3-butyric acid.

Persoonia hirsuta was very slow to respond to both types of culture media but initial results indicated a preference for WPM. The species has now been successfully established in tissue culture (Figures 9.9 and 9.10) and is multiplying well on this medium – an encouraging step towards long-term conservation of a highly threatened plant. Protocols for transferring cultured *P. hirsuta* plantlets to potting mix are now under investigation.

53 https://en.wikipedia.org/wiki/Sodium_hypochlorite

Figure 9.7: *Persoonia hirsuta* (Hairy Geebung). (Image: Nathan Emery)

Figure 9.8: A mature drupe. Seed production and recruitment events are rare in *P. hirsuta.* (Image: Nathan Emery)

Figure 9.9: *P. hirsuta* explants for subculturing. (Image: Amanda Rollason)

Figure 9.10: *P. hirsuta* explants in tissue culture. (Image: Amanda Rollason)

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Case Study 9.3: Translocation of micropropagated *Eremophila resinosa*

Eric Bunn and Shane R. Turner

The 'Sticky Eremophila' or *Eremophila resinosa* (Myoporaceae) is a critically endangered species from the Wheatbelt region in Western Australia which was listed as declared Rare Flora in 1982 under the Western Australian *Wildlife Conservation Act* 1950. It is ranked as Endangered (EN) under IUCN (1994) Red List criteria and was also listed as Endangered under the Commonwealth *Environment Protection and Biodiversity Conservation Act* 1999 (EPBC Act). Consequently, to assist in its management and recovery an interim recovery plan (IRP No. 266) was implemented in 2008 which set out a clear set of conservation actions including propagation and translocation.

The Westonia remnant of *Eremophila resinosa* existed as, 24 natural populations totalling 697 plants, most of which occurred on a **rehabilitated** mine site surrounded by farms near Westonia in the Eastern Wheatbelt in Western Australia. *Eremophila resinosa* was also known from a few other smaller sites in the Yilgarn district but some of these populations were in decline/**extinct** by 2009. *Eremophila resinosa* mostly frequents open Eucalypt woodland with an open scrub understorey in sandy loam/clay soils. The restricted distribution of *Eremophila resinosa* was considered to be due mainly to clearing of native vegetation for agricultural and mining activity. Some populations on road verges were under threat from weed infestation, frequent road maintenance activities, altered fire regimes, farming activities including herbicide and fertiliser drift and grazing by rabbits. In addition, future mining operations threatened plants on rehabilitated mined sites.

Vegetative material was collected in 2003 from five genotypes earmarked for removal as part of the re-commencing of mining activities and established successfully in tissue culture at Kings Park and Botanic Garden. Shoots were multiplied and eventually induced to form roots which were successfully transferred to soil demonstrating that all stages of the micropropagation process were feasible for this rare species. A translocation proposal was prepared by October 2003, a modified proposal accepted by Evolution Mining in March 2004 and after submission and consideration by the Dept of Conservation and Land Management (CALM), the program began (Figure 9.11a).

Due in part to the success of the first translocation of micropropagated plants (derived from the original five genotypes), seeds were able to be collected, germinated and seedlings used for later translocations (Figure 9.11b). Cutting propagation was also tried but was not initially very successful, with material of only one genotype producing viable plants. Plants from the original (**in situ**) wild populations and translocated plants produced harvestable seeds which were able to be utilised with great success for further translocations, after follow-up studies on how to maximise seed germination were completed. However, the initial plantings using micropropagated plants were invaluable in establishing a founder population (Figure 9.11c) that provided valuable information for later seedling translocations as well as seeds for off-site (ex situ) seed storage. Shoots from tissue cultured plants were also able to be cryostored (see Chapter 10) in liquid nitrogen to add to the overall ex situ **germplasm** conservation collections for *Eremophila resinosa*.

Figure 9.11: **(a)** Site of the first translocation at Westonia minesite, **(b)** Micropropagated rooted shoots of *Eremophila resinosa* ready for transplanting to soil and **(c)** A micropropagated plant of *Eremophila resinosa* about 12 months after transplantation. By the second year following transplanting most plants were able to produce harvestable quantities of seed. (Images: Eric Bunn and Bob Dixon)

9.4.2 Callus culture

Plant callus is a mass of unorganised plant cells that grow and expand in an unstructured way (Figure 9.12) instead of forming leaves, shoots or roots (Figure 9.6). In the natural environment, callus cells are commonly formed in response to a wound; the callus cells are produced, rapidly expand and eventually seal the damaged area. In plant tissue culture, callus formation may be induced from different types of explants by placing the material onto an appropriate tissue culture medium (George 1993, 1996; Davies *et al*. 2017). The culture medium is supplemented with specific plant growth regulators that act individually, or in combination, to promote a callus response (Anthony *et al*. 2004a, b; Panaia *et al*. 2004a, b; Panaia *et al*. 2011; Kodym *et al*. 2012). Different combinations of growth regulators may then be used to sustain callus formation, or promote the development of organised plant structures (i.e., shoots or embryos) that can be harvested and treated as new plants (Anthony *et al*. 2004a, b; Panaia *et al*. 2004a, b; Panaia *et al*. 2011; Kodym *et al*. 2012). Callus initiation and growth has been reported for a broad range of plant species including many different Australian native species (Anthony *et al*. 2004a, b; Panaia *et al*. 2004a, b; Ashmore *et al*. 2011; Bunn *et al*. 2011; Panaia *et al*. 2011; Kodym *et al*. 2012; Corredoira *et al*. 2015; Whiteley *et al*. 2016).

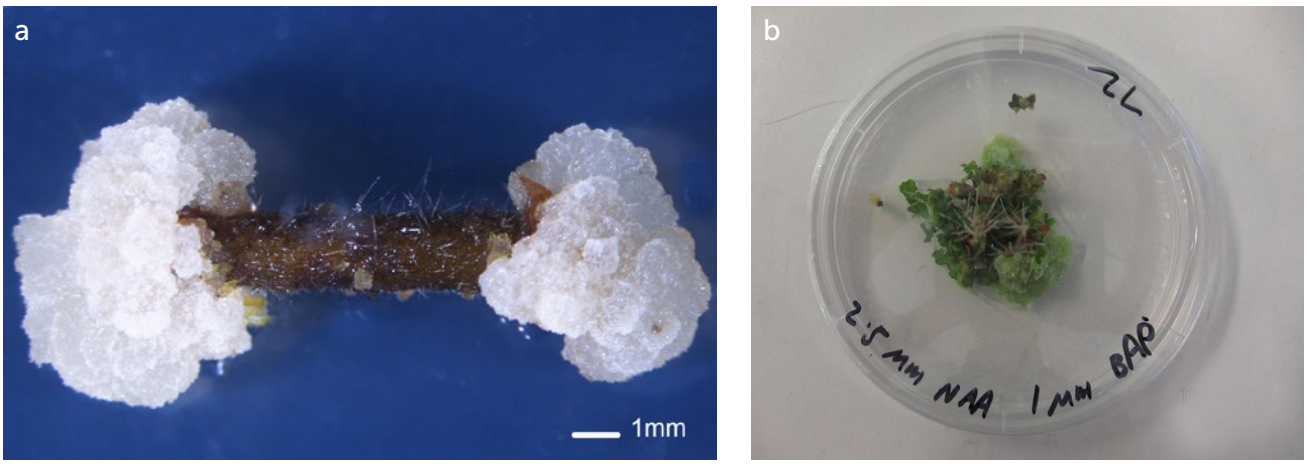

Figure 9.12: **(a)** Callus forming on an explant of the threatened *Androcalva perlaria* and **(b)** shoots regenerating from callus of the same species. (Images: S. Turner)

9.4.3 Suspension cell culture

A suspension cell culture is normally initiated by placing loose clumps of callus into a sterile liquid tissue culture medium. Callus is initiated as described above (Section 9.4.2) then placed in the liquid medium where it is gently agitated on an orbital shaker to keep the callus in suspension rather than settling on the bottom. Floating in the solution exposes the cells to more nutrients, increases oxygen availability and lessens the impact of waste compounds produced during metabolism. As the callus fragments break up, they form a cell suspension consisting of either individual cells or small agglomerates of cells (George 1993, 1996). A sample of the solution is removed under sterile conditions and placed into fresh liquid media at regular intervals to continue to grow and expand the cellular suspension. When desired, a sample of the suspension solution can be placed into a modified liquid medium formulated to stimulate the production of somatic embryos (Section 9.4.4; George 1993, 1996). Alternatively, small amounts of the suspension solution can be placed on semi solid growth media formulated to stimulate either more callus production, or the development of organised plant structures such as adventitious shoots or somatic embryos. New growth will usually appear within several weeks after transfer to this medium (George 1993, 1996).

9.4.4 Somatic embryogenesis

Somatic embryogenesis is the formation of embryos, that are similar in structure and function to zygotic embryos, from somatic (non-sexual) tissues. The advantage of this process over conventional shoot culture is that each somatic embryo has both a shoot and root axis. The embryo develops into a seedling similar to that produced by a seed. There is no need, therefore, for separate multiplication and rooting stages as in standard shoot culture systems. For many species, root induction is the most difficult phase in micropropagation and with somatic embryogenesis this phase is completely by-passed. In addition, a single gram of embryogenic tissue can produce thousands of somatic embryos, resulting in a huge increase in efficiency compared to standard shoot culture systems. In more advanced systems, somatic embryos can be desiccated, coated and treated like true seeds through direct sowing into nursery punnets or even field soil (Fujii *et al*. 1992).

See Case Study 9.4 for discussion of the prospects of somatic embryogenesis for mass production of native species.

9.4.4.1 Production of somatic embryos

The most common way to induce somatic embryogenesis is to apply the synthetic auxin (and herbicide) 2,4-dichlorophenoxyacetic acid (2,4-D) to appropriate explant material. Thidiazuron (TDZ) – a compound developed originally as a cotton defoliant in the 1970s – is also used for this purpose. The juvenility of the explant material is very important; seed embryos are commonly used as starting explants as they respond well to low concentrations of 2,4-D. Monocotyledon species are generally more responsive to 2,4-D than dicotyledon species for somatic embryo production, while dicotyledon species respond well to TDZ; however, some native monocotyledons also respond well to TDZ (Panaia *et al*. 2004a).

Somatic embryogenesis may be direct or indirect. Direct somatic embryogenesis can be induced from highly responsive explant material such as seed embryos or hypocotyl segments of seeds germinated in vitro (Figure 9.13). Clusters of somatic embryos develop directly from the explant following application of an embryo-inducing compound (2,4-D or TDZ). Indirect somatic embryogenesis involves the generation of callus from the primary explant (Section 9.4.2). This tissue can be cultured separately and later induced to form somatic embryos following treatment with an embryo-induction compound. The formation of a distinct callus phase is essential for plant cell culture (Section 9.4.3) in flasks, or bioreactors that can be automated, to provide vast numbers of cells for mass production of somatic embryos. However, the use of a callus stage may increase the risk of somaclonal variation (see Section 9.2) and is generally not recommended for conservation purposes.

9.4.4.2 Applications for somatic embryos

The potential of somatic embryogenesis is enormous as it is the most efficient high volume plant culture system; however, while an important role for somatic embryogenesis in conservation and restoration of native plants in Australia can be readily envisaged, progress with developing this technology is likely to remain slow. The methodology can be difficult at the best of times and considerable optimisation for individual species is often required. This leads to lengthy research phases, escalating research costs and therefore difficulties in securing ongoing research and development funding. Despite these problems, some progress has been made with the development of somatic embryogenesis protocols for Australian sedge species (Panaia *et al*. 2004b; Kodym *et al*. 2012; see Figure 9.14), native citrus (Hamilton 2007), species of Ericaceae (Anthony *et al*. 2004a, b), *Eucalyptus* species (Corredoira *et al*. 2015) and wax flowers (Ratanasanobon and Seaton 2010), with potential for both conservation and commercial production of plants. There is also potential for somatic embryos to be desiccated and coated to produce 'artificial seed' that can be treated as a real seed, i.e., sown into soil to generate a seedling. This system has only been developed for a restricted number of high value forestry, amenity or crop species to date (Jain *et al*. 1995; Zimny *et al*. 2003); however, as a 'proof of concept', these are powerful examples of the potential of this technology. For more information about somatic embryogenesis on Australian species please refer to Case Study 9.4.

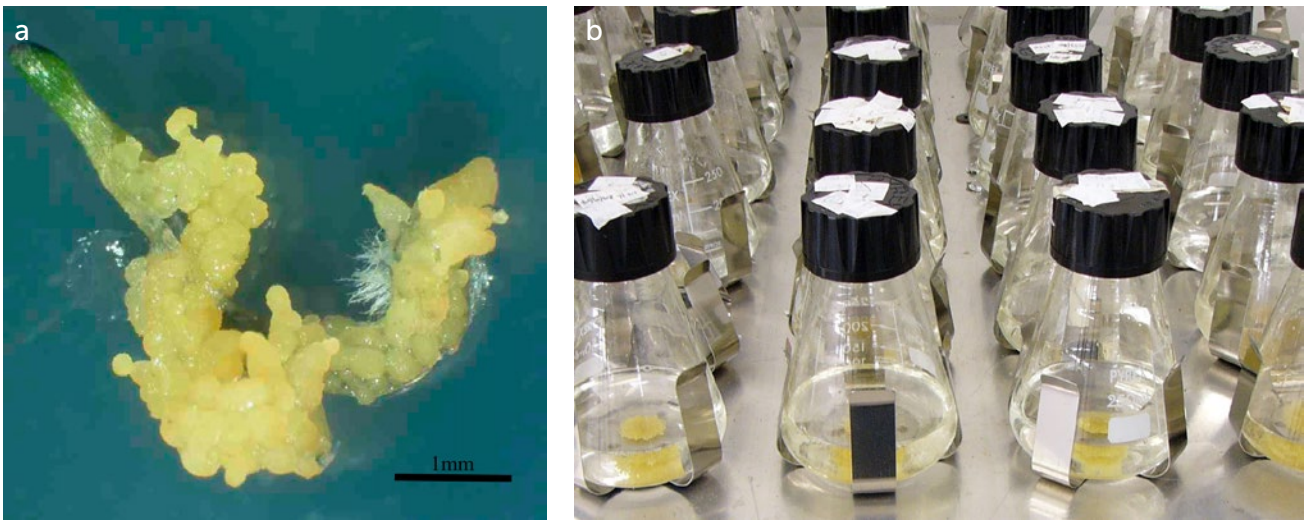

Figure 9.13: Somatic embryos initiated from: **(a)** a coleoptile segment of *Anigozanthos manglesii* (Image: E Bunn) and **(b)** seed tissue of *Citrus inodora.* (Image: K Hamilton).

Case Study 9.4: Somatic embryogenesis for mass production of native species

Eric Bunn and Shane R. Turner

Somatic embryogenesis is the process in which structures similar to seed embryos are produced in culture from non-sexual plant tissues. These structures may develop directly from explant surfaces or indirectly via an intervening callus phase. Somatic embryos can be induced to develop and 'germinate' like a zygotic embryo into an '**embling**' (so called to distinguish it from a normal seedling) that is capable of development into a whole plant.

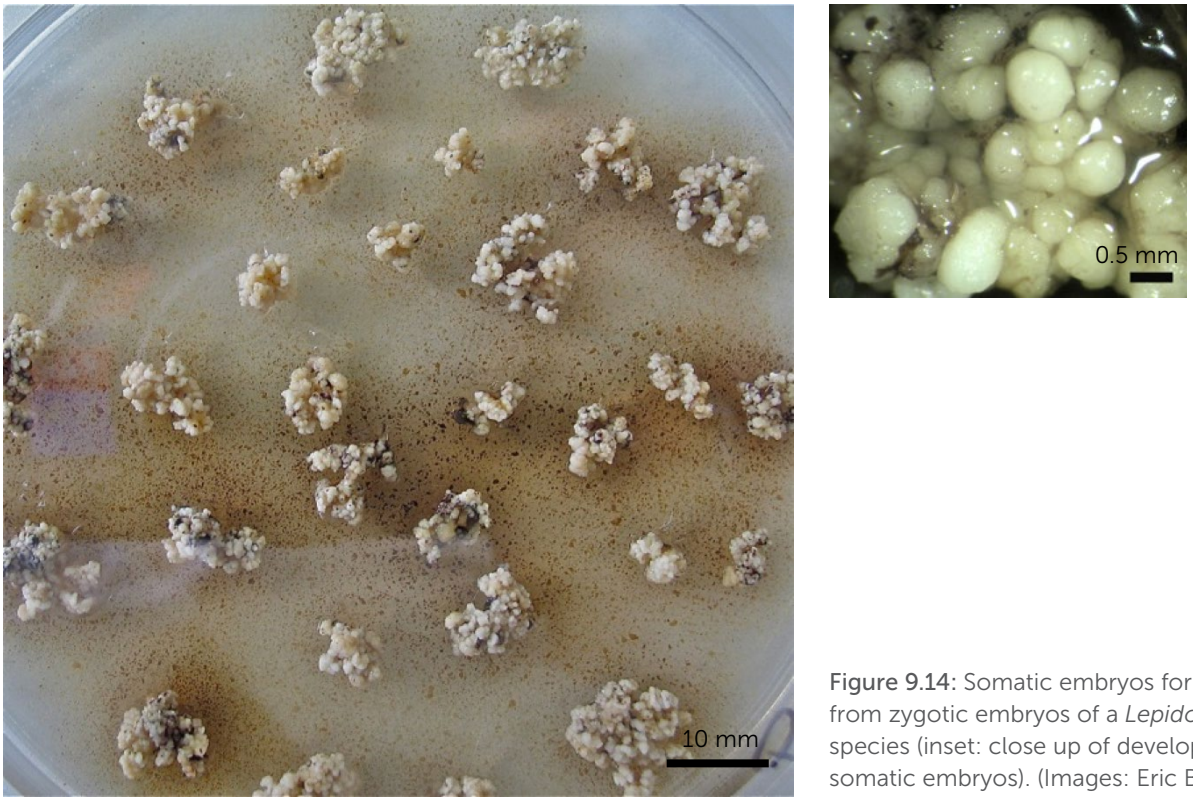

Figure 9.14: Somatic embryos forming from zygotic embryos of a *Lepidosperma* species (inset: close up of developing somatic embryos). (Images: Eric Bunn)

Some species are difficult to establish in tissue culture from vegetative material due to the type of tissues available for initiation. For example, monocotyledonous species which grow primarily from underground stems, **rhizomes** or **tubers** can be especially difficult to sterilise, as soil holds enormous numbers of fungi and bacteria that live in and on these vegetative structures. Some species rely principally on vegetative propagation to persist and can be established in tissue culture with considerable effort, but these frequently harbour latent contaminating microbes that are extremely difficult or impossible to remove. Other species produce sufficient seeds to allow the use of whole seeds, seedling explants (e.g., hypocotyl sections) or seed embryos as the starting material and this will often be the only feasible means to establish microbe-free cultures. Somatic embryogenesis is the most efficient way to mass propagate these types of species.

Australian native species which have been produced by somatic embryogenesis are few, but several new protocols have been published since 2008, including Ratanasanobon and Seaton (2010), Panaia *et al*. (2011), and Corredoira *et al*. (2015), see also Figure 9.15. However, examples where native species produced via somatic embryogenesis have been used in translocation programs are rare and the prospects for greater utilisation of this technique remain to be fully exploited (Ashmore *et al*. 2011; Bunn *et al*. 2011).

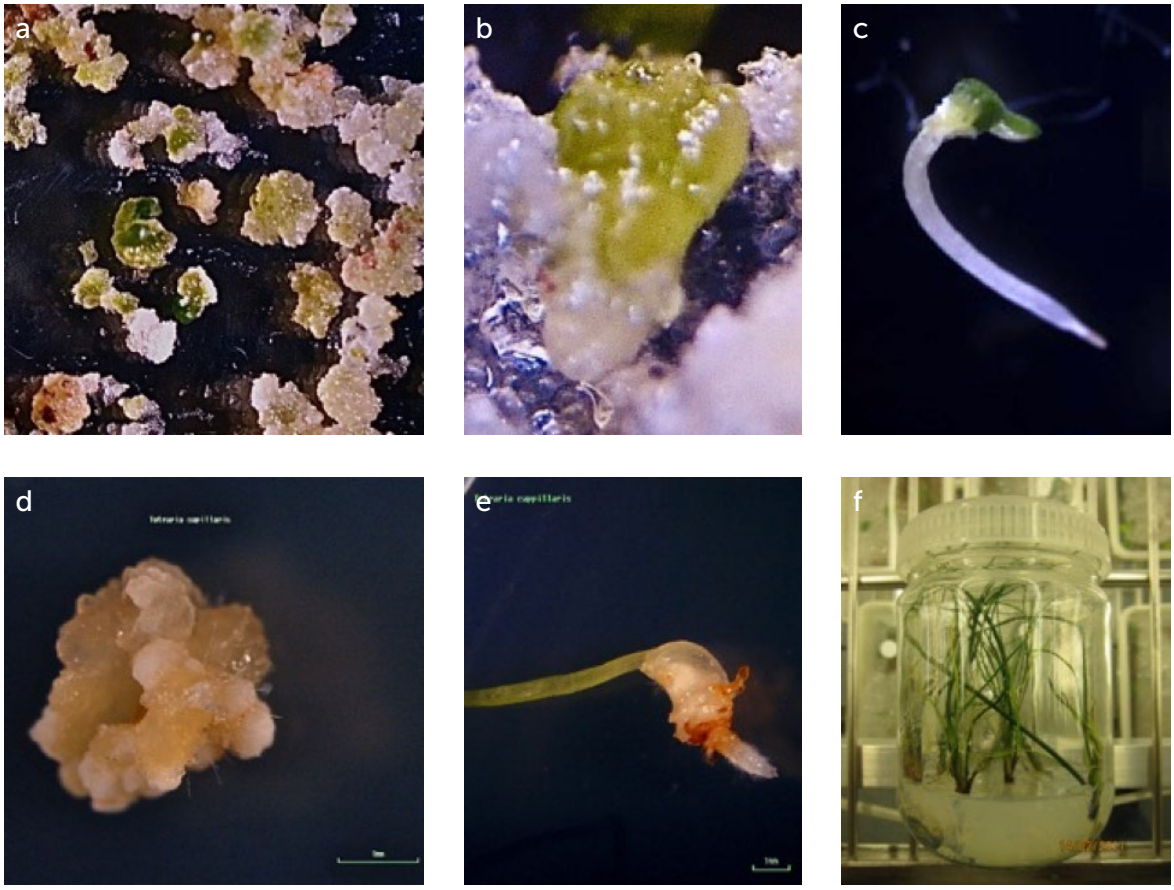

Figure 9.15: Examples of native spp produced by somatic embryogenesis: **(a-c)** *Philotheca basistyla*; **(a)** embryogenic callus, **(b)** a cotyledonary stage somatic embryo, and **(c)** a germinated somatic embryo ('embling') **(d-f)** *Tetraria capillaris* **(d)** globular embryos forming on callus, **(e)** an embling, and **(f)** whole plants from somatic embryos in a culture container. (Images: Eric Bunn)

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9.5 Common difficulties encountered in tissue culture

Tissue culture should be viewed as complementary to other forms of plant conservation, and should only be used as a replacement under some circumstances, as it is potentially expensive, resource intensive and – due to the complexity of the procedures involved – can be very challenging as there are many points in the tissue culture cycle where the process may fail (Koch 2007; Ashmore *et al*. 2011; Bunn *et al*. 2011; Kodym *et al*. 2014; Whiteley *et al*. 2016). Some of the more common difficulties that may be encountered when utilising plant tissue cultures are outlined below.

9.5.1 Microbial contamination during initiation

The success of the initiation phase often relies on the source of the plant material. Material collected from the wild can be difficult to initiate due to the large number of associated microbes present upon (surface contaminants) and within (endogenous contaminants) plant tissues. These microbes can contaminate cultures even after stringent surface sterilisation protocols have been applied (Bunn and Tan 2002). This is particularly problematic with material from warm and humid habitats such as those in tropical regions, or for species that mainly grow underground shoots (Bunn and Tan 2002; Sugii 2011; Drew 2013). Quite often, it is necessary to establish these plants from seeds or cuttings in a managed potted collection before attempting tissue culture. Steps can then be implemented to reduce the microbial load on the plant tissues (e.g., by treating the plants with fungicide) as well as stimulating the production of healthy and rapidly growing shoots that may be more resilient to the harsher surface sterilisation approaches that may need to be adopted. Nevertheless, there are many techniques for reducing contamination of material collected from wild sources (see Pence 2005), and these can be utilised if a species cannot be effectively propagated using more conventional approaches (Panaia *et al*. 2000; Bunn & Tan 2002; Bunn 2005; Whiteley *et al*. 2016).

9.5.2 Recalcitrance to tissue culture

In addition to the heavy microbial load, wild grown plants may be in poor condition due to low rainfall, low soil nutrient availability, or the impact of diseases such as Myrtle Rust or Phytophthora Dieback. Tissue culture (and any other type of propagation) is most successful with material that is young, healthy and actively growing. Where possible, then, it is best to establish plants from seeds or cuttings before attempting to initiate them into tissue culture. Some species (or individual genotypes), however, are simply very difficult to establish in culture. These species may respond poorly to the decontamination procedure, may fail to grow at all in the tissue culture environment, may grow but fail to produce multiple shoots, or may fail to grow roots even if leaves and shoots are easily produced (Bunn *et al*. 2007; Bunn *et al*. 2011). Such cases require much experimentation with media components or unusual tissue types – such as immature **ovules** or immature embryos – for successful initiation and growth (Drew 2013); however, in many (if not most) cases, the difficulties can eventually be overcome (Panaia *et al*. 2000; Bunn 2005).

9.5.3 Hyperhydricity

Hyperhydricity is a condition in which tissue cultured shoots develop a glassy or watery appearance. These shoots are very fragile and can be difficult to transfer out of culture. The condition can be controlled by manipulating the growing environment in the culture vessel, through increasing the amount of agar or sucrose in the medium, decreasing the concentration of cytokinin, or reducing humidity by growing in a vessel with a vented lid (Rossetto *et al*. 1992).

9.5.4 Difficulty establishing plants ex vitro

Some plants are very difficult to transfer out of the tissue culture environment. The difficulty may be overcome by manipulation of the explants prior to removal from the flask (Offord and Campbell 1992; Newell *et al*. 2003), and careful control of humidity post-removal. Successful acclimatisation of plantlets involves 'switching on' the photosynthetic apparatus so that the plant can function independently of the in vitro environment. This requires vascular connection of the roots and shoots, and protection from moisture loss through functioning stomata and development of a leaf cuticle. Plants transferred to potting mix may need intensive management for the first few weeks as they adjust to the new environment; variables such as light, temperature, humidity and soil moisture need to be carefully controlled using specialised plant propagation equipment and facilities (Beyl and Trigiano 2015; Davies *et al*. 2017). Plants at this point may also be unusually sensitive to commonly encountered plant diseases such as *Botrytis*, *Fusarium* and *Pythium* (i.e., damping off diseases; Bunn and Tan 2002).

9.6 Other considerations

9.6.1 Recording of information

It is very easy to mix up tissue culture collections, especially when they are held for many years. To avoid this, efficient recording systems need to be developed and meticulously followed to record the details of the material in the collection including the history in, and prior to, culture. Each subculture flask should also be coded to reflect the following details:

- Species;
- Clone/**Accession**;
- Date, location and collector of wild material or accession number;
- Date put into culture;
- Subculture number;
- Treatments/medium used.

9.6.2 Duplication at other facilities

As with seeds and other germplasm collections, it is advisable to duplicate important cultures at other sites. This is easily achieved by sending cultures in sealed plastic containers, providing the support medium (agar) is firm enough to resist breakage during transport. A phytosanitary certificate may be needed for transport between states or overseas. Transport of tissue cultured material is usually simpler than for seeds or whole plants as the risk of also transporting microbial or insect pests has been eliminated by the sterile growing environment. Check with the relevant authorities, however, before sending material outside of your state or country. As the cultures will be sensitive to temperature extremes or light exclusion, it is best to fast track their transport using express post or dedicated couriers so that they arrive at their destination within a few days.

9.7 Summary

Tissue culture provides a useful option for conservation of species that can't be conserved by seed banking. There is a vast body of literature relating to the techniques involved, reflecting the extent and complexity of its uses, so only a cursory examination of the basic procedures has been given in this chapter. References such as George (1993, 1996), Beyl and Trigiano (2015) and Davies *et al*. (2017) should be consulted for general tissue culture procedures, media formulations, and working concentrations of plant growth regulators, as well as more general requirements for setting up a tissue culture laboratory and integrating this within a modern plant nursery. Taji and Williams (1996) and Burchett and Johnson (1996) provide more specific information on, and examples of, the tissue culture of Australian plants. There is also now a large body of published work highlighting different aspects of the tissue culture approach, and describing various techniques in detail, for a broad range of Australian native plant species (e.g., Bunn *et al*. 2007; Ashmore *et al*. 2011; Bunn *et al*. 2011). It is important to understand that there are large differences in the response of various plant types to tissue culture, i.e., dicotyledons vs. monocotyledons and woody vs. non-woody plants. Literature specific to these types should be sought when investigating a new species for tissue culture.

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Chapter 10
Cryopreservation

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This chapter is a revision of the previous edition chapter by Kim N. Hamilton, Shane R. Turner and Sarah E. Ashmore, 2nd edition, pp. 129-148.

10.1 Introduction

Cryopreservation involves the storage of **germplasm** (e.g., **seeds**, **embryo** axes or tissue cultured shoot tips) at very low temperatures, typically utilising liquid nitrogen (LN) (-196 °C), or its vapour (-130 to -192 °C), to preserve living tissue in a state of suspended animation. Cryopreservation has become a viable long-term **conservation** tool for many threatened and economically valuable species (Walters and Pence 2020).

Cryopreservation provides a relatively low maintenance and space efficient conservation option for very long-term storage (i.e., >25 years), particularly for germplasm that is not suitable for conventional dry storage at -20 °C that must be maintained as living collections (Dulloo *et al.* 2009). This chapter provides guidelines on when to use cryopreservation, and what tissues to use. It also provides methods that may be used to establish a cryogenic germplasm bank of Australian plant species.

10.2 Cryopreservation as a long-term conservation tool

Cryopreservation can be considered a relatively new tool for conservation, with some of the oldest cryopreserved samples still showing good viability and regrowth after 20–40 years in storage (Ballesteros and Pence 2017; Ballesteros and Pence 2019; Pence *et al.* 2017). A wide range of plant species have been successfully cryo-stored, from agricultural species to rare and threatened native species (Reed 2008; Wolkers and Oldenhof 2021). However, as with other forms of germplasm storage used for conservation collections, cryopreservation must ensure the survival and recovery of true-to-type plants encompassing the diversity of the species, without degradation or **morphological** changes that would affect the species' survival in the wild.

Cryopreservation should:

- Supplement and complement conventional germplasm storage methods to risk-manage valuable collections;
- Be used as the priority storage method when conventional methods are inadequate or unable to provide long-term storage options;

- Be the preferred method of germplasm storage for critically endangered plants:
  - when only a small quantity of material is available; or,
  - when very long-term storage (multi-decadal) is desired, such as for critical **base collections**.

Depending on the species and tissue to be cryopreserved, new protocols may need to be developed and optimised through empirical experimentation, altering factors such as degree of desiccation, length of exposure to cryoprotective agents (CPAs), and preculture and recovery environment (Bettoni *et al.* 2021). Once a robust approach has been developed, some screening needs to be undertaken on different **accessions**, prior to the establishment of a long-term base collection, to make sure that the techniques and approaches adopted are working across the whole group of interest and that the results achieved are reproducible. The majority of costs associated with cryopreservation are involved in this protocol development; however, once cryopreserved, maintenance costs are comparable to seed banking (Li and Pritchard 2009; Pence 2011). A more recent approach has been to utilise **cryobiotechnology** (incorporating modern technologies such as transcriptomics, proteomics, metabolomics, molecular modelling and thermal analysis) to gain a greater understanding of the cryopreservation process, thereby advancing and optimising cryopreservation as a conservation tool (Pence *et al.* 2020; Pritchard 2018).

An on-going problem for many plant germplasm collections is the loss of critical accessions through equipment failure (of freezers and incubators), pests and disease, microbial contamination and human error (e.g., through mislabelling, or using poor sterile technique). Through adopting cryogenic storage, many of these risks (apart from equipment failure) are greatly minimised and, in some cases, eliminated. For example, for vegetative germplasm (i.e., **tissue culture** collections), LN storage can eliminate pests and diseases, **somaclonal variation**, microbial contamination due to mites, and can reduce the chance of human error as the material, once frozen, is virtually maintenance free.

10.3 Conservation of 'exceptional species'

While seed banking is an effective **ex situ conservation** approach for many plant species, there is growing awareness of '**exceptional species**' that cannot be adequately preserved long-term using conventional seed banking methods (Pence 2013). 'Exceptional species' are defined as: those that do not produce seed or produce seeds that are extremely difficult to harvest, do not survive conventional seed bank conditions (i.e., dry storage at subzero temperatures), do not remain viable for a reasonable length of time in seed bank conditions, or do not have the ability to germinate and produce plants upon removal from a seed bank even though they are viable (Pence 2013). This group includes **non-orthodox** seeds as described in Chapter 6.

Species with any of the above-mentioned characteristics require alternative ex situ conservation approaches via living plant (Chapter 11), tissue culture (Chapter 9), or cryopreserved collections (this chapter). These approaches are much more costly and time-consuming than seed banking, and often require novel research to develop successful long-term preservation protocols (see Case Studies 10.1 and 10.2). Cryopreservation brings a number of practical and economic benefits by reducing the need to perform labour-intensive tasks such as regular monitoring of viability and subculturing of tissue cultures, and reducing the space required to maintain these collections (Reed 2008). However, significant labour, time and expertise are required for the initial steps needed to develop a successful cryopreservation protocol, many of which are species specific.

10.4 What forms of germplasm can be cryopreserved

Protocols for the cryopreservation of a number of plant species and germplasm types have been reported in the literature (Reed 2008; Wolkers and Oldenhof 2021). Almost any form of plant germplasm can be cryopreserved, including:

- Seeds;
- **Zygotic embryos**, embryonic axes, or **protocorms**;
- Dormant vegetative tissue (dormant buds, bulbs);
- Actively growing vegetative tissue (cultured shoot tips, **callus**, cells, somatic embryos, **protocorm-like bodies**, hairy roots);
- Pollen (see also Chapter 13);
- Fern spores (see also Chapter 13);
- **Mycorrhizal** fungi (e.g., for orchids, see also Chapter 12).

It is critical to consider the desired purpose of the collection when deciding which form of germplasm to use. Collections should be made from multiple individuals, with the aim to obtain a genetically diverse collection representing the species (see Chapter 2). It is vital that the collections made are correctly stored and transported to the laboratory to ensure the samples are in their optimum condition (see Chapters 5 and 6 for handling seed collections, Chapter 9 for handling material for tissue culture initiation and Chapter 13 for pollen and spore samples).

Also consider the advantages, limitations and restrictions of each form using the following points as a guide:

- **Orthodox seeds** are the easiest form of germplasm to store and should be used where possible, particularly for species that are extremely threatened and when conservation strategies are urgently required. Use of seed eliminates the need to develop a complex storage protocol, avoids any potential damage caused by extraction of seed parts, and eliminates the need for sterile plant material as the seeds can be sown directly into seed raising mix rather than tissue culture media following retrieval from storage.
- Zygotic embryos and embryonic axes may be the best type of tissue to use for species that produce non-orthodox seeds (see Chapter 6 and Case Study 10.2). A zygotic embryo is the immature plant found within a seed (the result of fertilisation) and consists of the cotyledon(s), plumule (rudimentary shoot), radicle and hypocotyl. If the cotyledons are excised, the remaining parts of the embryo are known as the embryonic axis. Where the whole embryo is too large for cryopreservation (e.g., *Castanospermum australe*), the embryonic axis may be used (Box 10.2, see also Case Study 10.1). In cases where the embryonic axis itself is very large or presents differential survival of the root and shoot tips (e.g., *Cocos* and *Quercus* species), it may be necessary to extract just the plumule for cryostorage.
- Shoot tips are an important form of germplasm to store for critically endangered species as they have the highest potential to produce true-to-type plantlets, i.e., to represent the parental **genotypes** from the **extant population**. This is critical in small **gene** pools where the loss of even a single genotype can genetically compromise that population. Therefore, where species are reduced to very small populations, investigations into the cryopreservation of shoot tips should continue even when seed collections have been established. Cryopreservation of vegetative material may also be used for those species that cannot be stored for long periods of time as seeds, including species that produce non-orthodox seeds, produce few or no seeds, or produce seeds that are deeply dormant and therefore difficult to germinate.

Other types of cultured tissue (including single cell, callus, somatic embryo, and hairy root cultures) can be used for cryopreservation when alternative approaches are not possible. Cryopreservation is a multi-step process (as seen in Figure 10.1), and the specific combination of desiccation, cryoprotection and recovery conditions required to achieve optimal post-cryogenic survival is dependent on the germplasm selected.

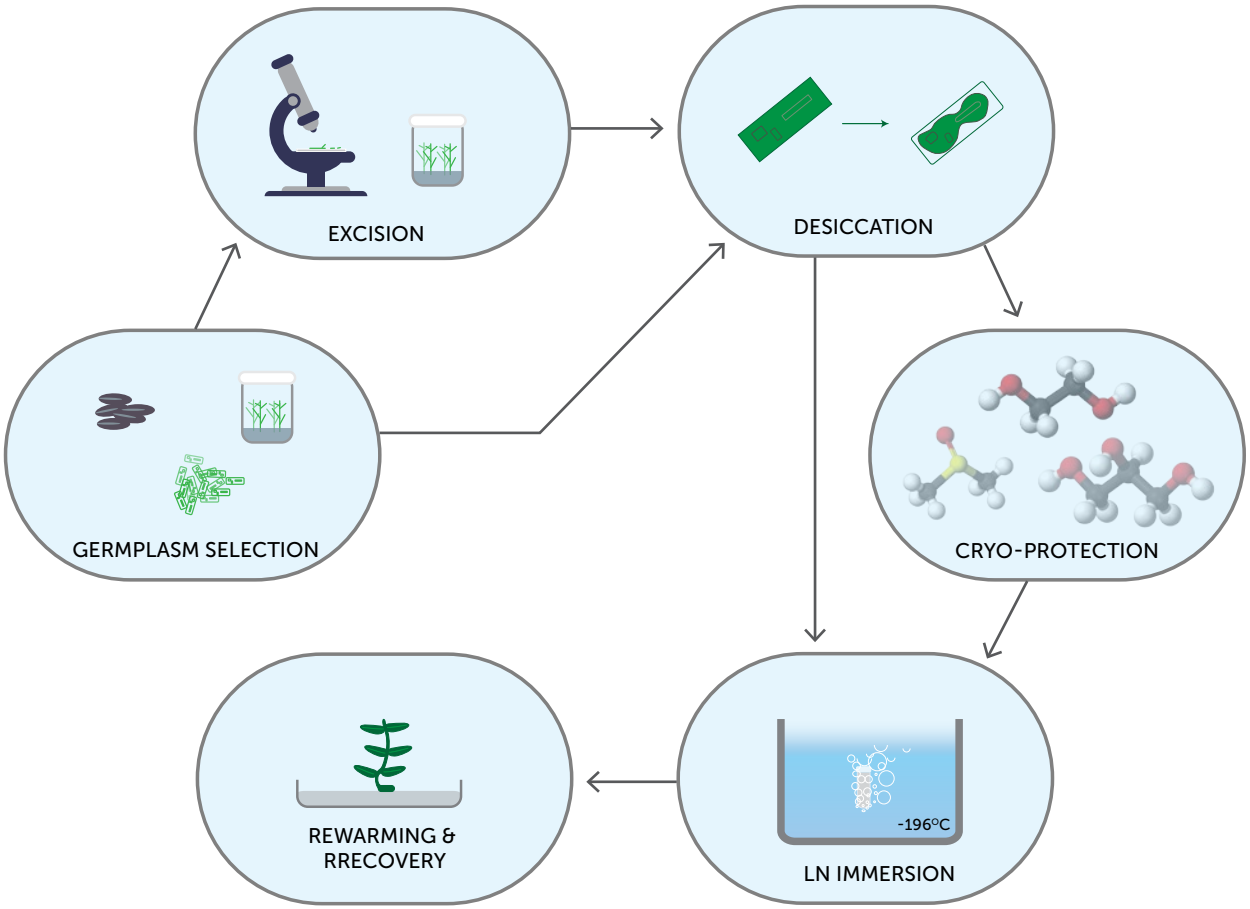

Figure 10.1: Overview of the common steps in the cryopreservation protocol. Each stage of cryopreservation can be altered and optimised to improve and enhance cryogenic success. Steps such as cryo-protection can be skipped if samples can be sufficiently desiccated, while germplasm such as shoot tips and embryonic axes require the additional step of excision from their source material.

Case Study 10.1: Developing a cryopreservation protocol for Bunya Pine (*Araucaria bidwillii* Hook.)

Lyndle K. Hardstaff

The Bunya Pine is an evergreen conifer belonging to the ancient family Araucariaceae. It is an emergent rainforest species found in only two fragmented populations in Queensland, with rapid decline recently noted in one population (Shuey *et al*. 2019). This species exhibits cryptogeal **germination**, in which the true roots and shoots emerge from a **tuber** formed by the swollen hypocotyl (Burrows *et al*. 1992). This may occur as long as two years after the hypocotyl emerges from the seed. Bunya Pine seeds cannot be stored using conventional seed-banking methods due to their high **moisture content**, limited drying tolerance, and rapid germination (Capocchi *et al*. 2011; Burrows *et al*. 2017). Development of a cryostorage protocol is therefore required to safeguard this species in ex situ collections as insurance against further loss **in situ**.

Pre-storage testing

Before cryostorage could be attempted, seeds were first tested to determine the processing, storage and **in vitro** techniques most likely to result in successful post-storage recovery.

- Processing: a number of different methods were trialled to open cones and seeds while preserving embryo quality. To open cones, use of a mattock to prise apart the top scales was most effective (Figure 10.2a). Seeds were best opened using a coping saw and bench-mounted vice (Figure 10.2b).

Figure 10.2a: Bunya Pine cones are opened to extract seeds. (Image: Lyndle Hardstaff)

Figure 10.2b: Bunya Pine seeds are held in a bench-mounted vice and opened with a coping saw. (Image: Lyndle Hardstaff)

- Short-term storage: seeds were stored in paper bags within plastic zip-lock bags (to stop condensation pooling, Figure 10.2c) at 22 °C, 15 °C, and 4 °C and tested for capacity to germinate after 1, 3, and 6 months. Seeds were found to survive at least 6 months when stored at 4 °C, with far fewer lost to precocious germination or fungal contamination than those stored at higher temperatures.

- In vitro growth: trials of whole seed (seed coat removed), whole embryo, partial embryo (cotyledon tube excised), embryonic axis, pseudo-radicle tip, and shoot tips determined that partial embryo was the most effective tissue to use for cryostorage (Figure 10.2d). Partial embryos are capable of producing both roots and shoots in vitro. Establishment of a shoot tip collection has been slow, with further testing still to be done (Figure 10.2e).

Figure 10.2c: Bunya Pine seeds were stored in paper bags within plastic zip-lock bags to stop condensation pooling. (Image: Lyndle Hardstaff)

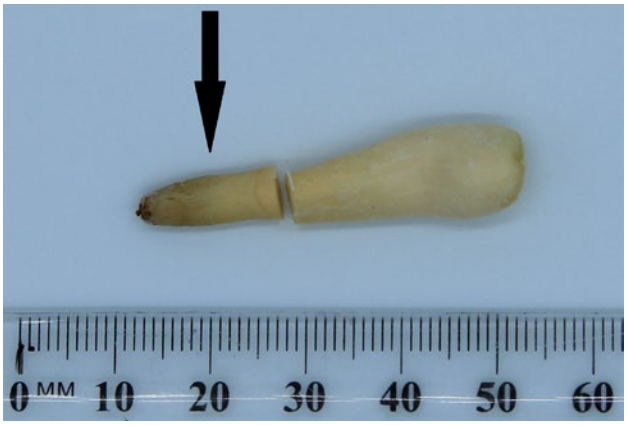

Figure 10.2d: Entire Bunya Pine embryo with arrow pointing to partial embryo. (Image: Lyndle Hardstaff)

Figure 10.2e: Establishment of a Bunya Pine shoot tip collection is possible, but the process is slow. (Image: Lyndle Hardstaff)

- Sterilisation: all embryo tissues were found to be sensitive to both sodium hypochlorite (NaClO) and sodium dichloroisocyanurate (NaDCC solutions). Figure 10.2f shows whole embryos with discolouration of embryo tip after sterilisation. Submersion in 100 mL 0.5 % NaClO solution plus a drop of Tween 20® for 10 minutes was found to be effective in most cases. Higher concentrations, even for shorter periods, resulted in lower survival rates.

Figure 10.2f: Whole Bunya Pine embryos with discolouration of embryo tip after sterilisation. (Image: Lyndle Hardstaff)

Cryostorage experimentation

Results of pre-storage testing suggest that while there are many difficulties to overcome, the Bunya Pine is a good candidate for cryostorage. A **vitrification** protocol has been chosen because the large size of the partial embryos makes droplet vitrification and encapsulation methods impractical. Initial results have been very promising, and optimisation of each step continues.

Embryos demonstrated some tolerance to desiccation, whether on a basal medium containing 0.4 to 1.2 M sucrose for up to four days, in a sterile airflow for between 30 and 240 minutes, or over silica gel for the same time period. Both entire and partial embryos were found to survive exposure of at least 60 minutes in Plant Vitrification Solution 2 (PVS2), a cryoprotective agent. Survival of cryostorage is demonstrated by growth after retrieval from liquid nitrogen.

Figure 10.3: Pseudo-radicle growth and initial tuber formation in partial embryos of *Araucaria bidwillii* 4 weeks after cryostorage. Both were pre-treated on a 0.8 M sucrose basal medium for 48 hours, then 20 minutes incubation in each of loading solution, plant vitrification solution number 2, and washing solution. Only the left embryo was submerged in liquid nitrogen. (Image: Lyndle Hardstaff)

This case study demonstrates the lengthy process and myriad options for optimising cryopreservation techniques for Australian species that cannot be stored by conventional seed banking.

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Case Study 10.2: Investigating cryopreservation options for *Syzygium maire*, a threatened endemic New Zealand tree

Karin van der Walt

Introduction

Syzygium maire is a threatened (Nationally Critical) tree **endemic** to New Zealand where it grows in waterlogged ground or on margins of streams in lower lying areas (de Lange *et al*. 2018) (Figure 10.4). *Syzygium maire* has fleshy red berries which develop over 8–11 months, maturing during early summer in populations at low altitudes (< 200 m.a.s.l) or in autumn at higher altitudes (> 500 m.a.s.l; Figure 10.5).

As *S. maire* seeds are metabolically active and shed at high moisture contents, the only long-term seed storage option is likely through cryopreservation of zygotic embryos (van der Walt *et al*. 2020). Key to cryopreservation is the control of the dehydration process and limitation of injury from chemical toxicity during treatments involving concentrated **cryoprotectant** solutions such as Plant Vitrification Solution (PVS2) (Sakai 1990). In this study we aimed to (a) optimise **embryo culture**, (b) investigate the impact of desiccation on embryo viability, and (c) examine the effect of PVS2 exposure on embryo survival and plantlet development.

Figure 10.4: Swamps dominated by *Syzygium maire* at Fensham Reserve, Wairarapa, North Island. (Image: Karin van der Walt)

Figure 10.5: Mature *Syzygium maire* berries in habitat. (Image: Karin van der Walt)

Materials and methods

Syzygium maire **fruits** were collected over three consecutive summers (2018–20) from Fensham Reserve (Wairarapa region) and Midhirst (Taranaki region). Prior to embryo excision, fruits were surface sterilised by submersion in 50 % Janola® (4 % sodium hypochlorite) for five minutes. The pulp and seed coat were removed **aseptically** to expose the radicle tip enabling the excision of the embryonic axes (hereafter referred to as embryos). Sterilised embryos were cultured on solid Murashige and Skoog (MS) medium supplemented with 3 % w/v sucrose and incubated at 15/25 °C with a 16 hr dark/8 hr light cycle.

To determine the effect of desiccation on embryo survival, excised embryos were desiccated in a **laminar air flow cabinet** for 0, 1, 2, 3, 4, 5 and 6 hours. For each desiccation treatment embryo viability was determined through germination as described above, while moisture content was calculated after drying at 103 °C for 17 hours and expressed on wet weight basis.

Solutions used for the vitrification protocol included loading solution (LS), PVS2 and washing solution (WS) (van der Walt 2021). Sterilised embryos were placed in LS for 20 min then transferred to PVS2 for various incubation times (30, 60 or 90 min). The LS and PVS2 steps were conducted at room temperature and 0 °C. After PVS2 incubation, embryos were soaked in WS for 20 min and transferred to culture medium for viability assessment. All experiments were conducted using 10 embryos and replicated four times.

Results

Untreated embryos (controls) had high moisture content (64.38 $\pm$ 0. 5 %) and 100 % viability. Radicle growth was observed after seven days and the first set of leaves were fully developed within 20 days. Desiccating embryos for up to 2 hr had no significant impact on viability or moisture content. However, embryos desiccated for 3 hr had a significant reduction in moisture content (25.4 $\pm$ 1.3 %) and although viability loss was recorded (86.6 $\pm$ 15.3 %), it was not significant. Low survival was associated with further desiccation (Figure 10.6).

Exposure to PVS2 had a significant effect on embryo survival with lowest survival (63.3 %) associated with 90 min exposure to PVS2. Survival rate was not affected by temperature (0 °C or 20 °C). Plantlet development was also negatively affected by PVS2 exposure, with some embryos showing radicle, but not shoot development following exposure to PVS2 for 60 min or longer (Figure 10.7).

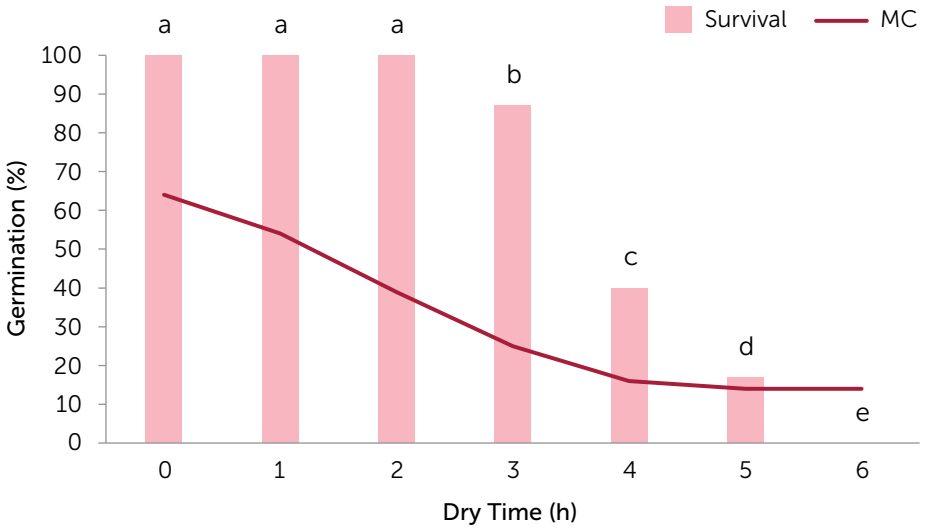

Figure 10.6: Germination (%) of *Syzygium maire* embryos desiccated for 0–6 h. Values followed by the same letter do not differ significantly (Fisher's, $P < 0.05$; $N = 10$)

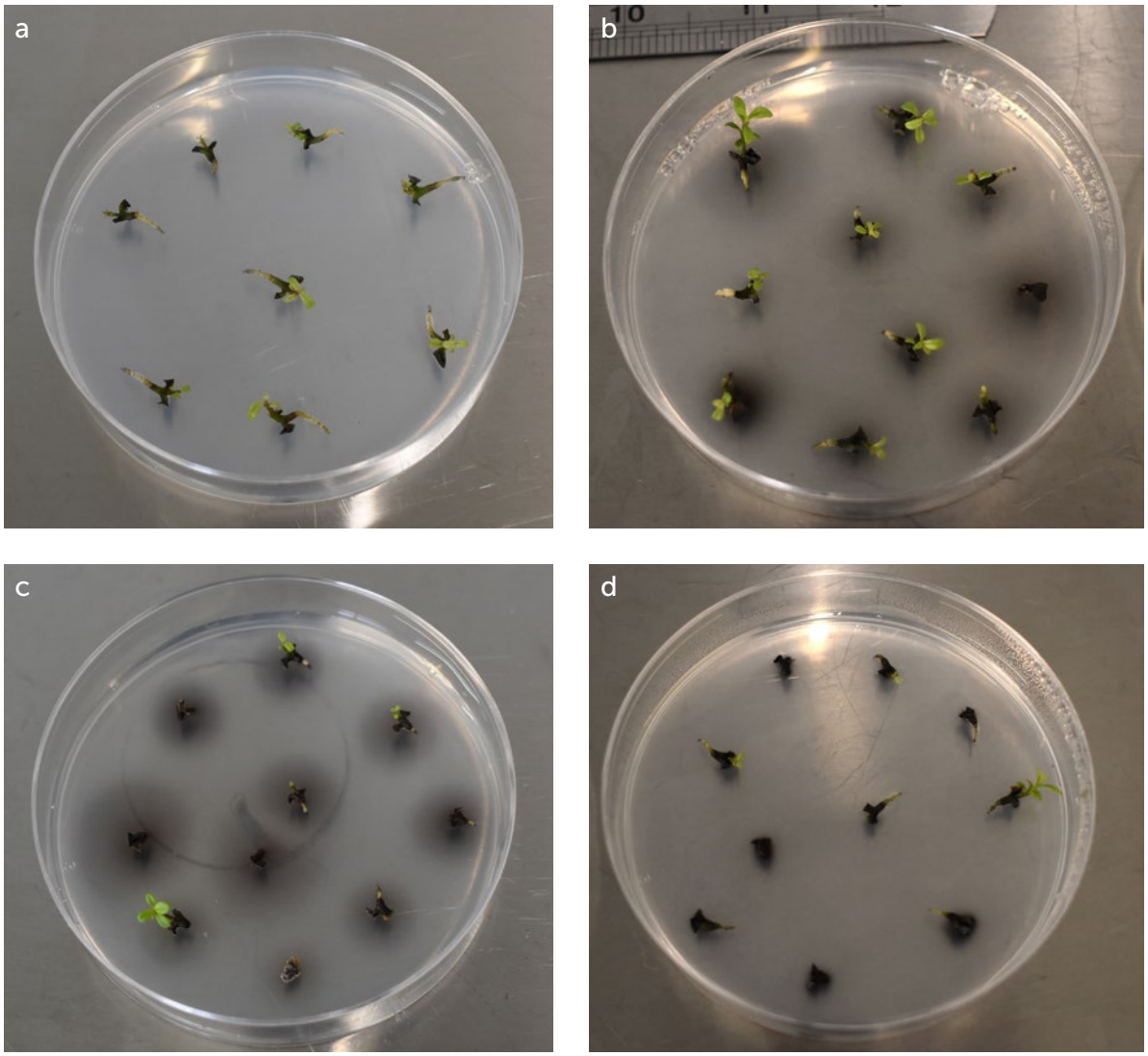

Figure 10.7: *Syzygium maire* embryo survival and plantlet development 10 weeks after exposure to PVS2 at room temperature for **(a)** 0 min, **(b)** 30 min, **(c)** 60 min and **(d)** 90 min.

Conclusion

These results demonstrate that *S. maire* embryos are metabolically active, shed at high moisture contents and although initial viability is high, desiccation is detrimental to survival. Exposure to PVS2 for longer than 30 min negatively impacted embryo viability and plantlet development; therefore, additional steps to optimise embryo survival could include the use of the novel droplet vitrification method which will limit exposure to PVS2 (van der Walt *et al*. 2021).

Acknowledgements

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10.5 The cryopreservation process

10.5.1 Cooling to liquid nitrogen (LN) temperatures

The main aim of cryopreservation is to cool the germplasm sufficiently to slow all biochemical processes and metabolic functions to such an extent that these reactions are in essence halted. To achieve cryopreservation survival requires the successful manipulating of water between its liquid, crystalline solid (i.e., ice) and amorphous solid (i.e., **glass**) physical states. With sufficient dehydration and fast cooling, ice crystals do not have enough time to grow and, through a process called 'vitrification', a 'glassy' (or vitrified) cytoplasm is formed that preserves cellular structure. Alternatively, a slower cooling rate can be applied in which extracellular ice forms, desiccating the cells due to the osmotic gradient, thereby increasing the viscosity of the cytoplasm and promoting vitrification (Kaczmarczyk *et al.* 2012). Optimising the cooling rate can

be a vital step for achieving the best cryopreservation success, while there are large technical and conceptual differences between slow vs fast cooling rate (i.e., external ice formation vs vitrification). In terms of fast cooling rates, different rates of cooling (i.e., 0.3 °C/s up to 80 °C/s) can be achieved using diverse approaches (Xia *et al.* 2014). Cooling rates above 1 °C/s seem to permit good vitrification and lower intracellular ice formation in many species; however, some authors have suggested that cooling rates need to be over 200 °C/s, expanding the hydration window and allowing species with higher water contents to be cryopreserved (Wesley-Smith *et al.* 2001).

10.5.2 Ice, desiccation and cryoprotective agents

Ice formation is the major impediment to achieving cryogenic success. The formation of ice crystals is often lethal as the crystals grow and pierce through organelles and cell membranes. The simplest solution to reduce ice formation is to desiccate the tissues; as the water content within the cells drops, less free water molecules are available to form ice crystals. **Desiccation-tolerant** germplasm, such as seeds, can be dried to such an extent that the cellular contents will naturally vitrify on cooling (Walters and Pence 2020).

However, this is not feasible for many **desiccation sensitive** tissues, including **recalcitrant** seeds (Chapter 6) and tissue cultured material (Chapter 9), as severe tissue damage can occur when the material is over-desiccated (Walters 2015). These types of germplasm require the use of cryoprotective agents (CPAs, also known as cryoprotectants). CPAs are small molecules that interact with water molecules through hydrogen bonding, interfering with ice nucleation, binding up free water molecules, and promoting the vitrification of the cellular contents (Benson 2008). Common CPAs include sugars, sugar alcohols, glycerol, ethylene glycol and dimethyl sulfoxide (DMSO), and these are used in various combinations to make vitrification solutions (Table 10.1).

Table 10.1: The two most common vitrification solutions and their CPA components. Modifications of each solution with different proportions of glycerol, sucrose, and dimethyl sulfoxide (DMSO) have also been used to optimise cryopreservation outcomes (Kim *et al.* 2009).

Solution	Components	Reference
Plant Vitrification Solution 2 (PVS2)	30 % (3.25 M) glycerol 15 % (2.42 M) ethylene glycol 15 % (1.92 M) DMSO 13.5 % (0.4 M) sucrose	Sakai *et al.* 1990
Plant Vitrification Solution 3 (PVS3)	50 % (5.43 M) glycerol 50 % (1.46 M) sucrose	Nishizawa *et al.* 1993

10.5.3 The recovery process

As with tissue culture, cryopreservation involves a series of stresses to plant material that may lead to somaclonal variation under some circumstances. While not a common occurrence, collections need to be managed accordingly. Thus, while not practical for every accession or indeed every species, validation of genetic integrity following cryopreservation (e.g., normal morphology for recovered plants) is essential for the long-term utilisation of cryopreservation. This may be confirmed using a variety of methods including studies of growth and morphology, genetic analysis or biochemical analysis.

Individual genotypes of a species can show differing recovery rates after cryopreservation, and poorly performing genotypes may require additional numbers to be cryopreserved, or additional optimisation of the cryopreservation procedure, to ensure adequate survival (see Section 10.7.2).

10.5.3.1 Viability testing

To monitor the effects of different cryopreservation treatments, regular testing of the plant's ability to regenerate needs to be undertaken at critical stages in any protocol (Figure 10.1). For instance, with tissue cultured material, the ability to generate new shoots is tested:

- After shoot extraction from mother plants;
- After preculture on a desiccating medium;
- After cryoprotection;
- After immersion in LN;
- After the transfer of material to different storage vessels (**dewars**) or institutions;
- After initial storage; and
- After many years (or decades) in storage (see Case Study 10.3).

Case Study 10.3: Longevity in cryopreservation: testing the theory

Valerie C. Pence and Abby Meyer

Although **longevity** in LN is projected to be decades or even centuries, there are fewer reports of extended storage times. This is due, in part, to practical considerations and because of the relatively young age of the field of plant tissue cryopreservation, which means that most reports document survival after short exposure times, e.g., 1 hour to a few days.

However, reports on longevity of cryopreserved material are important in empirically demonstrating the effectiveness of cryopreservation as a tool for long-term storage of plant germplasm (Pence *et al*. 2020). A recent study funded by the Institute of Museum and Library Services utilised the Frozen Garden of the CryoBioBank at the Center for Conservation and Research of Endangered Wildlife (CREW) (Cincinnati Zoo & Botanical Garden, USA), which contains a diverse collection of plant species and tissue types banked in LN over the course of over two decades. The collection includes multi-species examples of seeds, zygotic embryos, pollen, gametophytes, spores and shoot tips. Samples of all of these were removed and evaluated for viability. Overall, good survival was observed from most representatives of all tissue types, including shoot tips of threatened species, such as *Hedeoma todsenii,* native to the southwestern U.S. that is not producing any seeds and is thus, considered exceptional (Pence *et al*. 2017).

While more long-term studies are needed to demonstrate the efficacy of LN storage, long-term studies comparing LN storage with conventional storage are also needed for evaluating the role of cryopreservation in conservation. Some tissues, such as seeds, zygotic embryos, pollen, and spores, are able to survive at the temperature of conventional seed banking (-18 ° to -20 °C), but some species may be relatively short-lived under such conditions. Storing enough replicate samples at -20 °C and in LN for testing after longer storage times (e.g., 5, 10, or 20 years) can provide insight into which method will be the most efficient and effective for a particular species and tissue. Such studies with seeds, zygotic embryos, pollen, and fern spores have shown that in some cases -20 °C can effectively maintain viability for decades (Walters *et al*. 2005; Desheva 2016). In the CREW study, while some species lost viability in less than 20 years at -20 °C,

viability was maintained during that time in LN (Ballesteros and Pence 2017; 2018; 2019; Philpott *et al*. 2015). Such long-term and comparative studies will be critical to developing the most appropriate species-specific protocols for long-term ex situ conservation.

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10.6 Germplasm specific protocols

10.6.1 Orthodox seeds

Seeds in this category (see Chapter 5 Section 5.1.1 for determination) have naturally low moisture content and can be stored in LN without adjustment of moisture or chemical cryoprotection. In the previous version of these guidelines (Offord and Meagher 2009), it was recommended that the moisture content should first be equilibrated to 15 % RH at 15 °C, as it is for standard seed banking. More recent recommendations by the Center for Plant Conservation in the USA and the Royal Botanic Gardens Kew in the UK indicate it is better to equilibrate the seeds at higher RH (e.g., 25–32 % RH at temperatures ranging from 5 to 20 °C) prior to storing in LN (Ballesteros *et al.* 2021; Center for Plant Conservation 2019). The seeds are then placed into a **cryovial** and immersed in LN or nitrogen vapour with no further pre-treatment. Alternatively, larger seeds may be hermetically sealed in a laminated foil packet. Once a sample of seeds has been placed into LN, it is advisable to remove a small sub-sample for viability/germinability testing within a few days to make sure that the temperature changes during LN cooling and warming are not deleterious. When required, seeds should be thawed slowly for approximately 20 minutes at ambient room temperature. Use of a dry box containing silica gel is recommended when thawing seeds in cryovials to prevent the penetration of water into the vial if ice forms on the exterior. Additionally, small 'single use' subsamples can be stored for longer-term viability testing, to avoid freeze/thaw cycles that may damage the samples.

10.6.2 Non-orthodox seeds

This category includes seeds with recalcitrant qualities (i.e., desiccation sensitive) and those that display other storage constraints (e.g., **intermediate seeded species**, see Chapter 6 Section 6.6.4 Box 6.2). Seeds that tolerate desiccation and initial freezing but are short-lived in storage at -20 °C may be cryopreserved in the same manner as orthodox seeds.

Seeds that are desiccation or freezing sensitive require more complex protocols which may include the following:

10.6.2.1 For whole seeds

- Determination of initial seed viability (see Chapter 5 Section 5.5, Chapter 7.3).
- Desiccation, typically using higher RH and temperatures (50–75 % RH and 20 °C), to a pre-determined moisture content (Ballesteros *et al.* 2021). The extent of drying tolerated is first determined by drying the seeds to different moisture contents and re-checking viability. Seeds may be desiccated in a drying room, over silica gel, or over a saturated salt solution at a specific **relative humidity** for a few days to 1–4 weeks. Note: seeds should not be desiccated if embryos are to be extracted.
- Freezing. For some species, direct immersion of seeds in LN may be suitable. However, slow cooling procedures may need to be employed for difficult-to-store species (Xia *et al.* 2014). This is done with a programmable controlled-rate freezer or by placing the seeds in a container designed to slow the rate of freezing in a standard mechanical freezer (Box 10.1). Optimal cooling rates vary between species, but a cooling rate of 0.5–1 °C/min is generally applicable to a broad range of species.

Box 10.1: Controlled-rate cooling containers

Where a programmable freezer is not available, controlled-rate cooling can be achieved using insulated containers. While improvised foam containers can be used to house cryovials and slow the rate of cooling in a mechanical freezer, the exact cooling rate for each vial may be difficult to determine due to variable foam density. Commercial products have been developed to ensure reliable performance and a cooling rate of -1 °C/minute in a -80 °C freezer. These containers typically hold 12–30 cryovials and may utilise either a specialised polyethylene foam with vents to disperse cold air (e.g., Corning® CoolCell® Containers) or may be filled with isopropyl alcohol (e.g., Thermo Scientific™ Mr. Frosty™ Freezing Container).

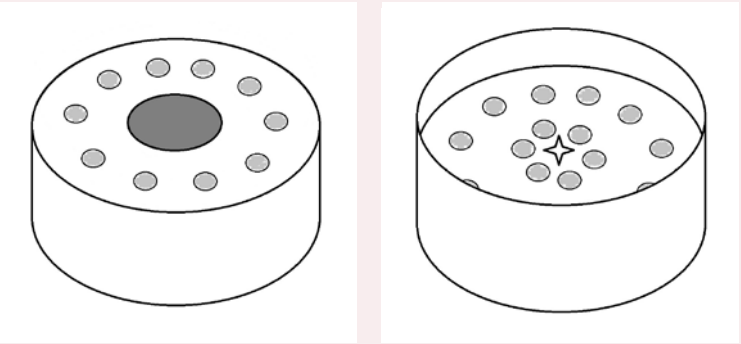

Figure 10.8: Examples of cooling containers. Foam-based containers have a simple push-on lid and either have a hollow core surrounded by holes for the cryovials (left) or use an insert to suspend cryovials over a larger hollow space. Containers using isopropyl alcohol (right) are made of plastic, with a screw-on lid, and contain an insert with holes for the cryovials.

10.6.2.2 For embryos and embryonic axes

This technique is used mainly for desiccation sensitive seeds (e.g., *Syzygium* species) or large seeds with intermediate storage behaviour (e.g., *Macadamia* species). Embryos or embryonic axes (EAs) must be small enough to fit within a cryovial, with smaller material (1–5 mm^3) preferred.

Box 10.2: Embryo variation

Seed and embryo size and morphology vary greatly between species. These variations will affect the ways in which seeds need to be processed and, ultimately, stored. An initial exploration of the seed is recommended to determine the approximate location of the embryonic axis within the embryo and susceptibility to damage (physical or oxidative) during extraction.

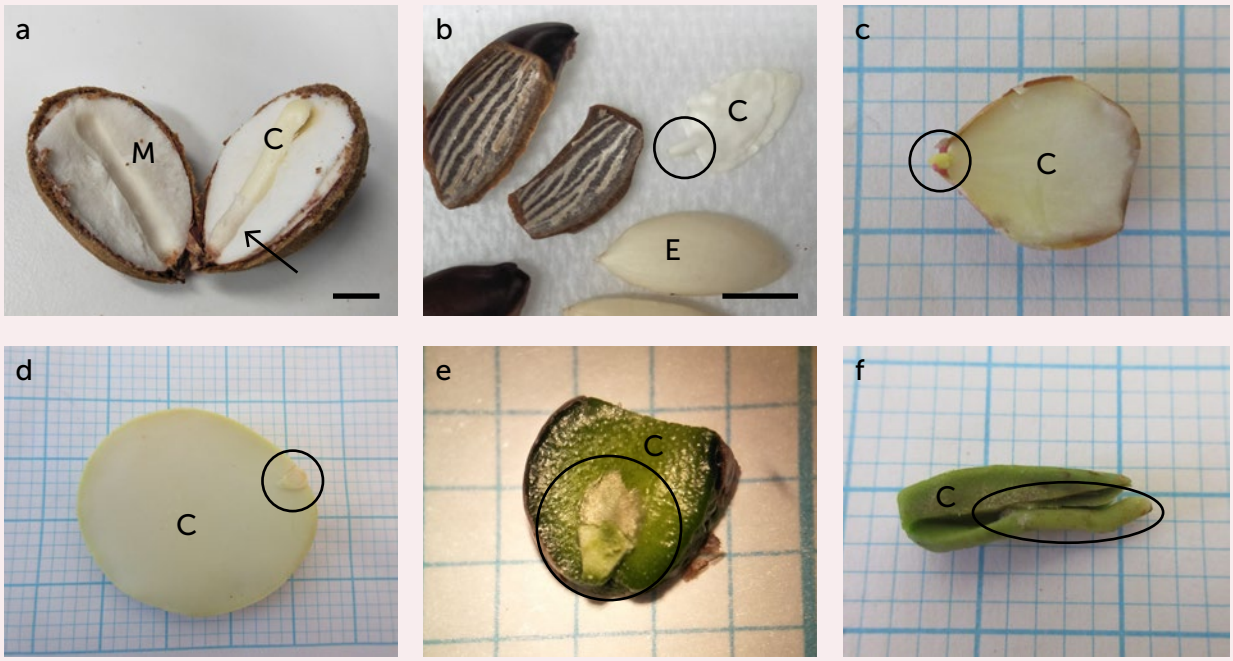

Figure 10.9: Embryonic axes (circled) from a range of Australian rainforest species: **(a)** *Araucaria bidwillii* (Araucariaceae) – arrow indicates approximate location of the embryonic axis within the embryo; **(b)** *Planchonella australis* (Sapotaceae); **(c)** *Davidsonia pruriens* (Cunoniaceae); **(d)** *Castanospermum australe* (Fabaceae); **(e)** *Glycosmis trifoliata* (Rutaceae); **(f)** *Syzygium fullagarii* (Myrtaceae). Scale bar = 10 mm, thin gridlines 2 mm apart. C – cotyledon, E – **endosperm**, M – megagametophyte. (Images: Lyndle Hardstaff)

The cryopreservation of embryos or embryonic axes (EAs, Box 10.2) for such species involves the following key steps:

- Excision of embryos or axes from whole seeds. The excised parts should be tested for viability before proceeding further as the excision process can result in mechanical damage or rapid oxidation. It may be beneficial to excise EAs under a solution of antioxidants (e.g., ascorbic or citric acids) to decrease oxidative damage (Ballesteros *et al.* 2021). EAs may be encapsulated in alginic acid at this point for encapsulation-vitrification or encapsulation-dehydration protocols (Gaidamashvili *et al.* 2021).

- Desiccation. There are a number of reported methods for desiccating embryos and embryonic axes (Normah and Makeen 2008). These include:
  - Exposure to low relative humidity over silica gel or a saturated salt solution;
  - Exposure to a flow of sterile air in a laminar flow cabinet for 0.5–8 hours;
  - Flash drying (Ballesteros *et al.* 2021), which combines airflow and low humidity to achieve rapid desiccation; or
  - Exposure to liquid or solid media containing high concentrations of sucrose, for 12–72 hours, which leads to osmotic desiccation.
- Cryoprotection. This is achieved by placing the embryos or EAs in a solution of cryoprotective agents (CPAs) for 0.5 to 2 hours. Common CPA solutions include PVS2 and PVS3 (Table 10.1). As solutions containing DMSO can be toxic to many embryos and EAs, media containing only glycerol and sucrose (e.g., PVS3) may be more effective. Depending on the cryopreservation protocol chosen, cryoprotected embryos or EAs may be placed directly in cryovials, with or without the cryoprotective solution, or placed in droplets of cryoprotective solution on foil strips. To reduce exposure time to CPAs, a vacuum can be used to increase the speed at which the CPAs infiltrate the embryos in a technique known as Vacuum Infiltration Vitrification (Table 10.2)
- Freezing. This is usually achieved by direct immersion of cryovials or foil strips containing the embryos or EAs in LN.
  - Increased cooling rates can be achieved with the use of LN slush (-210 °C, formed by applying a vacuum to LN), shown to be beneficial for *Quercus* embryos (Xia *et al.* 2014).
  - Alternatively, slow cooling may need to be employed where direct immersion proves to be deleterious to survival.
- Thawing and recovery. Embryos in cryovials are thawed in a 40 °C water bath. The cryoprotective medium is removed, typically with a sucrose-based washing solution (foil strips are placed directly into this washing solution for thawing if used), before embryos are incubated on a recovery medium. The recovery medium is usually species-specific but is generally based on half-strength Murashige and Skoog (1962) minerals or Woody Plant Medium (McCown 1981).

10.6.3 Orchids

10.6.3.1 Seed

The tiny, undifferentiated seeds of many orchid species are regarded as orthodox but are comparatively short-lived in storage. These may be cryopreserved without any pre-treatment (other than drying), though treatment with cryoprotectants may improve longevity (Merritt *et al.* 2014) (see Section 10.6.1 and Chapter 12).

10.6.3.2 Orchid mycorrhizal fungi

Storage of a range of Basidiomycota, which includes orchid mycorrhizal fungi (OMF) have been found to benefit from cryoprotective treatment with 10 % (w/w) glycerol (Nagai *et al.* 2005). However, Australian OMF cultures can be placed directly into cryo-storage without the use of CPAs with no apparent deleterious effect other than a lag phase in initiating **hyphal** growth (Batty *et al.* 2001) using the following steps:

- Sample cubes of fungi and growing medium from clean, growing cultures on potato dextrose agar;

- Place up to five 1 cm fungal cubes into cryovials under aseptic conditions;
- Rapidly cool the cryovials by plunging into LN;
- Thaw fungal cultures slowly at room temperature;
- Retrieve fungal cubes under aseptic conditions, particularly if the vial containing the other replicate cubes is to be replaced in cryostorage;
- Place fungal cubes on a suitable growth medium. While recovery of fungal cultures has been shown to occur on both fungal isolating media (FIM) and Potato Dextrose agar (PDA) media (Batty *et al.* 2001), initial recovery on FIM may be prudent to inhibit potential contamination. Oatmeal agar is also a suitable medium (Sommerville and Offord 2014; Sommerville *et al.* 2008).

10.6.3.3 Protocorms and PLBs (protocorm-like bodies)

A protocorm is the structure produced in the early stage of development of an orchid seedling. The few studies available that investigate the cryopreservation of protocorms use asymbiotically grown material (non-mycorrhizal) (Bustam *et al.* 2016; Watanawikkit *et al.* 2012). Generally, larger protocorms survive cryopreservation best with protocorms at stage four (leaf primordia formed but non-photosynthetic) for the Australian terrestrial species *Caladenia latifolia* showing the highest recovery and survival (Bustam *et al.* 2016; Watanawikkit *et al.* 2012). Further optimisation of the cryopreservation of Australian terrestrial orchid protocorms was achieved by Bustam *et al.* (2016) by using PVS2 in conjunction with a temperature pre-conditioning step, which increased recovery and decreased the time to plantlet formation. Further work is required to understand how transferrable these results are to a wider range of orchid species.

Protocorm-like bodies (PLBs) are differentiated organs initiated from **explants** or callus and are widely used for large-scale orchid in vitro propagation. Protocorm-like bodies differ from protocorms as multiple plants can be proliferated from each PLB. For orchid species where the time to maturation is impractically long or germination is poor, PLBs offer an alternative pathway to mass production and storage of genetic material. Cryopreservation methods for PLBs are typically by direct vitrification, or the encapsulation-dehydration/vitrification methods (see Section 10.6.4 below for further details on these methods, and Chapter 12 Case Study 12.3 for encapsulation discussion). The use of vitrification or encapsulation-dehydration methods has variable success among species and the development of species-specific protocols is likely to be required (Poobathy *et al.* 2013; Popova *et al.* 2010).

10.6.4 Tissue cultured material

Tissue cultured material is the most studied type of plant germplasm for plant cryopreservation, with a wide range of methods developed and optimised over many years (Wang *et al.* 2021). Tissue culture can provide a continuous supply of contamination free shoots for experimental purposes (see Chapter 9) and cryopreservation is the only long-term storage option for this type of material. However, as tissue cultured material is **clonal**, multiple accessions will need to be collected and stored to ensure a genetically diverse collection is conserved.

Cultured shoot tips are the most important form of germplasm to store for critically endangered species as they have the highest potential to produce true-to-type plantlets, i.e., they represent the parental genotypes from the extant population, which is critical in small gene pools where even the loss of only one genotype can genetically compromise that population. See Case Study 10.1. Other **cultured tissues, cells and adventitious propagules** (e.g., callus, **suspension cell cultures**, root cultures, adventitious shoots or somatic embryos; see Chapter 9) can be cryopreserved when alternative approaches are not possible.

Techniques for preserving tissue cultured material include the following:

10.6.4.1 Desiccation

This method involves simple desiccation of plant material prior to immersion in LN. Prevention of cryo-injury is often achieved by the removal of the bulk of the 'free water', and consequently the prevention of lethal ice formation.

- Moisture contents of between 10 to 20 % (fresh weight basis) are often optimal for survival of freezing (Engelmann 2011). Thus, this technique is only viable for **somatic tissue** that will tolerate this level of desiccation (e.g., somatic embryos) and, as such, is species/tissue dependent (Hoekstra *et al.* 2001).
- Samples may be cooled at a controlled rate to induce extracellular ice formation, reducing the possibility of intracellular ice forming (Box 10.1).

10.6.4.2 Encapsulation-dehydration

This method was developed by Fabre and Dereuddre (1990) and involves the encapsulation of tissue in a calcium alginate bead, followed by immersion in a concentrated sucrose solution and desiccation prior to direct immersion in LN. This method has been applied to many species using various tissue types including embryonic axes, somatic embryos and shoot tips (Streczynski *et al.* 2019). Niino *et al*. (2014) developed a new D cryo-plate variation of this method, combining the cryo-plate and encapsulation-dehydration methods to successfully cryopreserve mat rush buds.

10.6.4.3 Vitrification

Vitrification-based methods involving pre-treatment of samples with concentrated cryoprotectant solutions leads to the formation of a highly viscous intracellular solution which, on rapid cooling, forms an amorphous solid (glass) thus avoiding lethal ice crystal formation. The most widely used vitrification solution, for cryopreservation of a range of diverse tissues, is plant vitrification solution 2 (PVS2) developed by Sakai *et al.* (1990) (Table 10.1). However, there are many other vitrification solutions, e.g., PVS1 and PVS3, that have proven to be just as effective for particular species (Turner *et al.* 2001). Over time, the original vitrification methods have undergone various modifications (Table 10.2), each providing their own advantages such as improved cooling and rewarming rates or reduced physical handling of the samples.

The most successful cryopreservation method to date for Australian species is a droplet vitrification method (Panis *et al.* 2005), adapted for Australian species (Funnekotter *et al.* 2017c; Streczynski *et al.* 2019). Droplet vitrification procedures use PVS2 as the vitrification solution and have very fast cooling and re-warming rates.

The general procedure involves (as seen in Figure 10.1):

- Growth of plant cultures for approximately 21 days on standard tissue culture media to promote rapid and healthy growth.
  - Optimal growth conditions vary between species and depend on culture temperature, day-length, light intensity, age of cultures, hormone balances, mineral salts and vitamin combinations and concentrations, and adequate sucrose availability.
- Excision of shoot tips from vigorous tissue cultured plants.
  - These are typically 0.5–2 mm long (species specific) and consist of several leaf primordia and the apical **meristem**.

- Preculture (or pre-treatment) of shoot tips on a tissue culture medium containing 0.4–1.2 M sorbitol, sucrose or glycerol for 24–72 hours under standard growth conditions, with zero to low-light exposure, to begin cell desiccation.
- Exposure of precultured shoot tips to a 'loading solution' of 2 M glycerol, 0.4 M sucrose in half-strength Murashige and Skoog (MS) medium for 20 min at room temperature.
- Incubation of shoot tips in 100 % PVS2 (Table 10.1, 15 % dimethyl sulfoxide (DMSO), 15 % ethylene glycol and 30 % glycerol) for 10–60 min (depending on species tolerance) at either room temperature or 0 °C.
- Transfer of cryoprotected shoot tips to droplets of PVS2 on a strip of aluminium foil.
- Direct immersion of the foil strip containing the shoot tips in LN.
- Thawing of shoot tips at room temperature in a 1.0 M sucrose unloading (or washing) solution.
  - The washing solution removes the concentrated vitrification solution to minimise toxicity.
- Culture of shoot tips on an optimised in vitro recovery medium under low light conditions (Funnekotter *et al.* 2017c).
  - Signs of recovery growth should begin within 7–28 days if the technique has been successful.

Table 10.2: Variations on the vitrification cryopreservation method.

Method	Description	Reference
Vitrification	Samples are placed into a cryovial containing 1 mL of vitrification solution that is plunged into LN.	Sakai *et al.* 1990
Droplet vitrification	After cryo-protection, samples are placed into droplets of vitrification solution on an aluminium foil strip; this foil strip is plunged into LN, achieving rapid cooling and rewarming rates.	Panis *et al.* 2005
Encapsulation-vitrification	A combination of the encapsulation-dehydration and vitrification methods. Samples are encapsulated in alginate beads, and then exposed to a vitrification solution, reducing osmotic stress on the samples. Once cryo-protected, encapsulated samples are placed into cryovials and plunged into LN.	Sakai and Engelmann 2007
Vacuum infiltration vitrification	During the incubation in a vitrification solution, samples are placed under a vacuum to remove trapped air bubbles around the samples, increasing the surface interaction between the sample and vitrification solutions. This reduces the incubation time needed in CPAs to achieve vitrification, reducing their toxic side effects.	Nadarajan and Pritchard 2014
Cryo-plate / Cryo-mesh	A combination of the encapsulation-vitrification and droplet vitrification methods. Samples are encapsulated in a droplet of alginate, anchoring them onto a mesh strip or aluminium plate, and are then cryo-protected and plunged into LN. This method reduces handling of the samples, while maintaining the benefit of rapid cooling and rewarming rates.	Funnekotter *et al.* 2017a; Yamamoto *et al.* 2011

10.6.5 Dormant buds

Cryopreservation of dormant vegetative buds has become a common protocol for the large-scale cryopreservation of diverse clonally propagated woody crop species, such as apple, mulberry and black currant (Pence *et al.* 2020; Tanner *et al.* 2021). The rate of controlled cooling depends on the species, and sometimes requires the additional use of CPAs. Dormant buds are later recovered by direct grafting of the bud on the tree, micrografting onto an in vitro-grown seedling, **micropropagation** of the bud or direct rooting of the cryopreserved branch section (Towill and Ellis 2008).

Cryopreservation of this type of material involves the following steps:

- Dormant buds are usually harvested in the field in mid-winter;
- The buds are partially desiccated (if needed) to 25–41 % moisture content (depending on species);
- The buds are cooled in a two-step phase that involves controlled cooling to -30 or -40 °C (see Box 10.1);
- The pre-cooled buds are stored in liquid nitrogen or in the vapour phase of liquid nitrogen.

For Australian species, cryopreservation of dormant buds has not yet been tested, and the frost tolerance of plants (vital for cryopreservation success) from the Southern Hemisphere is considerably lower than that of plants from the Northern Hemisphere (Bannister 2007). Subalpine and alpine conifers such as *Podocarpus nivalis*, *P. lawrencei* and *Dacrydium bidwillii* resist freezing to -20 to -23 °C (Sakai *et al.* 1981) so could potentially be good candidates for this technique. Still, the number of Australian species that will be sufficiently cold tolerant to be suitable for this method is potentially very low.

10.6.6 Cryopreservation of pollen and pteridophyte spores

Spores from most species of pteridophytes (ferns and fern allies, see Chapter 13), and pollen from a large proportion of seed-bearing plants, tolerate a large degree of desiccation (i.e., down to 5 % of water content on a fresh weight basis) (López-Pozo *et al.* 2018). This physiological trait allows them to be safely dried at relative humidities (RH) between 20 and 70 % and stored in liquid nitrogen without damage associated with ice crystallisation (Ballesteros and Walters 2007). See Chapter 13 for detailed information and protocols on spore and pollen storage.

Specific protocols for the cryogenic storage of dry pollen and pteridophyte spores have recently been published (Nebot *et al.* 2021) and recommend:

- Drying tolerant pollen and pteridophyte spores at about 30 % RH and 20 °C;
- Storage in cryovials in the vapour phase of LN.

These conditions may ensure pollen and spore longevity for over 20 years ex situ (Pence *et al.* 2020), including short-lived chlorophyllous spores such as those found in the King Fern (*Todea barbara* (L.) T. Moore.). However, some species with very short-lived spores (e.g., *Equisetum* sp.) have shown viability decline within 10 years of cryogenic storage (Ballesteros *et al.* 2019), suggesting that the ex situ conservation of other germplasm forms for these species may be required for the long term (e.g., gametophytes or shoot tips).

In addition, pollen from some important and widely distributed species (e.g., *Poaceae*) is desiccation sensitive and does not tolerate the drying needed for cryogenic storage. For these species, a few protocols involving partial drying have been described (Nebot *et al.* 2021).

For cryopreservation of *Luisia macranth* orchid pollinia (Ajeeshkumar and Decruse 2013), for example, a suitable protocol includes either desiccation or the use of CPAs:

- Partial drying of pollinia
  - in a laminar flow for 30 minutes;
  - over silica gel for 120 minutes (a viable alternative for field collections); or
  - through exposure to PVS2 for 10 minutes.
- Transfer of pollinia to a cryo-vial and plunging into LN;
- Long-term cryogenic storage in the vapour phase of LN;
- Fast warming in a water bath set at 40 °C for 30-40 seconds.

10.7 Important factors to consider

10.7.1 Record keeping

As with all germplasm collections, and similar to those of tissue culture collections (see Chapter 9) and seed banking (see Chapters 5 and 6), a key feature for any cryopreservation program is efficient record keeping (see Chapter 15). Cryopreservation is a very long-term conservation option (>25 years), so care must be taken that records are not lost, and electronic records are stored in a format that is readable in the future, avoiding proprietary file formats that may become obsolete. Maintaining both a physical paper record and electronic copy is vital to minimise these risks, as even a quick visual assessment of what is cryobanked in the dewar is not recommended. Visual inspection requires the samples to be lifted out of the dewar into room temperature, where there is a risk of warming to de-vitrification temperatures, leading to potential loss of viability.

To quickly and reliably find and track vials, record the location and information of each cryovial in a central database. All boxes, stacks and vials then need to be uniquely labelled using a permanent technique such as engraving, permanent marker or bar coding, and linked back to the database. As cryopreservation is optimised for each species, detailed records on the cryopreservation method used, and any additional considerations for the recovery phase, are vital for long-term assessments of viability.

The following information at a minimum should be recorded:

- Unique ID on vial;
- Date put into LN;
- Precise location in dewar;
- The person who put the material into LN;
- Name of **propagule**/germplasm type;
- **Taxa** information;
- Genotype/accession/batch number linking to where and how the sample was collected;
- Number of propagules per vial;
- Cryopreservation method;
- Standard growth/culture medium;
- Pre-treatment conditions;
- Cryo-protection conditions;
- Regrowth conditions;
- Control viability.

10.7.2 Minimum sample numbers

A sufficient number of samples needs to be cryopreserved to ensure that, at the very least, one sample per **maternal line** will recover and grow into a plant. Dussert *et al.* (2003) and Volk *et al.* (2017) developed probabilistic tools that assist in determining the minimum number of samples to be cryo-stored to recover at least one plant, taking into account the survival rates of a control experiment, the total number of samples cryopreserved and a confidence interval (Table 10.3). This is valuable information that can be used as a guide to decide between continuing the cryopreservation protocol development to improve viability (for species with very low survival, i.e., <20 %), or using this time to increase the number of samples in the cryobank, or – if there are sufficient viable samples stored – to begin the cryopreservation process for a new species.

Table 10.3: The number of viable cryopreserved samples predicted with a 90 % confidence level, dependent on the total number of propagules stored and their control viability (Volk *et al.* 2017)

	Number of propagules placed into storage									
Viability	10	20	30	40	50	60	70	80	90	100
0.1	0	0	1	2	2	3	4	5	5	6
0.2	0	2	3	5	6	8	10	11	13	15
0.3	1	3	6	8	11	14	16	19	21	24
0.4	2	5	9	12	16	19	23	26	30	34
0.5	3	7	11	16	20	25	30	34	39	44
0.6	4	9	15	20	26	31	37	42	48	54
0.7	5	11	18	24	31	37	44	51	57	64
0.8	6	14	21	29	36	44	52	59	67	75
0.9	8	16	25	34	42	51	60	68	77	86

10.7.3 Correct storage: containers

Germplasm should be stored in polypropylene, polyethylene or similar plastic vials resistant to LN. Some vials can leak when placed in LN and subsequently may explode when warmed rapidly. Vials fitted with o-rings in their lids significantly reduce the risk of LN leaks.

All vials need to be appropriately labelled, including a unique identification number or name that links to a database, the date of storage and the name of the sample. Care must be taken to minimise human error when labelling the vials, as mistakes are not easily rectified once the vials are stored.

10.7.4 Risk management

Before establishing a cryogenic collection, develop a management plan to reduce risks to the collection and eliminate accidental losses (see also Chapter 14). For example, in a pre-arranged exchange program, a small sub-sample of all accessions may be sent to a similar facility either nationally or internationally. This may be done using dry shippers that have the capacity to hold cryogenically stored material for up to 21 days at LN temperature and are safe to transport on aircraft. However, the single biggest risk to cryogenic collections is securing LN supplies and having a reliable LN replenishment schedule (on average once per week). Managers must be

mindful of this and plan refills accordingly, taking into account public holidays, employee sickness and other chance events that may affect LN delivery.

In addition to the risks associated with the maintenance of cryogenic collections, there are also some associated occupational health and safety standards that must be observed when working with LN. These include the provision of adequate ventilation and low-oxygen alarms, especially if LN dewars are located in enclosed areas, and the use of appropriate protection such as gloves, facemasks, lab coats and enclosed shoes to protect from LN burns. Guidelines exist for the safe handling and storage of LN (Standards Australia 1997).

10.8 Cryopreserving Australian plant germplasm – a reflection on progress from humble beginnings to an exciting future

The first documentation of germplasm cryopreservation of threatened native Australian plants was published by Touchell *et al.* (1992) on cryopreservation of *Grevillea scapigera* shoot tips. Further research by Touchell *et al.* (1993; 1994; 1992; 2002) resulted in several milestone discoveries that formed the foundations for future cryopreservation research with Australian plant species. Since the first forays into cryogenic storage in the early 1990's, 145 species from 35 different families of indigenous Australian plant species have been successfully stored and revived via the cryopreservation process (Table 10.4). Cryopreservation has been applied to seeds, seed axes, spores, protocorms, mycorrhizal fungi, callus tissues and shoot tips of various Australian plants (including many threatened species) across a wide range of taxonomic groups and life histories with varying levels of success (Table 10.4). Great improvements were made in the decade 2000–09 to the basic vitrification procedure utilised a decade earlier, with many new species successfully trialled in cryopreservation. However, the introduction of droplet vitrification technology in 2010 paved the way for major improvements in the efficiency of cryopreservation. This has been reflected in both the number of species preserved and the versatility of the droplet vitrification system in allowing important variations to techniques to evolve (e.g., cryo-mesh and vacuum infiltration (VIV) methods (Funnekotter *et al.* 2017a; Funnekotter *et al.* 2015)).

As mentioned in Section 10.2, studies have been performed on a number of plant species that show excellent survival following multi-decadal LN storage (see also Case Study 10.3), suggesting that the claims of longevity in cryostorage do stand up to scrutiny. However, to date this aspect remains to be extensively tested with Australian species and should be factored into future strategies for ensuring the efficacy of long-term cryo-collections. While tissues and seeds of temperate Australian species have generally been successfully cryo-stored, recalcitrant-seeded tropical and sub-tropical Australian species still pose many difficulties and the road ahead remains a challenging one with these taxa (Streczynski *et al.* 2019). Nevertheless, much progress has been made, with the scope of cryopreservation research now much wider than previously envisaged.

Cryopreservation is an invaluable, indeed an essential, tool for plant conservation with unprecedented interest worldwide as plant **biodiversity** decline accelerates. Options for saving threatened species in situ are currently severely impacted by **habitat** loss, introduced diseases and pests, declining rainfall and more frequent fire events – all of which have also impacted heavily on Australia in recent decades. The way forward for the next decade and beyond will be well served by the cryopreservation knowledge built up by a few small but dedicated groups of scientists from around Australia from the early 1990's to the present. The harnessing of powerful new technologies such as metabolomics studies, molecular simulation and membrane

modelling have provided new insights into fundamental processes in cryostorage (Funnekotter *et al.* 2017b; Funnekotter *et al.* 2013; Funnekotter *et al.* 2016; Hughes *et al.* 2013; Hughes and Mancera 2014; Kreck and Mancera 2014; Menon *et al.* 2014; Streczynski *et al.* 2019). In addition, vital new research avenues (e.g., transcriptomics) are also being investigated which hold the key to an exciting future for cryopreservation science in Australia and for a new generation of conservation scientists. Plant cryopreservation science in Australia has come a long way from the first tentative experiments three decades ago and we can look forward to more advancements in both fundamental cryo-science and practical but powerful applications for Australian plant conservation.

Table 10.4: Published reports of successfully cryopreserved Australian species.
Conservation code: T, threatened; 1-4, priority 1-4; LC, least concern (according to WA *Biodiversity Conservation Act* 2016 and NSW *Biodiversity Conservation Act* 2016). Propagules: EA, embryonic axes; SE, somatic embryo; ST, shoot tip. Cryopreservation method (see Table 10.2 and Box 10.1): CC, controlled cooling; D, desiccation; ED, encapsulation dehydration; V, vitrification; dV, droplet vitrification; VIV, vacuum infiltration vitrification. Taxon names have been updated to ensure they are current.

Family	Species	Code	Propagule	Method	Reference
Amarantaceae	*Gomphrena canescens* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Anarthriaceae	*Hopkinsia anoectocolea* (F.Muell.) D.F.Cutler	3	ST	V	Touchell *et al.* 2002
Araucariaceae	*Araucaria bidwillii* Hook.	LC	EA	V	Hardstaff *et al.* 2020
Asparagaceae	*Lomandra sonderi* (F.Muell.) Ewart	LC	ST	dV, VIV	Funnekotter *et al.* 2015; Menon *et al.* 2012
Asparagaceae	*Sowerbaea multicaulis* E.Pritz.	4	ST	V	Touchell *et al.* 2002
Asteraceae	*Lawrencella davenportii* (F.Muell.) Paul G.Wilson	LC	seed	CC, D	Touchell and Dixon 1993
Asteraceae	*Pembertonia latisquamea* (F.Muell.) P.S.Short	LC	seed	CC, D	Touchell and Dixon 1993
Asteraceae	*Rhodanthe chlorocephala* subsp. *rosea* (Hook.) Paul G.Wilson	LC	seed	CC, D	Touchell and Dixon 1993
Asteraceae	*Schoenia cassiniana* (Gaudich.) Steetz	LC	seed	CC, D	Touchell and Dixon 1993
Casuarinaceae	*Allocasuarina fraseriana* (Miq.) L.A.S.Johnson	LC	seed	CC, D	Touchell and Dixon 1993
Chenopodiaceae	*Atriplex nummularia* Lindl.	LC	seed	D	Touchell and Dixon 1993
Colchicaceae	*Burchardia congesta* Lindl.	LC	seed	CC, D	Touchell and Dixon 1993
Crassulaceae	*Crassula colorata* (Nees) Ostenf.	LC	seed	CC, D	Touchell and Dixon 1993
Crassulaceae	*Crassula natans* Thunb.	LC	seed	D	Tuckett *et al.* 2010
Cunoniaceae	*Ceratopetalum gummiferum* Sm.	LC	ST	ED	Shatnawi *et al.* 2004

Family	Species	Code	Propagule	Method	Reference
Cunoniaceae	*Davidsonia pruriens* F.Muell	LC	seed	D	Ashmore *et al.* 2011
Cyatheaceae	*Alsophila australis* R.Br.	LC	spore	D	Mikuła *et al.* 2009
Cyperaceae	*Mesomelaena tetragona* (R.Br.) Benth.	LC	seed	D	Merritt *et al.* 2003
Elaeocarpaceae	*Tetratheca deltoidea* Joy Thomps.	T	ST	V	Touchell *et al.* 2002
Ericaceae	*Lysinema ciliatum* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Ericaceae	*Styphelia obtecta* (Benth.) F.Muell.	T	ST	V	Touchell *et al.* 2002
Fabaceae	*Acacia acuminata* Benth.	LC	seed	CC	Touchell and Dixon 1993
Fabaceae	*Acacia bivenosa* DC.	LC	seed	D	Merritt *et al.* 2003
Fabaceae	*Bossiaea ornata* (Lindl.) Benth.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Chorizema dicksonii* Graham	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Daviesia cordata* Sm.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Gastrolobium bilobum* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Gastrolobium leakeanum* J.Drumm.	2	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Gompholobium marginatum* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Gompholobium scabrum* Sm.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Hardenbergia comptoniana* (Andrews) Benth.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Hovea elliptica* (Sm.) DC.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Indigofera australis* Willd.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Isotropis atropurpurea* F.Muell.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Jacksonia floribunda* Endl.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Kennedia stirlingii* Lindl.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Labichea teretifolia* C.A.Gardner	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Leptosema aphyllum* (Hook.) Crisp	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Mirbelia dilatata* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Petalostylis cassioides* (F.Muell.) Symon	LC	seed	CC, D	Touchell and Dixon 1993

Family	Species	Code	Propagule	Method	Reference
Fabaceae	*Ptychosema pusillum* Benth.	T	ST	V	Touchell *et al.* 2002
Fabaceae	*Senna venusta* (F.Muell.) Randell	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Sphaerolobium fornicatum* Benth.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Swainsona canescens* (Lindl.) F.Muell.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Swainsona formosa* (G.Don) Joy Thomps.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Templetonia retusa* (Vent.) R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Urodon capitatus* Turcz.	3	seed	CC, D	Touchell and Dixon 1993
Fabaceae	*Viminaria juncea* (Schrad. & J.C.Wendl.) Hoffmanns.	LC	seed	CC, D	Touchell and Dixon 1993
Goodeniaceae	*Goodenia berardiana* (Gaudich.) Carolin	LC	seed	CC, D	Touchell and Dixon 1993
Goodeniaceae	*Lechenaultia formosa* R.Br.	LC	ST	V	Touchell *et al.* 2002
Goodeniaceae	*Lechenaultia laricina* Lindl.	T	ST	V	Touchell *et al.* 2002
Haemodoraceae	*Anigozanthos humilis* subsp. *chrysanthus* Hopper	4	ST	V	Turner *et al.* 2001
Haemodoraceae	*Anigozanthos kalbarriensis* Hopper	LC	ST	V	Turner *et al.* 2001
Haemodoraceae	*Anigozanthos manglesii* D.Don	LC	seed, ST	D, V	Merritt *et al.* 2005; Touchell and Dixon 1993; Turner *et al.* 2001
Haemodoraceae	*Anigozanthos pulcherrimus* Hook.	LC	ST	V	Touchell *et al.* 2002
Haemodoraceae	*Anigozanthos rufus* Labill.	LC	ST	V	Touchell *et al.* 2002
Haemodoraceae	*Anigozanthos viridis* subsp. *terraspectans* Hopper	T	ST	V	Turner *et al.* 2001
Haemodoraceae	*Anigozanthos viridis* Endl. subsp. *viridis*	LC	ST	cryo-mesh, dV, V, VIV	Funnekotter *et al.* 2017a; Funnekotter *et al.* 2017b; Funnekotter *et al.* 2015; Turner *et al.* 2000a
Haemodoraceae	*Conostylis dielsii* subsp. *teres* Hopper	T	ST	V	Turner *et al.* 2001
Haemodoraceae	*Conostylis micrantha* Hopper	T	ST	V	Turner *et al.* 2001

Family	Species	Code	Propagule	Method	Reference
Haemodoraceae	*Conostylis wonganensis* Hopper	T	ST	V	Turner *et al.* 2000a
Haemodoraceae	*Macropidia fuliginosa* (Hook.) Druce	LC	callus, ST, SE	V	Turner *et al.* 2000a; Turner *et al.* 2000b
Haloragaceae	*Myriophyllum balladoniense* Orchard	4	seed	D	Tuckett *et al.* 2010
Haloragaceae	*Myriophyllum petraeum* Orchard	4	seed	D	Tuckett *et al.* 2010
Hydatellaceae	*Trithuria austinensis* D.D.Sokoloff, Remizowa, T.Macfarlane & Rudall	LC	seed	D	Tuckett *et al.* 2010
Hydatellaceae	*Trithuria submersa* Hook.f.	LC	seed	D	Tuckett *et al.* 2010
Lamiaceae	*Coleus scutellarioides* (L.) Benth.	LC	seed	CC, D	Touchell and Dixon 1993
Lamiaceae	*Hemiandra gardneri* O.H.Sarg.	T	ST	V	Touchell *et al.* 2002
Lamiaceae	*Pityrodia scabra* A.S.George	LC	ST	V	Touchell *et al.* 2002
Malvaceae	*Androcalva perlaria* C.F.Wilkins	T	ST	dV, VIV	Funnekotter *et al.* 2015; Whiteley *et al.* 2016
Malvaceae	*Commersonia erythrogyna* C.F.Wilkins	T	ST	V	Touchell *et al.* 2002
Malvaceae	*Guichenotia ledifolia* Gay	LC	seed	CC	Touchell and Dixon 1993
Malvaceae	*Lasiopetalum glutinosum* (Lindl.) F.Muell.	LC	seed	D	Touchell and Dixon 1993
Malvaceae	*Seringia hermanniifolia* (J.Gay) F.Muell.	LC	seed	CC, D	Touchell and Dixon 1993
Menyanthaceae	*Liparophyllum capitatum* (Lehm.) Tippery & Les	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Agonis flexuosa* (Willd.) Sweet	LC	seed	D	Touchell and Dixon 1993
Myrtaceae	*Backhousia citriodora* F.Muell.	LC	seed	V	Hardstaff *et al.* 2020
Myrtaceae	*Beaufortia incana* (Benth.) A.S.George	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Callistemon phoeniceus* Lindl.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Calothamnus tuberosus* Hawkeswood	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Eremaea beaufortioides* Benth.	LC	seed	D	Touchell and Dixon 1993
Myrtaceae	*Eucalyptus* ×*graniticola* Hopper	4	ST	V	Touchell *et al.* 2002

Family	Species	Code	Propagule	Method	Reference
Myrtaceae	*Eucalyptus burracoppinensis* Maiden & Blakely	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Eucalyptus lane-poolei* Maiden	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Eucalyptus loxophleba* Benth.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Hypocalymma angustifolium* (Endl.) Schauer	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Kunzea baxteri* (Klotzsch) Schauer	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Leptospermum spinescens* Endl.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Melaleuca cuticularis* Labill.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Melaleuca huegelii* Endl.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Melaleuca radula* Lindl.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Melaleuca uncinata* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Myrtaceae	*Syzygium francisii* (F.M.Bailey) L.A.S.Johnson	LC	ST	ED	Shatnawi *et al.* 2004
Nymphaeaceae	*Nymphaea immutabilis* S.W.L.Jacobs	2	seed	D	Dalziell *et al.* 2019
Nymphaeaceae	*Nymphaea lukei* S.W.L.Jacobs & Hellq.	LC	seed	D	Dalziell *et al.* 2019
Nymphaeaceae	*Nymphaea macrosperma* Merr. & L.M.Perry	LC	seed	D	Dalziell *et al.* 2019
Nymphaeaceae	*Nymphaea violacea* Lehm.	LC	seed	D	Dalziell *et al.* 2019
Orchidaceae	*Caladenia arenicola* Hopper & A.P.Br.	LC	seed	D	Batty *et al.* 2001
Orchidaceae	*Caladenia flava* R.Br.	LC	seed	D	Hay *et al.* 2010
Orchidaceae	*Caladenia huegelii* Rchb.f.	T	protocorm, seed	dV, D	Bustam *et al.* 2017; Hay *et al.* 2010
Orchidaceae	*Caladenia latifolia* R.Br.	LC	protocorm	V, dV	Bustam *et al.* 2016; Watanawikkit *et al.* 2012
Orchidaceae	*Diuris arenaria* D.L.Jones	T	seed+fungi	ED	Sommerville *et al.* 2008
Orchidaceae	*Diuris flavescens* D.L.Jones	T	seed+fungi	ED	Sommerville and Offord 2014
Orchidaceae	*Diuris fragrantissima* D.L.Jones & M.A.Clem.	T	seed	D	Hay *et al.* 2010
Orchidaceae	*Diuris laxiflora* Lindl.	LC	seed	D	Hay *et al.* 2010

Family	Species	Code	Propagule	Method	Reference
Orchidaceae	*Diuris magnifica* D.L.Jones	LC	seed	D	Batty *et al.* 2001
Orchidaceae	*Diuris tricolor* Fitzg.	LC	seed+fungi	ED	Sommerville and Offord 2014
Orchidaceae	*Microtis media* R.Br.	LC	seed	D	Hay *et al.* 2010
Orchidaceae	*Pterostylis recurva* Benth.	LC	seed	D	Hay *et al.* 2010
Orchidaceae	*Pterostylis sanguinea* D.L.Jones & M.A.Clem.	LC	seed	D	Batty *et al.* 2001
Orchidaceae	*Pterostylis saxicola* D.L.Jones & M.A.Clem.	T	seed+fungi	ED	Sommerville *et al.* 2008
Orchidaceae	*Thelymitra crinita* Lindl.	LC	seed	D	Batty *et al.* 2001
Orchidaceae	*Thelymitra macrophylla* Lindl.	LC	seed	D	Hay *et al.* 2010
Phrymaceae	*Glossostigma drummondii* Benth.	LC	seed	D	Tuckett *et al.* 2010
Proteaceae	*Banksia ashbyi* Baker f.	LC	seed	D	Merritt *et al.* 2003
Proteaceae	*Banksia cuneata* A.S.George	T	seed	CC, D	Touchell and Dixon 1993
Proteaceae	*Banksia occidentalis* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Proteaceae	*Conospermum stoechadis* Endl.	LC	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea cirsiifolia* Meisn.	LC	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea dryandroides* C.A.Gardner subsp. *dryandroides*	T	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea dryandroides* subsp. *hirsuta* Olde & Marriott	T	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea flexuosa* (Lindl.) Meisn.	T	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea maccutcheonii* Keighery & Cranfield	T	ST	V	Touchell *et al.* 2002
Proteaceae	*Grevillea scapigera* A.S.George	T	ST	dV, V	Funnekotter *et al.* 2013; Touchell *et al.* 1992
Proteaceae	*Hakea aculeata* A.S.George	T	ST	V	Touchell *et al.* 2002
Proteaceae	*Hakea costata* Meisn.	LC	seed	CC, D	Touchell and Dixon 1993
Proteaceae	*Isopogon cuneatus* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Proteaceae	*Lambertia orbifolia* C.A.Gardner	LC	ST	V	Touchell *et al.* 2002
Proteaceae	*Macadamia integrifolia* Maiden & Betche	T	EA	V	Hardstaff *et al.* 2020

Family	Species	Code	Propagule	Method	Reference
Ranunculaceae	*Clematis pubescens* Endl.	LC	seed	D	Touchell and Dixon 1993
Restionaceae	*Loxocarya cinerea* R.Br.	LC	callus, ST	dV, VIV	Funnekotter *et al.* 2015; Kaczmarczyk *et al.* 2013
Restionaceae	*Loxocarya gigas* B.G.Briggs & L.A.S.Johnson	2	embryo	D	Touchell and Dixon 1994
Rutaceae	*Citrus australasica* F.Muell.	LC	seed	D	Hamilton *et al.* 2009
Rutaceae	*Citrus garrawayi* F.M.Bailey	LC	seed	D	Hamilton *et al.* 2009
Rutaceae	*Citrus inodora* F.M.Bailey	LC	seed	D	Hamilton *et al.* 2009
Rutaceae	*Philotheca wonganensis* (Paul G.Wilson) Paul G.Wilson	T	ST	V	Touchell *et al.* 2002
Sapindaceae	*Diplopeltis huegelii* Endl.	LC	seed	CC	Touchell and Dixon 1993
Sapindaceae	*Dodonaea hackettiana* W.Fitzg.	4	seed	CC	Touchell and Dixon 1993
Scrophulariaceae	*Eremophila caerulea* (S.Moore) Diels	LC	ST	V	Touchell *et al.* 2002
Scrophulariaceae	*Eremophila resinosa* (Endl.) F.Muell.	T	ST	V	Bunn *et al.* 2007; Touchell *et al.* 2002
Stylidiaceae	*Stylidium adnatum* R.Br.	LC	seed	CC, D	Touchell and Dixon 1993
Stylidiaceae	*Stylidium coroniforme* F.L.Erickson & J.H.Willis	LC	ST	V	Bunn *et al.* 2007
Stylidiaceae	*Stylidium expeditionis* Carlquist	4	ST	V	Bunn *et al.* 2007
Surianaceae	*Stylobasium australe* (Hook.) Prance	LC	seed	CC, D	Touchell and Dixon 1993
Zygophyllaceae	*Roepera aurantiaca* Lindl.	LC	seed	CC, D	Touchell and Dixon 1993

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Chapter 11
Living plant collections

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11.1 Introduction

Living plant collections are one ex situ management strategy for plant species that require **conservation** action. **Seed** and/or vegetative material from these species may be readily available for use in **restoration**, **translocation**, research, education, horticultural development and display. Living plant collections can take various forms such as potted collections, botanic gardens, arboreta, plantations, field genebanks and seed production areas (Nevill *et al*. 2016). Living plant collections are increasingly used to bridge the gap between **ex situ conservation**, and recovery **in situ** (Volis and Blecher 2010). They are especially useful when continuing access to in situ **populations** places a species under additional pressure.

Living plant collections are like zoo animal collections in that they can support the management of threatened species and contribute to **habitat** conservation, as well as highlighting the plight of threatened species when used in public displays, education and extension programs (Price *et al*. 2004). Most importantly, living collections provide the opportunity for research into the conservation and management issues facing wild populations. They are also useful for evaluation of economic potential and provide material for various scientific investigations.

The conservation value of a living plant collection is largely determined by the extent of genetic representativeness of the species when compared to remaining wild populations. Whether conserving the species as a whole, or a particular population(s), establishing a living plant collection that is genetically representative of the target group requires considerably more time, effort and space than some other forms of ex situ conservation, such as seed banking (see Chapter 3). Ideally living collections should be used with one or more other conservation technique(s) such as ex situ seed storage and careful management of remaining in situ populations. There are circumstances however, particularly with Critically Endangered species where few individuals remain, or species for which seed storage is not feasible, where it may be the only practical conservation option available (see Chapter 6 and Case Study 11.1).

A species conservation strategy might include cultivation of living plants for the following reasons:

- to conserve species with **desiccation sensitive** seeds that cannot be maintained in a conventional seed bank (Chapter 6);
- to conserve species that produce non-**viable**, few or no seeds, or seeds that are difficult to germinate (Chapters 5 and 7);

- to ensure clonal replicates are maintained, where unique or elite **genotypes** need conservation;
- to conserve species that are threatened but are easily cultivated;
- to make material available for reference, education and display purposes;
- to regenerate some seed collections to determine correct identification and taxonomic integrity;
- to produce material (i.e., seeds or cuttings) to aid recovery planning for **ecological restoration** and translocation (see also Chapter 8);
- to generate breeding and/or planting material for species that have a long life cycle;
- to bulk up **germplasm** for storage in various other forms of ex situ conservation e.g., to produce starting material for **tissue culture**, or seeds for banking;
- to produce material for conservation biology research such as genetic studies and taxonomy;
- to supply material for various purposes to remove or reduce pressure on wild populations e.g., for commercialisation of whole plants or plant parts such as seeds or cut flowers;
- to provide parent material for the establishment of field genebanks;
- to initiate an ex situ collection for species where there is little known about their suitability for seed banking.

(adapted from Bioversity International[54]).

Case Study 11.1: The lonely King's Lomatia

Wendy Potts

Lomatia tasmanica (King's Lomatia, Proteaceae) is a Critically Endangered Tasmanian **endemic** (Threatened Species Section 2006, 2020). This species is thought to be a sterile **hybrid** which propagates by suckering and may be one of the oldest known living flowering plants in the world (Lynch *et al*. 1998).

The sole **extant** population is at risk from the root rot pathogen, *Phytophthora cinnamomi* (Phytophthora Dieback), as well as wildfire. A second recorded population is believed to have been lost due to the impact of fire. The species is difficult to maintain in cultivation for more than a few years. Nevertheless, plants are held at the Royal Tasmanian Botanical Gardens and Australian National Botanic Garden.

While the cost of maintaining sterile hybrids in long term storage may not be justified for many hybrid taxa, the likely **extinction** of at least one of the parent species means that this **taxon** cannot be recreated. This, as well as its intrinsic scientific interest, warrants the long-term storage of tissue for *Lomatia tasmanica*, perhaps through tissue culture or cryostorage. The lack of **genetic diversity** between individuals simplifies storage in this case.

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11.2 Benefits and risks

A major benefit of cultivated collections is that living plants of key genotypes can be secured with ready access. Material for further propagation and cultivation can be harvested from these plants, thus reducing the need to collect from wild populations. This is especially important for rare species or those from remote locations (see Case Study 11.2 Care for the Rare). Special or rare genotypes can be conserved and distributed to protect against the likely loss of the species in situ. The horticultural knowledge gained by maintaining threatened species in living collections can help plan successful translocation attempts, e.g., microsite selection, or need for augmented watering. Living collections therefore provide both a source of **propagules** and source of knowledge to support in situ conservation (Figure 1.4).

Another major advantage of living collections is that species that cannot be maintained in seed storage can be sustainably cultivated, e.g., rainforest species with desiccation sensitive seeds can be germinated and grown-on, in turn providing a source of seed or vegetative material (see Chapter 6). Additionally, living collections provide an alternative for supplying non-seed material for restoration and research purposes for those species with as yet undetermined **germination** methods.

Living collections are available for conservation or biological research (see Case Study 11.4 on *Rhodomyrtus* and Case Study 11.5 on orchids) and plant breeders may use these collections as sources of desirable genotypes if collection permits allow for this activity. Germplasm conservation can also be integrated with public or other horticultural collections, providing opportunities for public appreciation (see Case Study 11.3 Conservation display beds at Royal Botanic Gardens Victoria).

Living plant collections allow successive harvesting of plant material for propagation without impacting the original populations as well as reducing collection costs if the species is remote and hard to access. For longer lived species such as trees, collecting can occur for many years, providing a reliable ongoing source of propagation material (see Case Study 3.3 on *Eucalyptus benthamii*). In comparison, shorter-lived herbaceous species will require renewal every few seasons, but it has been demonstrated that growing plants under cultivated conditions can appreciably improve the amount of seed harvested for some species when compared with wild collection (see Case Study 11.6 on seed production areas and Case Study 11.7 on conservation seed orchards).

Despite the many benefits of living collections, these are not without limitations and risks. Major limitations include the costs and resources required in providing facilities for specialist propagation, establishing a physical space for the growth and maintenance of plants and facilities, and a sound understanding of the horticultural requirements necessary to keep the plants healthy and free of pests or diseases (see Section 11.8, including Case Study 11.8 Myrtle Rust management at Auckland BG). Adding to the cost is the consideration of the growing area and time to maturity required to establish a collection that meets the aims of the conservation plan, including capture of the desired genetic diversity. Consequently, prior to establishing a living collection,

it is important to carefully consider the reasons for the collection, how it will be produced and maintained (short-term as well as long-term), and the end-use of the material. Increasingly, genetic research enables practitioners to curate a living collection with appropriate representation of genetic diversity, to make the most of the available resources and better support conservation actions (Maschinski and Pence 2019; Bragg *et al.* 2021). A guide to the most important issues to be considered is found in Box 11.1.

Box 11.1: Checklist for establishment and maintenance of living collections

- Have you followed the decision flowchart for ex situ conservation options in Box 2.1?
- What are the end-uses for the collection?
- Is living plant conservation appropriate for this species?
- What are the benefits and risks?
- What is the sampling strategy?
- What representation of genetic variation is required?
- How many plants are required?
- What material will be used to establish the collection?
- How will the material be propagated?
- How will the plants be grown?
- How will the plants be maintained for the required time?
- Have you followed Box 4.1 Pre-collection checklist?
- Can the clonality (and therefore genetic diversity) be managed and tracked effectively and reliably?

Key questions relevant to seed production areas, including species biology, collection design and sourcing, are also worth considering for other types of living collections (Nevill *et al*. 2016 Figure 2 p. 7491).

Special attention is required to ensure maintenance of genetic integrity and continuity of curation if living collections are to be maintained over long periods, as some plants may live well over a century. Over time, genetic erosion of collections is likely in living plant collections, due to selection pressures imposed by the growing conditions which may favour some genotypes over others, or the selection of genotypes with relatively less dormant seeds that germinate more readily (e.g., native daisies). The result is that the collection may not reflect the genetic background of the original material or natural population, a situation which may be exacerbated over several generations under horticultural conditions (see Chapter 3 section 3.4.2).

In designing the layout of a living collection from which seeds might be collected, the possibility of unwanted hybridisation needs to be considered (see Chapter 3 section 3.3.4.4). Plantings near closely related taxa could lead to hybridisation, particularly if the species is wind pollinated or pollinated by generalists such as honeybees. For species requiring **cross-fertilisation**, natural

pollinators may be limiting, and these types of species may require artificial **pollination** strategies to be developed and employed, requiring an understanding of the reproductive biology of the species (Cochrane and Barrett 2009; see also Case Study 11.7 seed orchards at Royal Tasmanian BG).

Case Study 11.2: Care for the Rare: supporting metacollection development for rare and threatened species in regional botanic gardens

John Arnott

Care for the Rare is a Royal Botanic Gardens Victoria (RBGV) and Botanic Gardens of Australia and New Zealand (BGANZ) initiative to support regional botanic gardens in Victoria to actively participate in ex situ plant conservation and display.

The program has three main objectives.

1. A sector capacity-building project: While Victoria has a large network of botanic gardens (42), a 2018 survey of Victorian regional botanic gardens identified that many gardens perceived they had a 'lack of skills and resources' necessary to manage rare and threatened species in their collections. Gardens staff and volunteers specifically cited their major impediments as 'difficulties in accessing plant material and information about their cultural requirements'.
2. Establish a multi-site conservation collection of Victorian rare and threatened species across a range of regional botanic gardens throughout the State.
3. Communicate, through well considered interpretation, the importance of plant conservation, the role that botanic gardens play and local conservation stories/messages.

Through the generous support of the Helen Macpherson Smith Trust (HMSTrust), the RBGV has progressed the *Care for the Rare* project to build capacity for regional botanic gardens to establish well-considered and relevant conservation collections (Figure 11.1a and 11.1b). An invitation to lodge an expression of interest was circulated through the BGANZ Victoria network with an overwhelming positive response from 24 gardens.

The HMSTrust grant funding is supporting the RBGV to undertake a pilot program for an initial six gardens:

- Australian Botanic Gardens Shepparton (Greater Shepparton Shire) (Figure 11.1c and 11.1d)
- Ballarat Botanical Gardens (City of Ballarat)
- Colac Botanical Gardens (Colac Otway Shire)
- Dandenong Ranges Botanic Gardens (Parks Victoria)
- Sale Botanic Gardens (Wellington Shire)
- Wilson Botanic Park (City of Casey)

Care for the Rare working group members undertook a series of site inspections of each garden to further explore specific factors to guide the formation of collections. This led to the development of Conservation Collection Plans, which articulate the broad approach, aims and objectives for the development of a conservation collection for each garden, including a detailed species list appropriate to each garden and a planting schedule.

The HMSTrust funding has enabled the RBGV to employ a dedicated plant propagator to source, propagate and produce the living plant stocks identified in each Conservation Collection Plan, and for these plants to be delivered to each participating garden. Plant production is undertaken at the Cranbourne Gardens Nursery, with over 2,500 plants of 190 taxa produced, dispatched and planted so far, and additional 'hard to source' taxa yet to be produced.

Each garden has absorbed the costs of garden bed developments, with funding supporting technical elements including advice around plant set out, elements of garden design and ongoing collections maintenance and management.

A key element of the project is to assist each participating garden with the interpretation of their collections. With the support of the BGANZ Botanic Gardens Engagement Network, a Living Collections Interpretation Toolkit is being developed which will support each participating garden and be later rolled out to the broader network of Victorian regional botanic gardens (and beyond).

Figure 11.1: L-R, **(a)** Stock heading for Australian Botanic Gardens Shepparton, Victoria; **(b)** Royal Botanic Gardens Victoria, Cranbourne nursery staff; **(c)** Care for the Rare team at Australian Botanic Gardens Shepparton; **(d)** Unloading plants ready for planting at Australian Botanic Gardens Shepparton. (Images: John Arnott)

11.3 Types of living collections

11.3.1 Botanic gardens, arboreta and specialist horticultural gardens

Traditionally botanic gardens and arboreta are, through their seed banks and plant collections, the main living germplasm repositories for threatened species worldwide (Mounce *et al*. 2017). In more recent times however, many local communities are engaging in growing threatened species in public gardens or parks. Unfortunately, unlike seed bank collections, the genetic diversity represented within species maintained as living plant collections is often low, or unknown.

These types of plant collections are either displayed in horticulturally attractive settings or as part of nursery stock and can be categorised as follows:

- Species collections – where species are grown in pots or planted according to a certain taxonomic grouping, such as by genera or family;
- **Clonal collections** – which consist of genetically identical collections propagated asexually (see Chapter 9);
- Conservation collections – consisting of threatened species or species from threatened **ecosystems** (for example, see Case Study 11.5 on orchids).

An advantage of botanic garden and arboretum collections is that the source of the material and its end use is usually well documented. There are many cases where the last few remaining representatives of a species have been found in a botanic garden. Propagation material from botanic gardens can be used to assist in the recovery planning for threatened species (for example, see Case Study 2.3 on the Wollemi Pine).

Additionally, botanic gardens often have adjunct services, such as botanical identification through **morphological** and genetic analysis, and facilities for research into horticultural requirements, disease susceptibility and other conservation-related issues. Quite often, living plant collections are deliberately made to study the biology of a species (see Case Study 11.4 on *Rhodomyrtus*). In such cases, consideration should be given to the fate of the material once the experimental program is completed, such as incorporation into other types of germplasm conservation (e.g., seed or tissue culture), public display and education, or translocation to create or reinforce a wild population.

Botanic garden collections and the expertise housed within their host institutions, should be considered a major resource for recovering and restoring threatened species. They are also historically stable sites where plant collections can be secured and maintained for long periods of time.

Case Study 11.3: Conservation collections at the Royal Botanic Gardens Victoria, Melbourne

Neville Walsh

Five 'conservation beds' have been established at the Royal Botanic Gardens Victoria (RBGV), Melbourne Gardens, highlighting threatened species from five broad environmental regions in Victoria: Mallee; Grasslands of the Volcanic Plain; the Grampians; Alps and Sub Alps; and the Eastern Ranges (Figure 11.2). Species were selected to 'tell the stories' of how rare and threatened species come to be that way – ranging from those that are simply geographically restricted, or those that have been severely depleted by land clearance and/or degradation (as is the case particularly in the grasslands).

After selection of relevant species, material for these display beds was sourced from the wild, with horticulturists accompanying botanists from the National Herbarium of Victoria – a division of the RBGV. Source material was accompanied by herbarium **voucher specimens** documenting locality, habitat (e.g., geology, aspect, altitude, associated plant community) and abundance of the species. The plants 'performance' both in the nursery and in the garden beds is recorded in the RBGV Living Collections Database.

Generally, material was sourced from a number of individuals in a population to provide representation of some genetic diversity in the collection. This increased the likelihood of some genotypes being more able to cope with the very different growing conditions at RBGV and allowed the display beds to have a role as a genetically diverse source of seed/cuttings for further propagation and use in conservation actions, such as reinforcing depleted wild populations. The plants have also been a source of **DNA** for **molecular**-based taxonomic work undertaken at the herbarium and other research institutes around the world. Their value in this respect has been enhanced because parent material has been identified by herbarium botanists and preserved as herbarium specimens that can be referred to in the future should taxonomic revision render this necessary.

Bed and planting designs were developed in conjunction with the Garden's landscape architect, horticulturists and herbarium botanists. Most of the species had never been grown as 'garden' specimens previously and their performance in horticulture has the potential to inform future attempts for **reintroduction** to the wild. Planting commenced in April 2008, and despite the extreme Melbourne summers of 2008/09 and 2019/20, most plants have flourished with minimal irrigation (Figure 11.2).

Figure 11.2: The photographs illustrate the fledgling 2009 garden (left) and its advanced state in 2020 (right). (Images: Neville Walsh)

11.3.2 Field genebanks

A field genebank is a collection of genotypes of a taxon maintained and managed in a wild, semi-wild or cultivated situation for the purpose of conserving genetic resources. For those in botanic gardens or arboreta, the term has often been used interchangeably with: 'living collection', 'plantation', 'clonal repository' or 'orchard' (IBPGR 1991). From an ecological restoration perspective, field genebanks sit on the continuum between natural managed populations and cultivated production (Pedrini *et al.* 2020 Figure 1 p.S229), with the term 'seed production area' more commonly used to describe these seed production environments. For the purposes of this chapter, the field genebank concept follows that of FAO (2014) and Maschinski *et al.* (2019). Note that Maschinski *et al*. (2019) specify "plants grown in the ground for the purpose of conserving **genes**". However, this chapter includes a wider range of situations including pots in a nursery, planted rows in the open or managed wild stands. The definition of "field" by FAO (2014) i.e., "plot of land with defined boundaries within a place of production on which a commodity is grown" is more akin to cultivated production, in keeping with the agricultural setting of many field genebanks.

Field genebanks provide material for a variety of purposes, from specific genotypes for horticultural development (Said and Rao 2001; Reed *et al*. 2004) through to a wide range of taxa for ecological restoration. These are principally managed to produce large amounts of propagation material (see Gibson-Roy *et al*. 2021) but in some cases may be specifically managed for threatened species conservation as well.

A field genebank established for conservation purposes should be sufficiently large enough to represent the diversity required for the conservation end use. This means that a field genebank could be quite extensive and potentially resource intensive. A field genebank to produce seeds for translocation or restoration should maintain the taxon, in particular its genetic diversity, producing sufficient seed for the establishment of **self-sustaining** populations in the wild. Selection pressures likely to alter the genetic makeup of the taxon should be minimised and avoided as much as possible.

The level of management for field genebanking varies from minimal, such as the introduction of a species into a wild location; to intensive cultivation for high volume seed production; and long-term targeted seed production at a botanic garden as may be the case for some Critically Endangered species (see Case Study 11.7) or Endangered Ecological Communities (see Case Study 15.3 seed production at Australian Botanic Garden Mount Annan). Some factors to consider when developing a field genebank are highlighted in Case Study 11.7 (conservation seed orchards at the Royal Tasmanian Botanical Gardens).

11.3.2.1 Introduced populations grown inter situ

When new populations of a species are established in the wild under environmental conditions similar to the parent in situ populations, they are sometimes referred to as 'conservation introductions' which are grown '**inter situ**' (or more correctly, 'inter situs'). Generally, these populations are grown in a wild or semi-wild situation analogous to the original population and constitute a type of translocation (Commander *et al.* 2018). Access is managed and genetic diversity can be maintained as a self-sustaining population and manipulated to produce material for other conservation purposes (see Case Study 3.3, where this method is referred to as '***circa situm***').

Conservation introductions provide an appropriate methodology for the rescue and recovery of threatened species with the opportunity to replicate the genetic diversity of the taxon. Although not widely instituted, conservation introductions offer some advantages for growing species away from **threats** faced by the original wild populations and may allow the opportunity to observe their viability (and potential **adaptability**) in conditions slightly different from those where the in situ remnants exist (see Case Study 3.1 on *Hibbertia*). The plants are then a source of material for other ex situ conservation actions e.g., seed banking for species with low reproductive output, research, and/or a source of material for translocations to the original habitat.

11.3.2.2 Seed Production Areas

Seed Production Areas (SPAs) is a term used by the ecological restoration sector to refer to plant populations established under field or nursery conditions with the primary objective of seed production (see Pedrini *et al.* 2020; Gibson-Roy *et al*. 2021). SPAs offer potential for low cost, high volume production of propagation material, mainly seeds, for large scale restoration and translocation of threatened species. SPAs can produce seeds or plants reflective of a broad range of genetic traits or selected for a narrower range of traits, such as disease resistance, depending on the goal of the program and the end use of material.

Genetic **fitness** is an important consideration for foundation SPA material and can be equally as important as genetic diversity especially for the risk of contaminating in situ populations from translocations or reintroductions (e.g., ploidy variability) (see Chapter 3).

For more information refer to the Florabank Guidelines (Commander 2021).

Case Study 11.4: Ex situ management including seed orchard establishment for Native Guava (*Rhodomyrtus psidioides*) affected by Myrtle Rust

Veronica Viler and Catherine A. Offord

Myrtle Rust's impact on many native Myrtaceae species has been significant, particularly for *Rhodomyrtus psidioides* (G.Don) Benth. or the Native Guava. Once common from Broken Bay on the NSW coast to south east Queensland and up to 120 km inland, in February 2019 the species was listed as Critically Endangered in NSW. *Rhodomyrtus psidioides* is severely threatened by Myrtle Rust over its entire range and characterised as 'extremely susceptible' to infection (Pegg *et al*. 2014; NSW Scientific Committee 2017). All plant parts have been documented as being affected including leaves, stems, flowers and **fruits** (Pegg *et al*. 2014; Carnegie *et al*. 2016; NSW Scientific Committee 2017). Damage to new foliage and subsequent failure to replace older leaves progressively weakens the plant, ultimately causing death (Viler and Offord 2020). Flowers and fruits are often affected and seldom manage to produce any viable seed, therefore *R. psidioides* struggles to reproduce either asexually or sexually in the wild and has suffered serious decline as a result. Collecting seed or cuttings of *R. psidioides* was flagged as a high priority by Australian Plantbank collectors.

Conventional horticultural wisdom often prescribes ideals: plant material in good condition, pest- and disease-free, collected at a specific time of year, all supported by known data. The reality of working with many threatened species is that material available may be limited and of poor quality, access to plants is restricted due to rarity, location or other external factors, information on

propagation and cultivation is non-existent, and resources are limited. In addition, management of species susceptible to Myrtle Rust requires special attention to the cultivation environment, diligent monitoring and sound hygiene practices (Viler and Offord 2020).

Despite the best efforts of staff, by 2015 only 12 seeds of *R. psidioides* were held in the Australian PlantBank, and four whole-plant genotypes in the nursery of the Australian Botanic Garden, Mount Annan (ABGMA).

Later that year a program commenced to preserve the small nursery collection and develop propagation techniques for the species. In the first two years of growth, cutting-grown plants became fertile and developed fruit (Figure 11.3a, c). The resulting seed appeared viable and the idea for a seed production area was conceived.

In February 2018, a planting site suitable for seed production was selected in consultation with the ABGMA horticultural staff and three genotypes planted (Figure 11.3b). To optimise seed production and allow for Myrtle Rust control, plants were pruned to below two meters high. Watering was restricted to soil soaking to avoid moisture settling on foliage, a key requirement for Myrtle Rust spore germination (Makinson 2018a p42).

While waiting for the fruit to mature, the unripe crop sustained a large and unexpected loss to **predation** by birds. In April 2019, two maternal seed collections (i.e., seed collections from two of three plants) were processed yielding a modest 366 seeds. Seed storage testing for the species was possible for the first time. Germination of fresh viable seed was ≥ 87 % and the seed was found to be **desiccation tolerant** but freezing sensitive, suitable for storage at 4 °C rather than -18 °C (Sommerville *et al*. 2019).

Germination testing produced several hundred seedlings (Figure 11.3d), some of which were initiated into tissue culture for **cryopreservation** research, others given to partner botanic gardens as part of a broader conservation strategy for the species and the rest discarded. While it can be difficult to let go of surplus material, especially when it has been challenging to produce, prudent curation of research collections ensures time and resources are not wasted and duplication/ distribution of material is a sound risk management strategy.

The following season, developing fruit on all three plants were netted to prevent further predation and three maternal collections were made in 2020, totalling 7,945 seeds.

The actions described correspond to identified priority actions in the *National Action Plan* (NAP) for Myrtle Rust in Australia (Makinson *et al*. 2020). The establishment of reliable seed-production systems and the accumulation of seeds in turn enable other NAP actions around screening for rust-resistance traits and eventual selection or breeding of more resistant genotypes.

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Figure 11.3: (a) Potted plants of *Rhodomyrtus psidioides* developed flowers and fruit at a young age; **(b)** *Rhodomyrtus psidioides* under netting at the Australian Botanic Garden, Mount Annan, to prevent predation by birds; **(c)** *Rhodomyrtus psidioides* fruits; **(d)** *Rhodomyrtus psidioides* seedlings from cultivated plants in the nursery at the Australian Botanic Garden, Mount Annan. (Images: Veronica Viler)

SPAs fall into four categories:

1. *Commercial cultivation* – where the SPA is managed to provide seed/plant material to supply the restoration sector. These SPAs are mostly established using common colonising woody perennials e.g., Acacias and Eucalypts. Commercial SPAs have also been established to grow seeds for restoration of a large range of herbaceous species, e.g., grasses, daisies, lilies; such ecosystems often contain threatened species which may benefit from ex situ seed production (see Case Studies 11.6 and 15.1).
2. *Community cultivation* – commonly used by Landcare and not-for-profit groups to maintain an ex situ population for augmenting a restoration project, where the in situ population is degraded. This type of SPA is usually sited on secure public land with controlled access.
3. *Research cultivation* – these collections are often maintained in close proximity to major research stations. Such plantings facilitate the utilisation of these materials in national or international research programs. Examples of this type of SPA include plantings of species with desiccation sensitive seeds (e.g., some *Citrus* spp.) and species that do not readily produce seeds (e.g., *Lomatia tasmanica* – Case Study 11.1).
4. *Seed orchard* – For many years, the term 'seed orchard' has been used for agricultural, forestry, breeding or similar germplasm collections. Seed orchards are a traditional practice of forestry, where selection of material for propagation is made based on desired traits (e.g., rapid growth) or characteristics (e.g., straight trunks). Though source material is usually well-documented, this trait selection means that the plants are likely to be genetic outliers of natural populations. These are purpose-driven collections and may be sources of material with some conservation value (see Case Study 3.3 on *Eucalyptus benthamii*), particularly if sourced from populations that no longer exist. Under this previous definition, seed orcharding was not usually appropriate for the restoration of threatened species as the genetic composition did not reflect the wild population (Offord and North 2009).

   More recently, and in this chapter, seed orchards have been used to describe a seed production area for bulking of seed collections of threatened species, with due consideration of the genetic diversity of the founding germplasm. The term 'seed orchard' is in common use in Australian botanic gardens and applied to shrub species (see Case Study 11.4) as well herbaceous species (Case Study 11.7) and orchids (Case Study 11.5). These bulked collections allow for storage of larger quantities of seeds and enable research that is not possible for smaller collections.

Case Study 11.5: Saving orchids from extinction: the RBGV Orchid Conservation Program ex situ collection

Noushka Reiter, Richard Dimon and Marc Freestone

Australia has over 1,800 species of orchids, most of which are terrestrial. Orchids are disproportionately represented on Australia's threatened species list, making up 17 % of all nationally listed flora (~197 species) (Australian Government 2019). The Royal Botanic Gardens Victoria (RBGV) Orchid Conservation Program aims to prevent extinction by:

- Storing a genetically diverse representation of seeds and **mycorrhizal** fungi.
- Propagating suitable numbers of each of our threatened orchids for conservation translocation (where orchids are introduced back into former sites or new sites).
- Undertaking conservation translocations of these species to protected public and private land where the appropriate vegetation, climate conditions and pollinators are present.

The RBGV Orchid Conservation Program undertakes research on all aspects of orchid ecology, including pollination, mycorrhizal associations, propagation, demographics and translocation. For south eastern Australian orchids, the RBGV Orchid Conservation Program has established a permanent ex situ collection of seeds, mycorrhizal fungi and living plants propagated from seeds (Figure 11.4).

For each species, plants are hand-pollinated and the seed collected. For conservation purposes, a diverse array of seeds are collected from wild populations to enable the establishment of a genetically diverse representation of plants in ex situ collections for research into their biology and eventual conservation translocation. With the assistance of volunteers, seeds are cleaned, dried at 15 % RH for two weeks, and stored in sealed airtight packages at -20 °C. The ex situ seed collection now includes genetically diverse seed collections from over 150 orchid species and well over 2,500 **accessions** of seeds.

For each species of orchid, we also isolate the mycorrhizal fungi and store pure cultures permanently ex situ at -80 °C for use in propagation and molecular identification. The RBGV now has over 3,000 isolates of mycorrhizal fungi stored at -80 °C for use in future conservation introductions and for taxonomic studies into their identity, ecology and distribution.

Since 2014 the RBGV has grown a large ex situ potted collection of orchids for conservation, introduction and education at the Cranbourne site (Figure 11.4). Seeds are germinated symbiotically with their mycorrhizal fungi in the laboratory and grown on in flasks before being potted up in the nursery. This ex situ living plant conservation nursery now consists of over 20,000 plants from 165 species of orchid, including 68 state and nationally listed threatened species. These species are primarily from the genera *Caladenia*, *Diuris*, *Thelymitra*, *Pterostylis* and *Prasophyllum*. A dedicated group of volunteers assist research staff in the curation and management of the collection. This collection holds many species brought back from the brink of extinction including *Caladenia pumila*, *Thelymitra mackibbinii*, *Caladenia audasii*, *Diuris fragrantissima* and *Prasophyllum correctum*.

This ex situ collection is now used extensively for seed orcharding pollination studies, taxonomy and conservation introductions. To date, this ex situ collection of orchids has contributed to well over 50 introductions of threatened orchids across south eastern Australia including large scale introductions of 800–1,000 plants of nationally listed threatened species including *Caladenia colorata*, *Caladenia versicolor*, *Caladenia xanthochila* and *Caladenia fulva*.

Further reading

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Figure 11.4: Examples of ex situ living collections at RBGV grown from seeds with their mycorrhizal fungi and now flowering in the shade house: **(a)** *Caladenia cretacea* and *C. cruciformis* (top), **(b)** *C. colorata* (middle) and **(c)** *Thelymitra epipactoides* (bottom). (Images: Richard Dimon)

11.4 Number of individuals to collect and maintain

Wherever possible, the genetic composition of a living plant collection should replicate, or at least represent, the known genetic variation in the wild which may be at the species level or in some cases at the population level.

The number of individuals to collect and maintain depends on:

- the intended end use;
- the number of plants available;
- the phenotypic diversity observed;
- genetic diversity (if known);
- the likely erosion of the collection over time;
- the ability of the taxon to adapt to cultivation;
- the resources available to establish and manage the collection; and
- the genetic fitness of the available parent material.

Guidelines for the number of populations to sample and individuals to collect for conservation purposes are given in Chapter 3.

It is advisable to collect the broadest range of genotypes available and avoid skewing the ex situ population towards extremes such as unusually large or **fecund** individuals. The aim is to capture as much genetic diversity as possible in the species or population. This may require some form of genetic sampling and assessment prior to making collection decisions (Schoen and Brown 1995; Li *et al*. 2005). For ex situ conservation of an Endangered species with a limited number of individuals (genets), all individuals should be sampled, providing there is no negative impact on the population. If it is possible, genetic data should be used to help develop a sampling strategy that maximises the level of genetic diversity in as few as possible individuals (Figure 11.5). This means a more targeted approach to germplasm collection can be adopted (Hoban *et al.* 2020; see also Chapter 3). If the aim is to maintain individuals for a specified period of time, at least three replicates (**ramets**) of each individual should be maintained to allow for losses.

Figure 11.5: Genetic analysis of *Pimelea spicata* (Thymelaeaceae) was utilised to narrow down the ex situ potted collection of 1000 plants to around 100 plants, representing the extent of the species, in the nursery at the Australian Botanic Garden, Mount Annan. Pictured are botanist Trevor Wilson and conservation geneticist Samantha Yap with the nursery collection in 2021. Details can be found in Bragg *et al*. 2021. (Image: Nathan Emery)

Where resources are limited, a sensible precautionary approach is to disseminate plants to other organisations where access to information is retained (multi-site collection or metacollection, see Case Study 11.2 'Care for the Rare' and Case Study 8.3). This strategy ensures that collections have a greater chance of survival in the case of unanticipated unfavourable events e.g., watering failure or disease outbreak.

Botanic gardens and seed banks are well placed to facilitate such sharing of collections as these use a consistent method for tracking accessions, genetic diversity and origin.

In practice, maximum genetic representation is unlikely to be achieved by most living plant collections. There are, however, cases where the genetic diversity represented in ex situ collections of threatened species has equalled or exceeded that of populations in the wild (due to habitat loss, disease etc). As an example, more genotypes of the nationally threatened *Allocasuarina portuensis* were held in ex situ nursery collections than existed in situ, which has significantly aided in the conservation of this species (see Case Study 2.1). Care should be taken, especially in the case of critically threatened species, to ensure that adequate genetic sampling is made from wild populations before genetic diversity is lost (Chapters 3 and 4, Bragg *et al.* 2021; Figure 11.5), and to ensure that this diversity is maintained for the required life of the collection. In cases where only a handful of individuals remains in the wild, a collection strategy that targets all remaining individuals should be considered and where possible, initially targeting those individuals or populations that are most likely to be lost in the short term.

11.5 Propagation and cultivation

The major propagation types for living collection establishment are:

1. seeds;
2. cuttings or division;
3. tissue culture (which can be initiated from different parts of the plant, depending on the species);
4. grafting or budding;
5. transplanting/plant salvaging.

Cultivated collections can be derived from one or a number of these techniques, from existing ex situ collections, or directly from wild populations. To maximise genetic diversity, propagation from seed is preferable in most cases, and is also generally the cheapest option (Turner *et al*. 2021). Seeds collected from different plants in the wild or from field genebanks and subsequently propagated should be labelled and kept separately, to ensure that there are representatives from each parent plant in the living collection and that the origin of the material can be tracked over time (see Chapter 3 Box 3.2 'Why not pool **maternal lines**?').

Where seeds are unavailable, difficult to germinate or the genotype of the individual needs to be replicated, vegetative propagation through cuttings, grafting, budding, division or tissue culture may be appropriate (see Chapters 7, 8 and 9 and Turner *et al*. (2021). For conservation purposes, an ex situ population established using vegetatively propagated material should represent as many parent plants as possible, being mindful of the constraints imposed by the plant's interaction with the cultivation environment as well as physical and resource limitations e.g., staff to maintain large numbers of plants. Compared to seed-based propagation, the production of plants via cuttings and tissue culture can be significantly more expensive, but in some situations is warranted. For an overview of the various approaches that can be employed for the propagation and production of plants please refer to Chapter 2.

Most botanic gardens hold propagation data for native species which can be a useful resource for information, along with many references which detail the principles and practices of propagation, such as Beyl and Trigiano (2015) and Davies *et al*. (2017) for general principles, Bowes (1999) for conservation collections or Stewart (2012) for Australian plants.

Case Study 11.6: Seed production systems for restoration

Paul Gibson-Roy

Impacts of habitat clearing, fragmentation and altered climatic conditions, often mean that seed resources required for ecological restoration or functional **revegetation** are not available in the quantity, diversity or quality required. For this reason, seed production techniques are now increasingly used to supplement or replace wild collections. Effective seed production enables simplified seed harvest, increased seed yields, increased reliability and quantity of production, and improved seed quality. Seed production approaches have proved successful in providing seed resources for a number of restoration projects across Australia in the past decade by supplying seeds of difficult-to-source species from various plant functional types (grasses, forbs, climbers, sub-shrubs, shrubs and trees), threatened species and species from threatened ecological communities (Gibson-Roy *et al*. 2021, Figure 11.6).

Seed production systems growing seeds for ecological restoration typically aim to capture and retain a broad range of genetic characteristics from founding population/s into, through and out of the production system (by avoiding selection biases). Systems growing seeds for more general or functional revegetation (e.g., shelter belts, soil stabilisation, windbreaks, amenity plantings) sometimes select for desired traits in wild founding populations and aim to retain those throughout the production system (by maintaining selection biases).

Seed production areas (SPAs) are sites where seed crops are grown and maintained, and harvested seed can be processed and stored (Pedrini *et al*. 2020; Zinnen *et al*. 2021). They can be small or large in scale, set-up and running costs. Production systems most used in Australia (often in combination) are containerised systems, weed mat systems, trellis systems and field bed systems (Figure 11.6). Production crops are typically generated from seeds, but can also be created through cuttings, divisions, or transplants. Crops are established by planting or seeding (depending on scale and system) and maintained in production for varying periods. Timing of crop replacement depends on various factors which include seed yield (productivity), time in cultivation, plant vigour or health, and seed market value. Crop maintenance entails managing factors that might promote or inhibit plant health and seed production such as irrigation, pests and disease, fertilisation, weeds, herbivores and biomass. Ideally, production systems are designed to facilitate both effective crop maintenance and seed harvest (Gibson-Roy *et al*. 2021).

Seed production requires some degree of infrastructure for propagating plants (seeds or cuttings), processing, testing and storing seeds, housing machinery or staff. Its scale and complexity are typically related to the size of the cropping area (e.g., square meters to hectares), the number of species grown, and the amount of seeds produced. Small SPAs may only require basic infrastructure such as tools, storage, propagation facilities and seed processing areas, whereas large SPAs can require various types of permanent buildings, sheds and machinery typical of horticulture enterprises or hobby farms.

References

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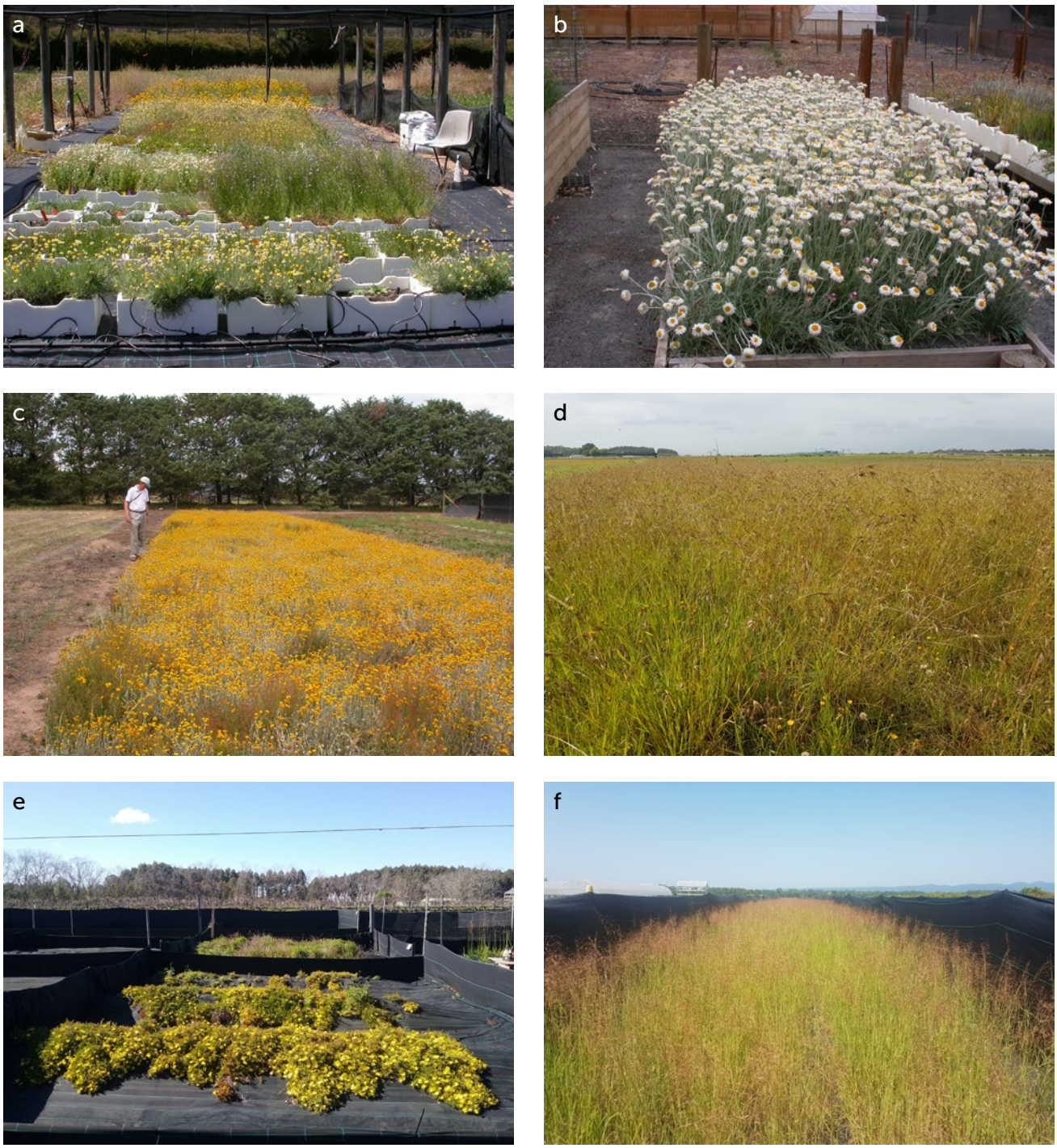

Figure 11.6: **(a)** Containerised system (foam box) growing various species for grassy woodland restoration. **(b)** Containerised system (raised wooden box) growing threatened daisy (*Leucochrysum albicans* ssp. *tricolour*). **(c)** Field bed system growing Common Everlasting (*Chrysocephalum apiculatum*). **(d)** Field bed system growing Kangaroo Grass (*Themeda triandra*). **(e)** Weed mat system (mounded) growing a spreading ground cover (*Hibbertia diffusa*). **(f)** Weed mat system (flat) growing Scent Top Grass (*Capillipedium spicigerum*). **(g)** Trellis system growing Blushing Bindweed (*Convolvulus erubescens*). **(h)** Trellis system growing Twining Glycine (*Glycine clandestina*). (Images: Paul Gibson-Roy)

11.6 Management of living collections

High conservation-value living plant collections should be considered separately from collections for other purposes such as horticultural display or plant development. Living collections are one of the most vulnerable means of maintaining germplasm and they can require intensive management to prevent loss of material through disease, irrigation failures or other issues. Plants may need to be regularly repropagated and this can lead to the accumulation of problems, including mislabelling or the propagation of 'off-types' (genetic aberrants) that may not be suitable representatives of the species. Meticulous record keeping is vital in the management of living collections. For other types of collections, as much of the information about the original collection must be maintained as possible (see Chapters 2 and 4), ensuring that the **provenance** and cultivation history of each plant in the collection is known. Mistakes made in record keeping can lead to poor conservation outcomes, such as translocation of inappropriate material. (See also Chapter 15 'Maintenance and Utilisation and Information Storage')

Various manuals for the management of living collections for conservation and related purposes are available from Botanic Gardens Conservation International (BGCI), e.g., Gratzfeld (2016)[55] and Bioversity International (undated)[56]; and the Center for Plant Conservation (Maschinski *et al.* 2019)[57]. Reference should also be made to the Australian Network for Plant Conservation publications, especially '*Guidelines for the Translocation of Threatened Plants in Australia*' (Commander *et al*. 2018)[58] where issues dealing with the management of translocation sites can also be applied to field genebank situations. Florabank Module 7 '*Seed Production*' is an excellent introduction to the topic (Gibson-Roy *et al*. 2021) as well as Pedrini *et al.* (2020).

11.7 Potential need to regenerate collections

Managing living collections of species with a short lifespan such as annuals that require regeneration be challenging and require a substantial level of resourcing. Some of these species may not be easy to regularly replace if fresh propagation material is required from wild populations, particularly if these are in remote or difficult to access sites. In such cases, sufficient plants (with appropriate levels of genetic diversity) need to be maintained until these flower and produce seeds to replace adult plants. Adult plants may be lost due to aging or could be used for the supply of cutting material if the plant can be propagated vegetatively. Indeed, to maintain an appropriate level of genetic diversity, it may be more effective to focus more on vegetative propagation of all remaining genotypes (so none are lost, and especially for tree species with long life spans) within the collection rather than relying on the production of seeds to replace plants as these are lost.

55 https://www.bgci.org/resources/bgci-tools-and-resources/bgcis-manual-on-planning-developing-and-managing-botanic-gardens/
56 https://www.bioversityinternational.org/
57 https://saveplants.org/wp-content/uploads/2020/12/CPC-Best-Practices-5.22.2019.pdf
58 https://www.anpc.asn.au/translocation/

Case Study 11.7: Challenges and triumphs of conservation seed orchards at the Royal Tasmanian Botanical Gardens

Lorraine Perrins

Banking on success

One goal of the Tasmanian Seed Conservation Centre, based at the Royal Tasmanian Botanical Gardens (RTBG), is to have all of Tasmania's rare and threatened species and key species of threatened plant communities secured as long-term collections.

Some major challenges in achieving this are:

- 20 % of Tasmania is remote Wilderness World Heritage with limited accessibility on foot, by air or sea.
- Many threatened species occur in remote or inaccessible areas, meaning monitoring for seed harvest can be difficult and very expensive.
- Some species occur in very small populations and/or occur sporadically across large areas making locating and harvesting seeds or cuttings difficult.

Developing 'Conservation Seed Orchards' can provide a way to overcome some of these obstacles by providing ready access to genetically diverse collections (Perrins 2020). Since the RTBG's Nursery Seed Orchard Program began in 2006, 45 orchards have commenced, with 30 so far completed. The average time to run orchards from cuttings or germinants to a 'good' seed harvest, i.e., minimum of 10,000 seeds, is currently 7 years, and costs approximately $7,000 per species (roughly the same amount as one helicopter trip). However as bulking of more easily grown species are completed, and trickier longer-term species are attempted, the cost per seed orchard is expected to increase.

Trials and tribulations

Examples of some issues impacting the success of orchards at the RTBG are:

1. *Understanding the biology*

   The endemic Tasmanian species *Tetratheca gunnii* requires buzz pollination by a suitably-sized native bee (European honeybees being too large). As RTBG did not have sufficient native bees within its boundaries (University of Tasmania 2017), activities to increase bee habitat and nesting sites within the nursery and introduce native bees are being enacted (Figure 11.7), as well as the temporary transportation of the seed orchard to higher bee density areas. Hand pollination, with the assistance of volunteers, was attempted but proved ineffectual (Figure 11.8, Perrins 2020).

   The **elaiosomes** (lipid-rich structures attached to seeds) of *Tetratheca* attract ants in an effective **dispersal** strategy called myrmecochory, so measures to prevent loss of seeds e.g., physical ant barriers, have also been implemented.

Figure 11.7: Under bench habitat plantings (left) and insect nest augmentation (right) to encourage pollinators to remain within the RTBG Nursery facility. (Image: Lorraine Perrins)

Figure 11.8: RTBG volunteer Laura Carvalho 'buzz' pollinating *T.gunnii.* (Image: Lorraine Perrins)

2. *Thinking outside the box*

The endemic cushion plant *Azorella macquariensis* on subantarctic Macquarie Island is a Critically Endangered species being impacted by a severe dieback (Perrins, 2012). The species is highly adapted to the harsh conditions on the island making it extremely difficult and costly to maintain in cultivation in Hobart. Despite several complex logistical challenges, including working on a remote World Heritage subantarctic island where supplies must be shipped via the annual Australian Antarctic Divisions (AAD) supply vessel, an ex situ collection/ seed orchard has been successfully developed on Macquarie Island but away from the wild populations (Figure 11.9; Perrins and Wood 2019). The collection is monitored via images sent from Parks and Wildlife Rangers based on the Island. Ten years on, this collection continues to thrive and is testament to the sustained cooperation between botanic garden staff, AAD, State Departmental scientists, and Tasmanian National Parks and Wildlife reserve managers.

Figure 11.9: *Azorella macquariensis* ex situ conservation collection and seed orchard on Macquarie Island. (Image: Andrea Turbett, TASPWS)

3. *Expecting the unexpected*

   Two Tasmanian coastal herbaceous species, *Lepidium flexicaule* and *Veronica novae-hollandiae* were easily propagated and grew rapidly, producing flowers within a few months. However, the *Lepidium flexicaule* orchard rapidly succumbed to an outbreak of White Rust (*Albugo candida),* a difficult-to-control disease which arrived in Australia in 2001 and affects members of the Brassicaceae family. *Veronica novae-hollandiae* proved to be very susceptible to Downy Mildew (*Peronospora* spp.), despite growing in a constantly wet dune environment on the West Coast of Tasmania.

Additional considerations made prior to the formation of a seed orchard include:

- Ensuring there is sufficient genetic diversity within the seed orchard.
- Providing suitable growing requirements for species.
- Ensuring differing species held do not hybridise, reducing the genetic integrity of the collections.
- Maintaining accurate records to monitor potential genetic erosion for longer term seed orchards if conditions favour more robust genotypes.
- Providing disease and pest controls, being mindful that pesticide usage to control insect pests may also kill pollinators.
- Understanding the reproductive biology of species to determine effective pollination requirements and harvesting techniques, for example, for explosive seed capsules.
- Appreciating the time required for harvesting. Not all species can be bagged after flowering. Many species continue to flower and release seeds over many weeks/months, requiring daily or more frequent hand collection, usually during peak summer holiday periods when volunteer assistance can be diminished.
- Understanding the 'end use' of the orchard once complete e.g., not using biological controls or soil inoculants on species that will be used for translocation purposes.

Satisfying rewards

The development of the orchard programs at RTBG from cuttings or seeds to productive, flourishing seed orchards has provided challenges. However, it has consistently bestowed much larger quantities of seeds than could be collected from wild populations. Additionally, the benefits of intimately observing and recording specific requirements of each species can reap much more than just the physical rewards.

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11.8 Biosecurity concerns

There are several biosecurity concerns for living collections. The focal species itself may have weed potential (even if it is a native species) so depending on where the living collection is located this may need to be considered and managed as required. Additionally, a species might be a host for a disease (see Case Study 11.8 Myrtle Rust) or through poor practices may end up being infected with a problematic plant pest or disease, (e.g., various scales, Myrtle Rust or Phytophthora Dieback) that can then be transferred to other local species.

Wild-collected vegetative material may be a vector for pests and diseases which could be introduced into an ex situ facility such as a nursery or garden (Stanley and Dymond 2020). Infected material may threaten existing conservation collections. In general, material should not be collected from the wild if it is visibly infected or infested by a pest or disease or collected from a site identified as harboring a disease. The use of quarantine zones within the nursery can mitigate some risk by observation of material in a separate area (such as a lockable greenhouse) for a set amount of time (weeks to months). Ideally, no vegetative material should be brought to a nursery or garden with natural soils attached as this is a proven pathway for the introduction of *Phytophthora* spp. or other potentially devastating soil-borne diseases. However, if there are no other options then appropriate steps need to be taken to isolate the material until it is considered safe or if not, then carefully disposed of in line with relevant guidelines, such as those set out by the Nursery Industry Accreditation Scheme Australia (NIASA)[59] and the International Plant Protection Convention (IPPC)[60]. The presence of soil pathogens, pests and diseases and even weeds in a living collection may prevent ex situ material being restored to wild sites. For plants destined for future field plantings, these problems need to be avoided at all costs and may require intensive management in the months leading up to in situ planting.

In exceptional circumstances it may be impossible to completely avoid dealing with a species that is infected with problematic pests or diseases (see Case Study 11.4 on *Rhodomyrtus* and Case Study 11.8 on Myrtle Rust at Auckland Botanic Garden). In these instances, material can be collected with appropriate care if the risk of extinction by not collecting is higher than managing the risk through judicious use of fungicides or quarantine e.g., a species with high threat status that may be infected but is asymptomatic.

Plants not native to a state or region may be grown for conservation purposes in an ex situ facility to protect a threatened species from a disease present in their home range, or to move the species to more suitable climatic conditions. Care must be taken to ensure that these species do not escape from cultivation and monitoring plans are required to mitigate this risk (see Case Study 11.8). State and territory biosecurity agencies may require inspections and phytosanitary certificates prior to transfer of living plant material interstate (see Chapter 15 section 15.4.3 for details).

If the end use of a collection is to provide plants for restoration or translocation, it is important to carefully consider the use of any biological control agents or soil inoculants, to avoid the risk of transporting foreign organisms into a natural area (see Commander *et al*. 2018; Stanley and Dymond 2020).

59 http://nurseryproductionfms.com.au/niasa-accreditation/
60 https://www.ippc.int/en/

Case Study 11.8: Managing ex situ plants highly susceptible to pathogens

Rebecca Stanley and Emma Bodley

Initiating an ex situ collection with a provider, such as a nursery or a botanic garden, is a well-established response in threatened plant recovery management. For a plant that is host to a devastating environmental pathogen, this type of acquisition by the recipient nursery could put other crops or collections at risk, or adversely affect other stakeholders and projects undertaken at the facility. Not all options for germplasm collection may be equally risky in terms of pathogen spread and accepting material is best done on a case-by-case basis. Methods of managing the risk include holding ex situ collections outside of the climatic range of the pathogen, treating plants e.g., with fungicides, housing plants in a place with physical barriers to pest and diseases, establishing survey and monitoring programs, and preparing incursion response plans including transfer of material to locations where no other plants or collections are put at risk (Stanley and Bodley 2020).

Since the arrival of Myrtle Rust in NZ, the Auckland Botanic Gardens duplicated a collection of the critically threatened *Metrosideros bartlettii* (rāta Moehau) to a botanic garden outside the expected climatic envelope of the rust. The remaining collection on site is now in a greenhouse separated from other crops to reduce inoculum reaching them, gathered together to enable effective and efficient fungicide application (fortnightly all year, apart from winter when treatment is monthly) and without overhead watering (likely to promote Myrtle Rust). Treatment and control regimes to maintain the health of a collection may require the facility committing to an ongoing, and potentially indefinite, program. Ex situ providers must weigh up the costs of ongoing management including time to apply treatments, staff training to use chemicals and extra responsibilities such as surveying and monitoring.

We ceased development of a seed orchard for wild collected *Lophomyrtus* species (*Lophomyrtus bullata* and *L. obcordata*) and their cultivars (including natural hybrids), as this genus is emerging as the most affected New Zealand native species in the wild, and may serve as a reservoir for Myrtle Rust at the Gardens. It was not feasible to treat plants in a garden setting with fungicide. When 13 cultivars of *Lophomyrtus* in our nursery became infected with Myrtle Rust, the plants were transferred to an offsite research collection managed by a plant pathologist undertaking research into Myrtle Rust resistance. An incursion response plan prepared ahead of time ensures that if, or when, a pathogen arrives at the ex situ facility, all stakeholders understand and have agreed to the disposal or movement of the material as action must be undertaken quickly.

We recommend:

- Discussion with an ex situ provider occur prior to delivery of a highly susceptible species, to ensure planning is in place to enable conservation of the affected germplasm and protection of existing plants, collections and projects at the facility.
- Ensuring the facility has time to prepare for the arrival and discuss implications with stakeholders of other projects on site.
- Deciding up front what happens if the material becomes infected to avoid disposing of genetic material which may be on the verge of extinction.

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11.9 Living collections compared with horticultural displays

Horticultural displays, through design, interpretation and species selection, are opportunities to highlight and educate the public about different species, ecological communities, taxonomic relationships, potential threats and other conservation issues and messages (see Case Study 11.3), and at a more basic level may simply be implemented for purely aesthetic reasons. These types of collections often only have a few representatives of certain species and are not specifically managed in terms of genetic diversity, or to produce seeds or cutting material.

In comparison, living collections maintained for conservation or restoration purposes may be deliberately selected to have representative genetic diversity, the appropriate sex ratios, certain planting densities, pruning regimes, and so on, that are more suited to production purposes rather than for display.

All types of living collections in botanic gardens can be a fantastic opportunity to educate and engage the public on the threats and challenges faced by individual species and the conservation work and/or research being undertaken to preserve them. Such displays can inspire people to become more connected with their plant **biodiversity** and can generate support, endorsement and even advocacy for efforts being made to secure their future. Even a small number of plants with recorded provenance and cultivation history can prove to be useful for conservation research if the records are sufficiently detailed and considered accurate. The difference is the data is linked to the plant and not necessarily the number of plants. Management of living collections for conservation and restoration purposes is quite different and far more intensive than amenity horticultural displays.

11.10 Metacollections

Metacollections can be formed across networks of locations such as several botanic gardens, to address the requirement for space and other resources that are required to conserve the genetic range of a taxon. By sharing a collection strategy and coordinating collections (see Case Study 8.3), a far greater range of genetic diversity can be conserved and the risk of loss of important genotypes reduced (Wood *et al*. 2020). Plant collection data is shared between ex situ facilities to ensure the provenance of the metacollection is managed for restoration planning purposes. This type of approach and coordination may be more appropriate for longer-lived larger taxa such as species of *Araucaria* or rare rainforest species such as Ribbonwood (*Idiospermum australiense*) where space requirements often preclude the establishment of large numbers of individuals at any one site (see Case Study 2.3 on the Wollemi Pine).

The transfer of pollen between metacollections, currently an under-utilised option, may also provide solutions to issues relating to genetic erosion or self-incompatibility. See Chapter 13 for more details of pollen collection and storage.

11.11 Stakeholders

Landowners and/or traditional owners are key partners in many living collection projects and should be consulted on the formation and management of any living collection. Stakeholders/owners may wish to be involved in collecting, deciding on ex situ locations and restoration sites as well as providing a cultural context for the species in question and its significance for traditional owners. The living collection may be a possible resource for ongoing engagement with stakeholders/owners as well as for wider community engagement in plant conservation.

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Chapter 12
Isolation, propagation and storage of orchid mycorrhiza and legume rhizobia

Noushka Reiter, Richard Dimon, Marc Freestone, Belinda Davis, Zoe-Joy Newby, Nigel Swarts, Karen D. Sommerville

12.1 Introduction

Symbiotic mutualisms between plants and fungi (Brundrett 2009; Balestrini and Lumini 2018) or plants and **rhizobia** (Long 1989) are often essential for their growth and survival in the wild. In particular, the Orchidaceae and Fabaceae (the second and third largest plant families in the world) are highly reliant on their symbioses with **mycorrhizal** fungi and rhizobia, respectively. In Australia, a number of unique and **endemic** species in these two plant families are at risk of extinction, with three species of each already becoming **extinct** since European settlement. For these species, **conservation** of the **symbionts** is critical for the long-term conservation and eventual **rewilding** of these plants. This chapter will therefore focus on techniques for collecting, growing and conserving symbionts, as well as **seeds**, to support conservation of threatened species in the Orchidaceae and Fabaceae.

12.1.1 Conservation of Orchidaceae

Most orchid species in Australia have a southern distribution, with the highest number of threatened orchid species being found in the south east of Australia (Wraith and Pickering 2019). Causes of orchid decline in Australia include **habitat** destruction, competition with weeds, grazing by introduced and native animals, and inappropriate fire regimes (Reiter *et al*. 2016; Wraith and Pickering 2019). Australia has over 1,800 species of mostly terrestrial orchids (Backhouse *et al*. 2019) many of which are highly threatened, making up 17 % of all nationally listed flora (197 species, Australian Government 2020). Several species of Australian orchid are now extinct including *Caladenia brachyscapa* G.W.Carr and *Diuris bracteata* Fitzg. Many more species teeter on the edge of extinction with only a handful of plants remaining in the wild e.g., *Caladenia pumila* R.S.Rogers, *Caladenia audasii* R.S.Rogers, *Prasophyllum correctum* D.L. Jones and *Caladenia busselliana* Hopper and A.P Br.

What makes orchids so unique in the plant kingdom is their complicated life history. Their flowers are often dependent on one or a few pollinator species (see examples in Johnson and Schiestl

2016 and Phillips *et al*. 2020), and the family exhibits a wide range of possible **pollination** mechanisms e.g., sexual deception (Reiter *et al*. 2017; 2019), food deception (Phillips and Batley 2020) and food reward (Reiter *et al*. 2018a). As such, the study of the identity and distribution of the pollinators can be challenging. Orchids have the smallest seeds of all Angiosperms, and these are like dust in appearance (Beer 1863). Though each capsule produces, in many cases, thousands of seeds (Arditti and Ghani 2000), the seeds lack **endosperm** so are entirely dependent on mycorrhizal fungi for **germination** in the wild. Mycorrhizal fungi also provide nutritional support to mature plants to a greater or lesser extent (Rasmussen 1995). Another unique factor in the life cycle of many terrestrial orchids is the ability to become dormant for a short period each year, and for longer periods if needed, to avoid seasonally unfavourable conditions (Tremblay *et al*. 2009). All these factors need to be considered for conservation and **translocation** success (see Case Study 12.1). The incorporation of orchid mycorrhizal fungi in the propagation of plants for conservation translocations is particularly important and has led to a higher success rate in translocations for several species (Reiter *et al*. 2016 and see papers cited in Phillips *et al*. 2020).

Case Study 12.1: Conservation translocation of the Endangered *Caladenia colorata*

Noushka Reiter

Caladenia colorata D.L.Jones is endemic to south eastern Australia and is now known from a handful of **populations** in South Australia and Victoria. The species is listed as Nationally Endangered *Environment Protection Biodiversity and Conservation Act* 1999. The total number of wild plants in Victoria is less than 600 with pressures from grazing and weed invasion. *Caladenia colorata* (Figure 12.1) typically has one to three flowers that range in colour from pale yellow to pink or yellow with a red lip, and a subtle sweet smell.

Thirty plants from across two populations were hand pollinated, using flowers greater than 10 m apart from each other. Seed for propagation was collected from pods four-six weeks after pollination. The seeds were cleaned and dried to 15 % **relative humidity** before being stored at -20 °C until further use. Plants were grown from seeds symbiotically to mature flowering individuals using the techniques of Reiter *et al*. (2016).

The pollinator was identified as the thynnine wasp species *Phymatothynous pygidialis* (Reiter *et al*. 2018a). The pollinator was found to be present at both the remaining wild sites in Victoria and the introduction sites (Reiter *et al*. 2018a). The mycorrhizal fungi were identified as *Serendipita* OTU (Operational Taxonomic Unit) A (Reiter *et al*. 2020b) and did not vary between the remaining sites of this species.

Introduction sites were selected that were greater than 100 hectares, had a vegetation and soil match to **extant** sites of *C. colorata*, were permanently protected as either National Park or covenanted private property, and had the pollinator present. In addition, each site was fenced with rabbit proof fencing and any weed or herbivore control was undertaken on each site as required.

Plants were translocated into *de novo* sites that were permanently reserved by generous private landholders through Trust for Nature with the assistance of the Australasian Native Orchid Society volunteers (Reiter and Thomson 2018). Each individual orchid was individually identified in the field with a numbered disc and pin, 10 cm to the north to aid in re-emergence monitoring and identification of the plants in subsequent years. The location of each plant was triangulated from stakes in the field to aid future detection. Each plant was caged and watered (up to monthly average

rainfall only if rainfall fell below average), until plants entered their first dormancy in the field. Between 2013 and 2017 a total of 883 plants were introduced of *C. colorata*.

Plants were monitored annually for emergence (July), flowering (September-November) and seed set (November-December). In addition, any recruits were recorded. A wild population was monitored using this method for comparison against the translocation sites.

Over 1,000 plants were propagated symbiotically with *Serendipita* OTU A for introduction and as a permanent **ex situ** collection at the Royal Botanic Gardens Victoria. Of the 883 plants that were introduced between 2013 and 2017, 80 % survived with an additional 580 recruits. The control wild population had on average 83 % emergence ± 18 % standard error. Seed orcharding has begun on the ex situ collection to ensure that an adequate number of seeds are available for future conservation work with this species.

This research highlights that introductions of endangered orchids on a large-scale incorporating knowledge of both the pollinators and mycorrhizal fungi is both possible and can provide significant conservation outcomes for threatened species. These conservation translocations have doubled the number of wild plants of *C. colorata* with substantial recruitment leading *to* self-sustaining populations with a population growth rate >1.

Figure 12.1: *Caladenia colorata* in nursery, and in field surrounded by recruits (red dots). Inset: *Caladenia colorata* in flower. (Images: Richard Dimon, Noushka Reiter)

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Orchid conservation is labour-intense and volunteers play a significant role in day-to-day maintenance of collections at several botanic gardens (see Case Study 12.2).

Case Study 12.2: Tasmanian Orchid Volunteer Conservation Program

Nigel Swarts and Magali Wright

Volunteer programs that implement ex situ recovery actions have become the frontline for orchid species conservation across Australia. Associated with each major botanical garden in southern Australia is an orchid conservation program that relies extensively on volunteers for the time-consuming work required for orchid conservation. With the considerable reduction in on-ground resources from Federal and State governments for threatened species conservation, volunteers increasingly fill the gap with their generosity and commitment (Mckinley *et al*. 2017; Tulloch *et al*. 2020). Volunteers have a passion and enthusiasm for our rich orchid flora which compliments their desire to learn new skills and develop new friendships with like-minded people. At the Royal Tasmanian Botanical Gardens (RTBG), volunteers from the Friends of the RTBG and Threatened Plants Tasmania Wildcare Inc. have come together to form the Tasmanian Orchid Conservation and Research Program. Supervised by orchid scientists, over 20 volunteers have participated in the program over the last 10 years working towards the propagation of Tasmania's most threatened orchids.

Working in a demountable site office donated by the RTBG, which has been converted to a dedicated orchid conservation laboratory, volunteers have learnt the skills of sterile technique and microscope use in CSIRO-donated **laminar flows** with equipment funded by small environmental grants schemes. This has led to the ability to extract pelotons from root material and make sure cultures of mycorrhizal fungi that are now stored in perpetuity in the RTBG's Tasmanian Seed Conservation Centres. Volunteers have learnt to dry and clean orchid seeds, sterilising them with a bleach solution and carefully sowing seeds on oatmeal agar Petri dishes.

There is always great enthusiasm when 6 to 8 weeks later, green shoots can be seen from a successful orchid seeds and fungi combinations, especially when the species germinated is particularly rare or has been challenging in previous seasons. Volunteers have learnt the art of carefully removing individual seedlings from Petri dishes and placing them into vermiculite over agar growth containers. Every year volunteers pot up the laboratory-raised seedlings, which sees hundreds of seedlings of Tasmania's most endangered orchids transferred into the glasshouse.

Complimenting the conservation program is the support from RTBG Horticulture staff who prepare potting mix, create space in the glasshouse, manage pest and diseases, control watering, and monitor plant health. Without the support of these staff, this program would not be possible as the day to day care of terrestrial orchids are beyond the capacity of a volunteer program.

The Tasmanian Orchid Conservation and Research Program could not have built up ex situ insurance collections and successful propagation methods for over 10 of the State's threatened orchid species without volunteer support. The time that volunteers provide to Australia's orchid conservation programs must be valued, coordinated and have clearly articulated conservation benefits for volunteering to remain a functional model for threatened species recovery.
While volunteer programs alone can't replace adequately resource conservation efforts, volunteers have stepped up around Australia to protect the species they love and we should celebrate their efforts.

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12.1.2 Rhizobia for the conservation of Fabaceae

In Australia, the Fabaceae are found throughout the continent and are present in most habitats from the coast to the alps and inland deserts (ALA 2020), with the highest diversity in south-west Western Australia. Australia has over 1,200 species of pea (VicFlora 2020), many of which are under **threat**, including 121 species listed under the *Environment Protection Biodiversity and Conservation Act,* 1999 (Australian Government 2020) and several species that are now extinct including *Acacia kingiana*, *A. prismifolia* E.Pritz and *Pultenaea maidenii*.

A large area of research comprises the symbiotic association of Fabaceae with nitrogen-fixing soil bacteria, collectively called rhizobia. In this symbiosis, the bacteria stimulate the plant to produce root nodules in which the bacteria reside. In a mutual exchange of resources, the rhizobia then turn molecular nitrogen into ammonia (suitable for plant uptake) and the plant produces sugars for the rhizobia (Sprent and Raven 1985). This mutualism has facilitated the colonisation of Fabaceae into areas that have poor soils, including much of Australia. While rhizobial root nodulation has the potential to increase agricultural yield while minimising resource use (Matiru and Dakora 2004), utilising this symbiosis for conservation of threatened species is relatively unknown.
A major factor contributing to this is the lack of knowledge concerning rhizobial diversity across the Australian landscape. Current consensus presents the genus *Bradyrhizobium* as the dominant

nodule-forming bacterial group within Australian native Fabaceae, compared to other known genera such as *Rhizobium* and *Mesorhizobium* (Lafay and Burdon 1998; Marsudi *et al.* 1999).

12.1.3 Chapter aims

As symbiotic organisms are so important in the life cycles of these two families, this chapter reviews methods for sampling, isolating, storing and inoculating threatened plants with appropriate symbionts to improve conservation outcomes. Before beginning a conservation project for a specific plant species, however, it is recommended that an effort is made to ensure that the plant **taxon** is described and accepted, by checking the Australian Plant Census[61] (see also Chapters 3 and 4; Commander *et al.* 2018 Chapter 2.3.1). Before attempting any field trips for mycorrhizal collections, pollen or seed collection, seek appropriate permits (see Chapter 2.3; Commander *et al.* 2018 Chapter 4.2). Appropriate hygiene measures should also be followed (see Chapter 4; Commander *et al.* 2018 Chapter 2.6) and consideration given to collection of a **voucher specimen** (plant, rhizobia or mycorrhizal fungi), habitat data, and appropriate methods for record keeping (see Chapters 4, 14 and 15; Commander *et al.* 2018 Chapter 6.3.4).

12.2 Orchidaceae

12.2.1 Hand pollination to ensure seed set

Most of Australia's orchids rely on insect floral visitors for cross pollination. A few species of Australian orchid, notably some species within the genus *Thelymitra*, are self-pollinating (Cheesman 1881), but other species of *Thelymitra* rely on insects for **outcrossing** between individuals (Dafni and Calder 1987). The use of a pollen vector generally results in higher rates of outcrossing and the **preservation** of greater **genetic diversity** within populations (Hamrick and Godt 1996). For the majority of orchid species that have had their pollination rates assessed, natural **fruit** set is pollinator-limited (Tremblay *et al*. 2005), i.e., a large proportion of flowers remain unpollinated due to the absence of interaction with a suitable pollinator. Orchids that exhibit a specialised strategy for pollination may also be limited by pollinator availability (Phillips *et al*. 2015; Reiter *et al*. 2017) or share a pollinator and form **hybrids** with co-occuring species (Reiter *et al*. 2020a).

Consequently, relying on naturally occurring pollination events to generate sufficient fruit set for seed collection may be a risky strategy for conservation planning and species recovery programs. Artificial pollination by hand, will increase the likelihood of fruit set and obtaining **viable seed**. Tremblay *et al*. (2005) demonstrated that between 2– and 20–fold increases in fruit set can be achieved with experimental hand pollination. Hand pollination will also be required for plants in ex situ collections where pollinators are unlikely to be present or do not have access to plants. Hand pollination also allows the tracking of parent plants, and the control of genetic diversity, in breeding and translocation programs.

In orchids, the pollen in each flower is usually bound in a coherent mass known as a pollinium (Johnson and Edwards 2000). These often occur in pairs (pollinia) joined by a sticky tab called a viscidium. Hand pollination can therefore be carried out fairly easily using fine forceps or a toothpick; the pollinia are removed from beneath the anther cap and transferred to the sticky surface of the stigma of another plant (Swarts and Dixon 2017). In general, the Orchidaceae have low levels of genetic differentiation between populations (see papers cited in Phillips *et al*. 2020). For some rare species, which have suffered from **inbreeding**, a drastic improvement in seed viability has been

61 https://www.anbg.gov.au/chah/apc/about-APC.html

achieved through crosses between populations (see the example of *Thelymitra mackibbinii* in Phillips *et al*. 2020). If hand pollinating flowers within a population, it is preferable to choose plants several meters apart to ensure outcrossing beyond **clones** or closely related individuals.

While hand pollination has been shown to generally produce more fruits than natural (insect-mediated) pollination, resource limitation, where flowers do not have sufficient nutrition or carbohydrate resources to set fruit (Roberts 2003), can occur. Thus, while seed production in the wild can be enhanced through hand pollination, it is advised to limit this to alternate years, or between different plants in different years, as repeated pollination of the same plant may lead to plant decline (Farnsworth *et al*. 2006).

Orchids vary substantially in flower **morphology** so care must be taken to ensure that the procedure is completed correctly (Table 1, Figure 12.2). In some cases, the flower may need to be carefully dissected to access the pollinia (male reproductive organ) or the stigmatic surface (female reproductive organ). Orchids within a population may flower at different times, and natural pollination (or pollen removal) can occur at any time within the flowering period, it can be difficult to correctly time hand pollination with the period when most of the plants in the population are fertile. An option for countering this problem is to cover flower buds with gauze bags just prior to flowering, thereby restricting access to flowers by insect visitors and increasing the chance of having virgin flowers to hand pollinate. However, as it is rare to have high pollination rates in orchids (see papers cited in Phillips *et al*. 2020), this is not usually necessary.

Table 12.1: Description of mechanism for hand pollination for different Australian orchid genera.

Genera	Description of hand pollination techniques
Acianthus, *Bulbophyllum, Burnettia*, *Caladenia* (Figure 12.2 a), *Calochilus*, *Chiloglottis*, *Corunostylis, Cymbidium, Cyrtostylis*, *Dipodium*, *Dendrobium* (Dendrobiinae), *Eriochilus*, *Genoplesium, Glossodia*, *Leptoceras*, *Lyperanthus*, *Orthocerus*, *Prasophyllum*, *Sarcochilus* (Aeridinae), *Thelymitra* (Figure 12.2 b), *Townsonia*	Pollinia can clearly be seen underneath the hood of the column (usually at the top of the flower) and the stigma can easily be seen below or deeper inside the flower. Remove the pollinia with tweezers (hard pollinia) or toothpick (soft pollinia) from one flower and place it onto the sticky stigmatic surface of another flower on a different plant. *Prasophyllum* have non-resupinate flowers so the stigma is above the column lobes, similar to *Cryptostylis* where the pollen sits directly below the stigma.
Pterostylis (Figure 12.2 c)	Hold down the labellum and then use a toothpick to access the pollen inside the top of the hood. Then on another plant, hold down the labellum and by looking through the hood you can see the stigmatic surface to then place the pollen. The labellum can be dissected for ease of access.
Gastrodia (Figure 12.2 d), *Spiranthes*	Due to their tubular floral segments, flowers may need to be dissected to access the pollinia and stigmatic surface.
Corybas (Figure 12.2 e)	When flowers are mature, use a needle to cut a hole on the lateral lower part of the flower to gain access to the pollinia and stigma. For cross-pollination, remove the pollinia and place on the stigma of a plant several meters apart as most *Corybas* species are clonal.

Genera	Description of hand pollination techniques
Arthrochilus, *Drakaea* (Figure 12.2 f)	*Arthrochilus* flowers are turned downwards towards the **ovary**, where pollinia can be accessed under two pairs of curved wings at the top of the column. Pollinia can be placed on the stigma just below the column. *Drakaea* contain their pollinia in a hood at the top of the stiffly held column opposite the labellum claw. These can be removed and placed on the circular stigma of another plant just below the column hood.
Caleana (Figure 12.2 g), *Paracaleana*	Duck orchids hold their pollinarium at the elongated column foot of the flowers (Hopper and Brown 2006). Pollinia can be removed from the upside-down hood and placed onto the stigma.
Diuris (Figure 12.2 h)	To obtain pollinia use a toothpick to access the area just behind the column lobes. Pollinia can then be placed in the sticky stigma of another plant in front of the column.

Figure 12.2: **(a)** *Caladenia crebra* **(b)** *Thelymitra mackibbinii* **(c)** *Pterostylis galgula* **(d)** *Gastrodia lacista* **(e)** *Corybas diemenicus* **(f)** *Drakaea concolor* **(g)** *Caleana major* **(h)** *Diuris ochroma.* (Images: Noushka Reiter, Ryan Phillips, Belinda Davis and Tobias Hayashi)

12.2.2 Seed collection, cleaning and drying

The timing of orchid seed collection has a strong influence on seed quality and therefore its **longevity** in subsequent storage (Merritt *et al.* 2014). Seed pods (capsules) need to be harvested as close as possible to the timing of natural **dispersal** to maximise seed longevity in storage (Merritt *et al*. 2014). Seed quality continues to increase up until the point of natural dispersal as seeds dry to equilibrium with ambient conditions (Hay *et al.* 2010). Orchid seeds are generally considered **orthodox**, meaning they have a predictable increase in seed longevity as seed **moisture content** and temperature is reduced (Pritchard *et al.* 1999). As it can be difficult to return frequently to field sites, and as different species vary in their time for capsule maturation, installing breathable fine mesh or cloth tea bags over the pollinated flowers may be an effective method for maximising the amount of seeds collected by ensuring any seeds dispersed before you can return to the site is captured. It is critical that the tea cloth material is sealed with micropore tape, to ensure that seeds do not escape and that plant material is able to respire (reducing fungal infection).

Once mature orchid capsules have been collected, preparation (cleaning and drying) for long-term storage can have a significant impact on the longevity of the seeds and their ability to germinate once removed from storage. Seed quality (and rate of deterioration) is influenced by the environmental conditions of the maternal environment, as well as by the handling of the capsules during harvesting, transport, processing and storage phases (Figure 12.3, Swarts and Dixon 2017). As seed quality will reduce from the moment of harvest, preparation for long term storage should commence as soon as possible. Removing seeds from capsules or placing capsules in conditions of low relative humidity (approximately 15 % RH) will minimise risks of contamination from bacteria and fungi. Prior to placing seeds in containers for long term storage, ensure that all capsule husk material has been carefully removed as these may also harbour contaminants. This can be achieved by emptying the seed capsules on a white sheet of paper and removing all husk material with forceps under an illuminated magnifying lamp. The remaining seeds can be carefully returned to the storage container for drying prior to placing in long-term storage. Whilst data on the **desiccation tolerance** of orchid seeds is still relatively scarce, drying seeds to 15 % relative humidity at 15 °C prior to storage appears to promote long-term survival. Merritt *et al*. (2014), in their review on the storage behaviour or orchid seeds following drying, support this approach. It is recommended seeds are not dried longer than 7–10 days (Millennium Seed Bank Partnership 2015).

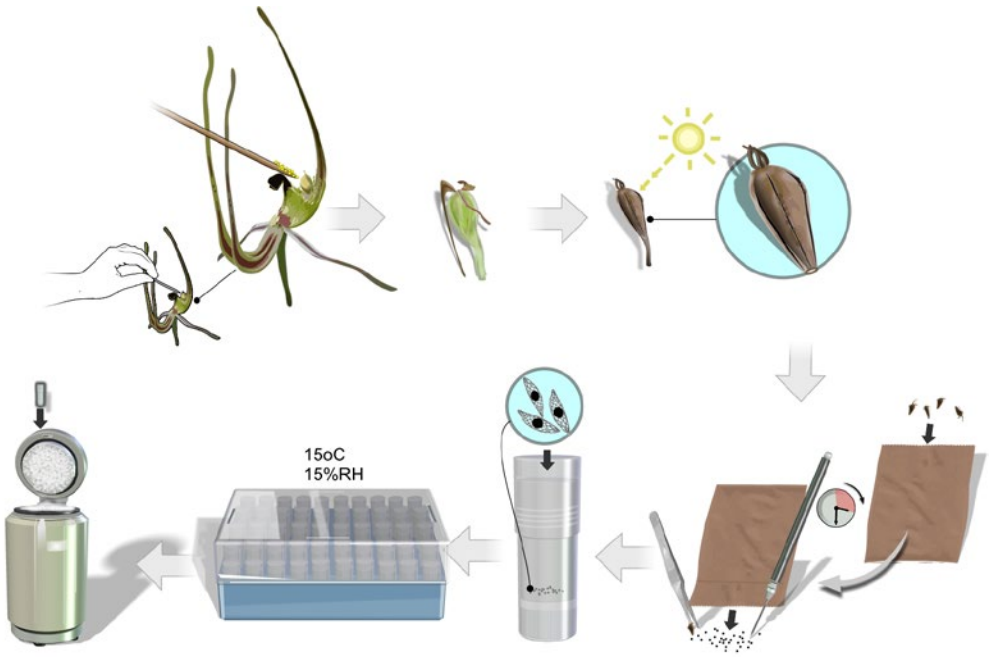

Figure 12.3: Overview illustrating hand pollination, capsule ripening, seed cleaning, drying and storage of Australian terrestrial orchids. Reproduced with permission from Swarts and Dixon (2017) Conservation methods for terrestrial orchids (J Ross Publishing: Florida USA).

12.2.3 Seed storage

Once seeds have been dried to an appropriate moisture content, they should be packaged in an airtight container to prevent moisture uptake during storage. Suitable containers are discussed in detail in Chapter 5 section 5.4.2 but can include vacuum-sealed laminated foil packets or jars with airtight lids. Due to the small size of orchid seed, do not store in plastic containers as seeds will become hydrostatic. Cool storage can then be utilised to extend seed longevity.

While orchid seeds are generally considered to be orthodox (Pritchard and Seaton 1993; Hay *et al*. 2010), there is considerable variation in their response to standard seed storage conditions (Neto and Custodio 2005). Storage at 4–8 °C is suitable for species that only survive a moderate amount of drying (desiccation-**intermediate**) or species that do not survive freezing (freeze sensitive) and has been found to keep seeds viable for periods greater than five years (Pritchard and Seaton 1993; Shoushtari *et al*. 1994). Orthodox species have also been successfully stored at 4 °C but are expected to have greater longevity when stored at -18 °C to -24 °C (Hay *et al*. 2010). Where suitable facilities are available, seeds may also be stored at -80 °C or in liquid nitrogen (or its vapour) at -196 °C (Chapter 10). Storage at -196 °C may increase seed longevity over storage at higher temperatures and may also serve to increase seed germination (Hay *et al*. 2010; Merritt *et al*. 2014). Seed of many orchid species may be stored at these lower temperatures without any pre-treatment other than drying. Pre-treatment with **cryoprotectants** may improve longevity and germination response post-storage (reviewed in Merritt *et al*. 2014) but this response can vary among species (Schofield *et al*. 2018) and further research in the area is needed (see Chapter 10).

If sufficient seeds are available, then storage at multiple temperatures (for example, -18 °C and -196 °C) is advisable (Chapter 15; Merritt *et al*. 2014) to spread the risk of viability loss at a given temperature. Ideally seeds should be stored in duplicate collections in more than one location, with collections routinely checked for viability, thereby providing invaluable data on longevity and appropriate recollection intervals (Seaton *et al*. 2018).

12.2.4 Seed viability testing

Assessing seed viability is an important part of conservation programs and seed banking for threatened species (Batty *et al*. 2001; Chapter 5). However, the seeds of orchids have several unique attributes that make testing their viability problematic; their tiny dust-like, endosperm-less seeds are dependent on infection by orchid mycorrhizal fungi for germination in nature (Smith and Read 2008) and they can be complex to germinate in ex situ culture. The small size of orchid seeds and the visibility of **embryos** under microscopy means X-ray imaging is of limited use for assessing seed viability in orchids (though an exception would be the hard-coated seeds of the genus *Rhizanthella*). Instead, seed viability testing of orchids focuses on germination trials and chemical staining.

12.2.4.1 Asymbiotic germination

The first method for orchid asymbiotic seed germination was developed in the 1800s (Moore 1849). Asymbiotic germination involves sowing orchid seeds on a nutrient medium containing a mix of sugars, micro- and macro-nutrients, growth factors and hormones, but without a mycorrhizal fungus, with the medium supplying all ingredients necessary for germination (Knudson 1922). There are many types of media used for asymbiotic orchid germination and these have often been optimised to suit particular genera or individual species.

Asymbiotic germination can be used for both propagation and assessing seed viability (Pritchard 1985; Van Waes and Debergh 1986a). This method avoids issues around incompatibility with mycorrhizal fungi (Pritchard 1985), although it is slower than symbiotic germination (Johnson *et al.* 2007; Jusaitis and Sorensen 2013) and the results can still be affected by factors such as temperature and light (Rasmussen 1992; Nikabadi *et al*. 2014). The main issue with asymbiotic germination is that the nutrient composition of an optimal asymbiotic germination medium is often genus specific (Rasmussen 1995), meaning that germination trials may be needed to establish the optimal medium for some genera before seed viability can be reliably tested.

The seeds of most epiphytic species and many species of terrestrial orchids can be germinated on asymbiotic media and there is a large body of literature detailing the history of work in this area and methods used (see papers cited in Knudson 1922; Arditti 2009). However, terrestrial orchid seedlings grown on asymbiotic media have been reported to be less vigorous, and to have a lower survival rate when transferred to soil in the shade house, than symbiotically raised seedlings (Clements *et al*. 1986; Ramsay and Dixon 2003). In addition, if asymbiotically grown seedlings are transplanted to a site in the wild from which the necessary fungal symbiont is absent, they will be unable to establish a self-sustaining population (Zettler *et al*. 2004). For this reason, it is recommended to germinate such species on a culture containing a suitable fungal symbiont (Phillips *et al*. 2020).

12.2.4.2 Symbiotic germination

The first symbiotic methods for propagation of orchids were developed by Noel Bernard (Bernard 1899; 1902). In Australia, the first published record of widespread symbiotic germination of Australian orchid species was by Warcup (1971; 1973). Warcup used a germination medium with the following components (given in g per Litre): 12 g agar; 0.3 g sodium nitrate; 0.2 g potassium dihydrogen phosphate; 0.1 g magnesium sulphate; 0.1 g potassium chloride; and 0.1 g yeast with 1 % powdered cellulose as a carbon source. This formula was later modified by Clements and Ellyard (1979) for use in symbiotic propagation of Australian terrestrial orchids, largely by replacing the powdered cellulose with oatmeal. Modifications of the basic medium developed by Warcup (1971), or of oatmeal agar, have been successful in germinating the majority of Australian terrestrial orchid genera (i.e., *Chiloglotis* (Roche *et al*. 2010), *Pterostylis* (Sommerville *et al*. 2008), *Thelymitra* (Reiter *et al*. 2018b), *Caladenia* (Reiter *et al*. 2020b), *Drakaea* (Phillips *et al*. 2011), *Diuris* (Sommerville *et al.* 2008; Sommerville and Offord 2014), and *Prasophyllum* (Freestone *et al*. 2021), though optimisation of these media may be required for a given genus or species.

Germination tests can provide accurate measures of actual seed viability when undertaken under optimal conditions (Dowling and Jusaitis 2012). However, symbiotic germination tests will be inaccurate if not appropriately controlled. When using symbiotic germination methods keep in mind that the majority of Australian orchids studied to-date have been shown to associate with more than one species of mycorrhizal fungi. For example in a study of 127 species of *Caladenia* (Spider-orchids), 10 *Serendipita* Operational Taxonomic Units (OTUs) (species of mycorrhizal *Serendipita*) that supported germination were found in the genus (Reiter *et al*. 2020). Though species generally associated with one OTU per site or within a species, other species were found to associate with up to 5 OTUs of *Serendipita*. In addition, different orchid mycorrhizal species can have different optimum temperatures for germination of seeds (Reiter *et al*. 2018b) and the germination efficacy of mycorrhizal fungi can vary between different media types (Rafter *et al.* 2016; Mala *et al.* 2017), replicates (Phillips *et al*. 2011), and isolates (Freestone *et al*. unpublished data). Seed burial trial methods developed by Rasmussen and Whigham (1993), have been suggested as alternatives to ex situ germination trials, but are not recommended with threatened species due to the large amount of seeds required and often poor results (see examples reviewed in Phillips *et al*. 2020).

12.2.4.3 Chemical staining

Fluorescein diacetate (Pritchard 1985), Evans blue (Baker and Mock 1994) and Acid Fuchsin (Vujanovic *et al.* 2000) have all been suggested as suitable stains for assessing the viability of orchid seed; however, the most popular stain for orchid seeds is 2,3,5-triphenyltetrazolium chloride (TZ or TTC; see also Chapter 5). First described by Lakon (1949) and adapted to orchid seeds by Van Waes and Debergh (1986b), the colourless aqueous **tetrazolium** solution is converted into a red substance (Formazan) in the presence of enzymes in the dehydrogenase group, which are responsible for reduction processes in living tissue (Singh 1981). In theory, living embryos stain red, but dead embryos remain colourless. However, despite the widespread adoption of TZ staining, there is ongoing debate around its accuracy. While some studies support TZ staining as an accurate method of assessing seed viability for orchids (Van Waes and Debergh 1986b; Vujanovic *et al.* 2000) others report that TZ staining over-estimates seed viability (Lauzer *et al.* 1994; Batty *et al.* 2001), or underestimates it (Stewart and Kane 2006; Lemay *et al.* 2015). Much of this uncertainty derives from the scoring of stained embryos, with a wide spectrum of different pinks, oranges and reds observed (M Freestone, *pers. obs*). The decision on what shade of red constitutes a viable embryo has a large impact on the reported seed viability. Therefore, TZ staining is most useful in providing information on relative rates of seed viability, but results need to be calibrated with germination trials to give a more accurate estimate of actual viability (Swarts and Dixon 2017).

Fluorescein diacetate (FDA) works in a similar way to TZ and follows a similar method, except that viable embryos fluoresce and therefore a microscope with a UV filter is required to assess the result of staining. While FDA staining has been shown to accurately reflect viability in European orchids (Pritchard 1985), Batty *et al.* (2001) observed that FDA frequently over-estimated seed viability when compared with symbiotic germination. Staining with FDA can also suffer from potential observer bias, with the observer required to decide what level of fluorescence in an embryo denotes it as viable (Batty *et al.* 2001).

12.2.5 Collection of tissue for fungal isolation

Orchid mycorrhizal fungi (OMF) can be isolated from parts of the plant the fungus has colonised. Mycorrhizal fungi form coils of **hyphae** (called 'pelotons') within the orchid's cells; these are easily visible under a microscope and can be extracted by disrupting the cell walls. In terrestrial orchids, OMF occur in the underground parts of adult plants and in germinating seeds. These tissues can be collected from the field (without harming the orchid) and utilised to isolate the mycorrhizal fungi necessary for germination.

12.2.5.1 Collection of tissue colonised by OMF

Tissue samples collected from the field can degrade rapidly once collected, and OMF will need to be isolated very soon after collection of the tissue, so advanced preparation is needed. Ideally, the time between collection and isolation should be a maximum of 24 hours, though some success up to four days has been observed. The tissue samples benefit from being kept cool but not frozen (4° C).

Once in the field, locate the target population and assess the number of individuals that are emergent for the season. If individuals have not been previously identified at that site, or if there are co-occurring species that could be mistaken for the target species when in leaf, it is prudent to consider collecting mycorrhiza only from flowering individuals. Collection of a voucher specimen is highly recommended. If a voucher cannot be collected due to low population numbers,

take photographs and make measurements of all the diagnostic features that could be used to confirm identification later.

The distribution of OMF within the roots, **rhizomes** or underground stems of orchids varies considerably (Figure 12.4) and thus the collection method used will vary slightly depending on the target genus. Terrestrial orchids have a much greater reliance on their OMF partner than epiphytic species typically do and, generally, OMF can be reliably obtained from any individual terrestrial orchid (Burgeff 1959; Rasmussen 1995). Methods of collection for the various tissue types are as follows.

Rhizome colonisation: OMF colonises the outer cortex of the underground rhizome. The most recent infection points tend to be closer to the growing apex. This colonisation pattern has been observed in the Australian **mycoheterotrophs** *Gastrodia* and *Rhizanthella* species. Soil should be carefully dug away from the orchid, starting at the flower/flowering spike and using shallow actions to avoid damaging the rhizome. Continue tracing the flower/flowering spike until the rhizome is reached (which may be anywhere between 5–10 cm depth) (see Figure 12.4 c). Rhizomes can be harvested whole and transported to the laboratory for peloton extraction; however, where the species is extremely rare and destructive harvesting is to be avoided, slices of the rhizome cortex can be taken leaving the rhizome **in situ** (Mursidawati 2004). Soil should be carefully replaced around the rhizome once the cortex slices have been removed.

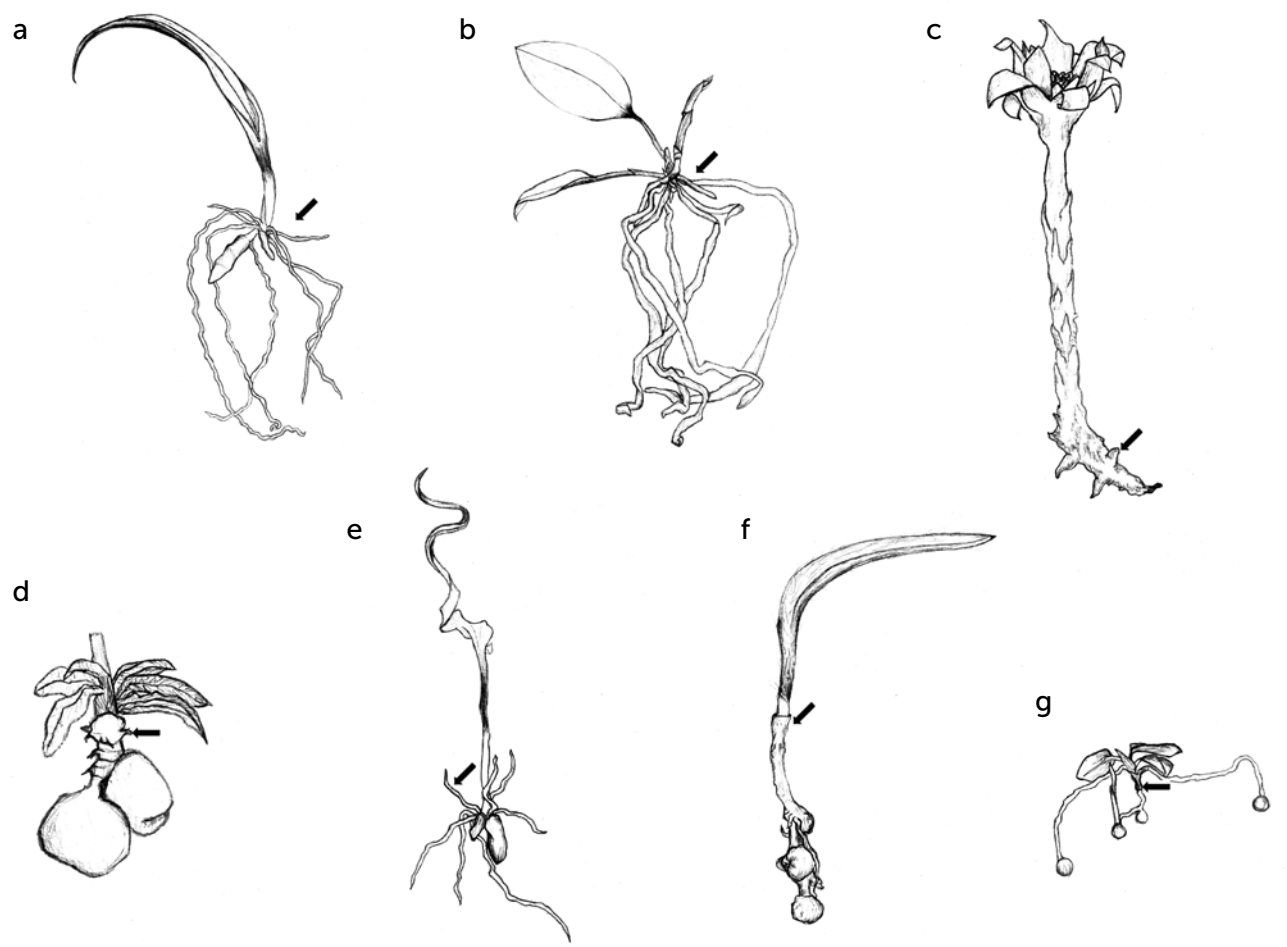

Figure 12.4: Arrows indicates sections of root, rhizome or collar region respectively where pelotons of mycorrhizal fungi were found within tissue **(a)** lateral roots of *Thelymitra epipactoides* **(b)** lateral roots of *Cryptostylis leptochila* **(c)** below ground rhizome of *Rhizanthella slateri* **(d)** collar region of *Pterostylis basaltica* **(e)** subsurface roots of *Thelymitra matthewsii* **(f)** collar region of *Caladenia rosella* **(g)** collar region of *Pterostylis tenuissima*. (Images: Dana Kitchener)

Underground stem colonisation: The vertical underground stem between the **tuber** and the soil surface is patchily colonised, with a concentration of OMF generally closer to the soil surface. Areas of colonisation may be identified by the presence of trichomes on the underground stem surface (Figure 12.4 d). This pattern of colonisation is typically seen in *Pterostylis* (Ramsay *et al*. 1986). Soil should be carefully removed around the stem, taking care not to separate the plant from its tubers. Longitudinal slices of the underground stem may be taken if destructive harvest of the entire stem is undesirable, concentrating on the cortex and taking care not to slice laterally through the entire stem. Ensure tubers remain in the ground and replace soil carefully.

Collar colonisation: OMF densely colonise a swollen region of the underground stem directly below the leaf (Ramsay *et al*. 1986). The OMF colonise the cortex of this swollen region which occurs within the first 1–2 cm of the soil surface and is recognisable by tufts of trichomes. This pattern of colonisation is seen in *Caladenia, Caleana, Chiloglottis, Cyanicula, Drakaea, Eriochilus, Ericksonella*, *Elythranthera, Glossodia, Paracaleana, Pheladenia* and *Leptoceras*. Soil can be removed close to the stem to a depth of 2 cm to reveal the swollen collar (Figure 12.4 f). Collars can be harvested entirely or by taking a longitudinal slice of the outer cortex (Phillips *et al*. 2016). Collar slices have been shown to be effective in obtaining OMF in *Caladenia*, without affecting subsequent plant emergence (Wright *et al*. 2007). The decision on whether a collar slice or whole collar is taken will depend on species rarity, distance being travelled (whole collars will fare better on a longer multi-day trip) and purpose of collection.

Root colonisation: Most terrestrial orchids display root colonisation by OMF, of which there are a few sub-types of note in terms of collection. The OMF colonise the adventitious roots (Figure 12.4 a) that arise from the central stem in clusters above the tubers (Ramsay *et al.* 1986). Terrestrial orchid genera displaying this colonisation pattern include *Calochilus*, *Cryptostylis, Diuris, Leporella, Lyperanthus, Microtis, Prasophyllum, Pyrorchis* and *Thelymitra* (Ramsay *et al*. 1986). Colonisation is typically patchy along these roots and can sometimes be identified in the field by slightly swollen cream-coloured areas or the presence of trichomes. If any of these features are observed, these roots should be targeted for harvest. Care should be taken to dig down at a short distance from the stem (approx. 5–10 cm) to avoid breaking the roots and losing them in the soil. Dig a trench along one side of the orchid, brush away the soil from the stem and trace it down to the point at which roots are growing out from the stem. Harvest multiple roots from each individual but do not remove all roots. Replace soil carefully, taking care to ensure the tuber remains attached to the emergent plant.

There are a few orchid genera and species which display slightly different root colonisation patterns. *Epiblema* and some members of *Prasophyllum* (e.g., *Prasophyllum drummondii, P. fimbria, P. parvifolium* and potentially other species of *Prasophyllum*) and *Thelymitra matthewsii* (Figure 12.4 e) possess apogeotrophic roots, known as 'pilot' roots, that grow up to the soil surface and become colonised by OMF (Ramsay *et al*. 1986). These 'pilot' roots should be targeted for collection. As they grow very close to the soil surface, digging should be undertaken carefully to be able to identify these particular roots. Scraping the surface around the adult to locate the root tips and then tracing them down to remove a root section may be the safest option here. Keep surface soil and deeper soil separate when digging and ensure the surface soil is replaced to the surface once harvest is completed, as this is the soil that contains the OMF.

Colonisation of rhizome-borne tuberous roots (Figure 12.4 b) is a colonisation pattern that is entirely confined to the genus *Cryptostylis* (Ramsay *et al*. 1986). Collection of root tubers requires digging down at a distance (approx. 10–15 cm) from the central stem of the plant as the target material is at the end of the roots. Digging a trench alongside the plant and teasing the soil by hand back towards the plant is a good option to ensure the root tubers remain intact and can be

verified as the right material for collection. Replace all soil once collection is made to ensure the plant is not damaged.

Root and collar colonisation: There are a few orchid genera which have adopted a combination of root and collar colonisation. This is seen in *Corybas* and *Cyrtostylis* (Ramsay *et al*. 1986). Collection of both collar and root sections will be important for species that have not been previously collected. It is generally accepted that collar collections are adequate for *Drakaea* (Phillips *et al*. 2011). *Corybas* roots are easily collected very close to the soil surface and can often be found adhering to the deep leaf litter they typically inhabit. Carefully pull away leaf litter from around the leaf to ascertain whether digging is required.

All material collected (collar slice, root section, rhizome) should be cleaned of visible dirt at the point of collection. Material should then be wrapped in clean paper towel and moistened with water. Small pieces of material can be placed in an airtight, 30 ml plastic tube to retain moisture. Larger pieces of material can be placed in a plastic ziplock bag. Individual plants must have their own collection receptacle, with the date, species, plant identifier and location clearly labelled. Multiple root sections from the same individual can be stored together. All material should be kept cool (4 °C) for the duration of the field trip and on return to the laboratory to prevent the sample degrading.

Research on some species of *Prasophyllum* suggests that the OMF residing in the cortical cells of adult plants may not be the same species required for symbiotic germination of the seeds (see McQualter *et al*. 2006). Some species of *Prasophyllum* associate with a diverse assemblage of OMF, many of which are not involved in seed germination of plants in the wild (Freestone *et al*. 2021). In these orchids, there can be some change in the relative abundance of OMF associates during the growing season (Freestone *et al*. unpubl. data.). When attempting to grow species with limited quantities of seed, small pilot germination trials are recommended to ascertain OMF isolates capable of germinating the orchid's seed, before larger scale germination trials are attempted.

12.2.5.2 Cleaning OMF-colonised tissue samples

Two methods are typically used to clean unwanted contaminants from tissue samples before extracting the fungi: rinsing and sterilising. Rinsing is the more cautious approach and can sometimes be adopted preferentially when working with a new species to culture.

For this method, place the tissue in a strainer and rinse it under running tap water to remove any remaining dirt. Place the tissue in a sterile plastic tube and agitate the tissue periodically and allow it to stay in the tube for approximately 5 minutes. With flame sterilised forceps, move the sample into a second tube containing sterile water in a laminar flow cabinet. Repeat agitation for 5 minutes then move the sample to a third tube containing sterile water for a final rinse.

It is not known how sterilisation affects orchid species which have a bacterial association with their OMF, as is seen in the pink cultures associated with the mycorrhizae of *Drakaea* (*Tulasnella secunda*) (Linde *et al*. 2017). When working with a species new to culture, if material is limited or if the aim of isolation is to capture the maximum diversity of fungal partners, it may be prudent to adopt the gentler method of repeated rinsing above.

The most common sterilisation method, involves cleaning the sample under running water as above, followed by soaking tissue in a solution of 0.05 % NaOCl for 3 minutes, followed by 1–2 rinses in sterile water.

12.2.6 Fungal isolation

12.2.6.1 Block method

This method is typically used for epiphytic or terrestrial species that are known or likely to carry a high diversity of orchid mycorrhizal fungi (OMF) (Fernandez Di Pardo *et al*. 2015) and follows the method outlined by Bonnardeaux *et al*. (2007). Once a root section or rhizome section has been sterilised and rinsed, remove the tissue using sterilised forceps and place it on a sterile Petri dish being used as a work surface. Cut the root or rhizome into small 1 cm sections using a sterile scalpel. Transfer a block of material onto plating media (see section 12.2.6.3), seal the Petri dish in parafilm and store in the dark. Check the plates regularly for hyphae growing out from the tissue block. When the hyphal strands are 1–3 cm in length, they may be sub-cultured by dissecting the tip of each hypha and transferring it to fresh media. The block method can yield multiple OMF and contaminants so it is particularly important to monitor plates very closely as some fungal strains can be over-run by faster growing isolates (Batty *et al*. 2002).

12.2.6.2 Peloton isolation method

The peloton isolation method largely follows the highly reliable method established by Warcup and Talbot (1967) and Rasmussen (1995). Place one side of a sterile Petri dish under the dissecting microscope over a black base. Using a sterile glass pipette or sterile plastic pipette tips and a pipette, place sterile water from a newly **autoclaved** 30 ml tube of deionised water in the middle of the Petri dish (approx. 4 cm diameter). Line up the material through the dissecting microscope so you can see the tissue in the field of view of your microscope. This will become your working area. Using sterile forceps, place the sample in the middle of the sterile water droplet. With the sterile forceps hold one end of the sample and with a sterile scalpel, use the blade to scrape open the cell walls of the tissue section releasing pelotons (fungal coils) into the water. The pelotons will look like small cream rods or grains. Once the root/collar sample has been exhausted of pelotons, remove the tissue from the working area and place it in the bin.

Pelotons now undergo serial dilution through five rinses to remove as many contaminants as possible. Using the other half of the sterile Petri dish and a clean pipette tip, plate out five droplets. Using the microscope to enhance visibility pick up pelotons using a pipette from the isolation droplet, trying to avoid picking up small dirt particles and other tissues. The aim is to take as many pelotons as possible, without transferring all of the initial water used in isolation. Drop the pelotons into the first of the five rinse droplets in the new Petri dish. Eject the pipette tip into the bin and replace it with a clean one or replace with a clean glass pipette. Place the rinsing Petri dish under the dissecting microscope to enhance the view, pick up the pelotons using a pipette from the first rinse and transfer them to the second rinse droplet. Again, try not to transfer all the water from the first rinse. The aim here is to ensure each rinse contains less and less of the original isolation water. Repeat serial rinses until pelotons are sitting in the fifth droplet ready for plating.

12.2.6.3 Seedling isolation method

Fungal isolates can also be obtained from seedlings using the 'seed baiting' technique. For rare species, this is not recommended due to substantial seed wastage (Phillips *et al*. 2020). In this method, seeds may be placed in small water-permeable packets and buried in situ close to an adult orchid (Rasmussen and Whigham 1993). Seed may also be sown in Petri dishes over soil collected from the vicinity of an adult orchid in the field (Brundrett *et al*. 2003) or over potting mix collected from a potted specimen of the target orchid (Sommerville *et al*. 2008). When the seeds begin to germinate (i.e., when they have produced a **protocorm** and a rudimentary shoot),

the seedlings can be removed from the bait and used for fungal isolation. The seedlings are rinsed several times in distilled water to remove adhering soil particles and contaminants and are then placed whole on a selective medium such as Fungal Isolation Medium (Warcup 1950). When the hyphae begin to grow out of the protocorm they can then be subcultured as described below.

12.2.6.4 Plating

Optimal plating media for OMF can differ depending on the family or genus of mycorrhizae you are working with. The most common medium used is often referred to as Fungal Isolation Medium (Clements and Ellyard 1979), which first appears as Czapek Dox Agar (Czapek (1901–02) and was first used in Australia for the isolation of mycorrhzal fungi by Warcup (1950 and subsequent publications). Soil Solution Equivalent Medium (Angle *et al*. 1991) has been useful in isolating slow growing, species of OMF such as *Tulasnella* from *Drakaea* (Phillips *et al*. 2011). To transfer the pelotons to the plating medium, use a clean pipette tip to pick up <10 pelotons and place them on the medium in 4–7 well-spaced droplets. Spacing droplets will allow some space for growth between pelotons to ensure you can subculture a clean growing hyphal tip. It also allows space to avoid subculturing any contaminants that may grow. Transfer as little water as possible to minimise contamination. Replace the lid on the plating dish between transfers. Repeat this process using the same pipette tip, plating pelotons onto different plates until all pelotons from the last rinse have been plated. Parafilm all dishes.

Typically, growing fungal cultures in plates are incubated at temperatures between 12 and 24 °C depending on the fungal genera and climatic conditions the fungal partner naturally occurs in (Batty *et al*. 2001; Reiter *et al*. 2018; Calevo *et al*. 2020). Experimental determination of the most appropriate temperature may be required if little is known about the fungal species in question. Plates should be checked within the first five days of isolation. Some species of fungi grow more quickly than others. Cultures derived from *Microtis* and *Pterostylis* can show hyphal growth within three days of plating, while cultures from *Caladenia* typically take 5–7 days and cultures from *Drakaea* and *Paracaleana* can take over a month. Ideally the first subculture should take the actively growing hyphal tip when it is only 3–5 mm from the peloton. Therefore, repeated monitoring of isolation plates is essential to capture that initial growth before it can be outgrown by contaminating fungi. Orchid mycorrhizal fungi are typified by their branching growth when viewed under the microscope and are generally white/cream in appearance, with the exception of OMF from *Drakaea* or *Caleana* which are typically pink.

12.2.7 Storage of mycorrhizal fungi

12.2.7.1 Continuous growth

Continuous growth methods are simple and inexpensive and are utilised in mycological collections all around the world (Homolka 2014). OMF cultures can be maintained on low-nutrient media (e.g., oatmeal agar, cornmeal agar) and are routinely subcultured (by transferring a small cube of agar and fungus to fresh medium) once the agar is depleted of nutrients. Subculturing is often required after around three months of storage (Zettler 1997). To increase the time between subcultures, plates can be placed in cool storage to reduce the metabolic rate of the fungus. Zettler (1997) found OMF stored for 2 years on low nutrient agar and held at 5 °C was still capable of germinating orchid seeds. Similarly, Ercole *et al*. (2013) stored ten OMF (including species of *Ceratobasidium* and *Tullasnella*) for 10–24 months at 4 °C on 2 % malt extract agar plates, with nine cultures subsequently able to stimulate germination.

12.2.7.2 Water and oil storage

The continuous growth approach has a relatively high risk of contamination by unwanted fungi or mites, and the frequent subculturing can encourage genetic or pleomorphic changes (i.e., changes in physical appearance or growth of the culture by always selecting the fastest growing hyphae), both of which can influence functional characteristics of the fungi (Gams 2002; Homolka 2014). These risks can be reduced by storing the cultures under sterile liquid. The storage under water method can maintain viable mycorrhizal fungi for up to 10 years, is cost effective and requires minimal energy and labour input. However, it is not always reliable, as rates of retrieval from water storage can be poor and the time fungi can be kept in storage is variable.

In this method, the OMF is initially cultured on low nutrient agar, then 10–30 pieces of colonised agar (discs or plugs ~0.5 cm^2 in size) are added to a sterile container half filled with sterile distilled water or sterile mineral/paraffin oil. The containers are held at room temperature or lower, with cultures remaining viable for long periods (potentially decades; Homolka 2014). To revive cultures stored in this way, a piece of colonised agar is removed from the container **aseptically**, with excess water or oil drained away by holding the piece against the side of the vial or by placing the agar piece on the outer edge of a fresh agar plate and moving it over the agar surface in a spiral motion towards the centre of the plate. Typically, several pieces will be removed at once in case one fails to produce fungal growth, but the remainder can be re-sealed and returned to storage.

While liquid storage can reduce the rate of fungal growth, slow growth is still occurring and therefore **mutations** in the OMF are still possible (Homolka 2014). Further, the method may not be suitable for all species, with Richter and Bruhn (1989) reporting only 54 % of mycorrhizal basidiomycetes surviving 4–48 months storage in cold sterile water. It is thus not the recommended way of storing Australian orchid mycorrhizae.

12.2.7.3 Cryostorage

Cryostorage is the storage or preservation of tissue at extremely low temperatures and includes storage at -80 °C or -192 °C. Cryostorage is a more reliable method for the storage and retrieval of mycorrhizal fungi and it is proposed that threatened orchid seeds and associated mycorrhizal fungi should be stored in this more secure system. The Royal Botanic Gardens Victoria has been storing OMF at -80 °C for the last ten years, with reliable retrieval of cultures that are regularly used for germination and propagation of threatened orchids from *Serendipita*, *Tullasnella* and *Ceratobasidium* (Reiter pers obs). This system requires no maintenance and has less operational safety risks associated with it than storage on liquid nitrogen or its vapour.

Liquid nitrogen can be used for both seeds and fungal collections; however, the method can be reliant on regular labour to maintain an adequate liquid nitrogen level if the storage system is not automated. In addition there are significant health and safety concerns regarding the storage and handling of liquid nitrogen. It also relies on regular funding for the supply and delivery of the liquid nitrogen, which is expensive. An alternative to this is storage in nitrogen vapour, which uses much less liquid nitrogen but can maintain a temperature of -192 °C in a well-insulated tank. This is the system currently in use at the Australian PlantBank. While expensive to set up, it requires no on-going maintenance other than periodic refilling of a liquid nitrogen storage tank by a contracted supplier.

12.2.7.4 Storage at -80 °C

After the initial expense, a -80 °C freezer offers a reliable, effective and economical system, that requires little maintenance, labour input, energy and funding. It is more likely to be maintained within an organisation over the long term due to the lack of labour cost, which is an important goal for a conservation collection. As for any storage method, a monitoring and alarm system is recommended.

Trials at the Royal Botanic Gardens Victoria over the last 10 years have shown that mycorrhizal fungi can retain their viability after storage at -80 °C if they are stored and retrieved correctly using the following method:

1. Inoculate the agar plate with fungi, usually 1 piece in centre of the plate but more pieces if the OMF is slow growing (any growth agar is suitable).
2. Incubate until culture is at least half covering the plate, preferably not reaching the sides of the plate.
3. Using a sterile scalpel, cut plugs of agar with fungal growth from the growing edge. Add plugs to each cryotube (e.g Nalgene® cryogenic 2 ml vials). Insert cryotubes into a Corning® freezing container or equivalent (these are containers that slow the rate of cooling to 1 °C per minute).
4. Place cryofreezing container with isolates into -80 °C freezer for at least 4 hours, but preferably overnight.
5. Remove frozen cryotubes from the freezing container in the -80 °C freezer and place in a storage box and immediately return to -80 °C freezer.
6. After at least 1 week, open one ampoule and test for growth and lack of contamination. If the culture fails to grow, open a second one and if this fails, repeat the process again from the beginning.
7. Use a chart or a database to keep a record of where each culture is kept in each box so that the cultures can be found and retrieved quickly.

12.2.7.5 Concurrent storage of seeds and fungi using encapsulation

An alternative storage technique that enables the concurrent storage of seeds and fungi is 'encapsulation'. In this technique, orchid seeds and a fungus appropriate for initiating germination are bound together with sodium alginate to form a jelly-like bead (Wood *et al*. 2000). The bead is then dehydrated, either by immersion in a sucrose solution followed by air-drying (known as 'encapsulation-dehydration') or by immersion in a solution of cryoprotectants such as PVS2 (known as 'encapsulation-**vitrification**'; see Chapter 10 for further information on PVS2). The encapsulated seeds and fungi can then be stored in small plastic vials at -20 °C, -80 °C or -196 °C (see Case Study 12.3). When needed, the beads can be thawed at room temperature (Sommerville *et al*. 2008) or more rapidly in a heated water bath.

The encapsulation-dehydration technique has the benefit of reducing the steps required to germinate the seeds following removal from storage. The drawbacks are the need for a storage protocol that suits both seeds and mycorrhizal fungi. Beads produced by encapsulation-vitrification need to first be removed from the storage solution thawed and then washed to remove cryoprotectants before transfer to a suitable medium (such as oatmeal agar) for germination. The thawed beads are then incubated at a suitable temperature until germination occurs. Seedlings produced in this way have been found to be very robust (Sommerville and Offord 2014).

Encapsulation has the benefit of enabling the seeds and fungi to be stored in a single location, simplifying the maintenance of collection records and ensuring a suitable fungus is always available to germinate the seeds when required. The encapsulation techniques are also suitable for preserving protocorms (partially germinated orchid seeds) and **protocorm-like bodies** generated from shoot tissue (Saiprasad and Polisetty 2003; Bustam *et al*. 2013; Gantait and Sinniah 2013). While encapsulation-dehydration has been found to maintain seeds and fungal viability for up to two years in liquid nitrogen storage (Sommerville *et al*. 2008), research to confirm the suitability of the encapsulation methods for longer term storage is required.

Case study 12.3: Encapsulation of orchid seed and mycorrhizal fungi for conservation and propagation

Karen D. Sommerville, John P. Siemon, Catherine A. Offord

Effective seed banking for terrestrial orchids requires storage of both the seeds and the fungal symbiont/s necessary to germinate that seed. Such collections are often stored separately using very different techniques. An alternative method, that shows promise for simplifying both storage and subsequent propagation, incorporates the simultaneous encapsulation of seeds and fungus in a sodium alginate bead (Wood *et al*. 2000). We applied this protocol to two threatened orchid species from NSW to determine the combined effects of storage temperature and storage duration on recovery of the fungal symbionts and germinability of the seeds (Sommerville *et al*. 2008). We monitored survival following transfer to potting mix to investigate whether the process would assist in producing seedlings fit for transfer to a field environment (Sommerville and Offord 2014) and also tested the protocol on two additional threatened species (Figure 12.5).

Seed and fungal hyphae were obtained from ex situ collections of *Pterostylis saxicola* and *Diuris arenaria* held at The Australian PlantBank. The seeds were sterilised then added to a solution of 2 % w/v sodium alginate (Sigma®) along with fungal hyphae scraped off the surface of several oatmeal agar plates (Figure 12.6a-c). The resulting suspension was pipetted drop by drop into a solution of 100 mM calcium chloride to form individual beads (Figure 12.6d-e). The beads were transferred to a solution of 0.75 M sucrose for 24 h, then dried for 18 $\pm$ 1 h in a laminar flow cabinet (Figure 12.6f). Dried beads were transferred to sterile **cryopreservation** vials for storage at 23, 4, -18 or -196 °C. The beads were withdrawn from storage at intervals of 0, 3, 6, 12 and 24 months and were sown individually into 60 mm plastic Petri dishes containing 10 mL oatmeal agar. The Petri dishes were arranged in five stacks of ten (each stack constituting one replicate) and incubated in the dark at 23 $\pm$ 2 °C for 12 weeks. Beads were inspected fortnightly for fungal growth and germination.

The fungal recovery rate declined from 100 % (immediately after bead drying) to 0–2 % after three months' storage at 23 °C for both orchid species, and after 12- and 24-months' storage at 4 °C for *D. arenaria* and *P. saxicola*, respectively. No germination occurred in the absence of fungal growth; therefore, a reduction in fungal recovery was accompanied by a reduction in germination rate for both species. Storage for up to 24 months at -196 °C for *D. arenaria*, and at -18 or -196 °C for *P. saxicola*, caused no significant reduction in fungal recovery or germination ($P > 0.05$; Figure 12.7). Seedlings germinated **in vitro** were successfully transferred directly to potting mix; those transferred to potting mix in late autumn (May) showed 100 % survival and produced tuberoids of a good size by the end of the growing season (October – November).

The encapsulation-dehydration protocol was also successfully tested on *D. flavescens* and *D. tricolor*, indicating that the technique has great potential for use in conserving terrestrial orchids and their fungal symbionts. The success of transferring germinated beads directly to potting mix was particularly promising as the transition from in vitro to ex vitro environments can be difficult for some species and has often resulted in poor survival for orchid species in the past.

Figure 12.5: Terrestrial orchids preserved by encapsulation-dehydration of seeds and compatible mycorrhizal fungi: **(a)** *Pterostylis saxicola*, **(b)** *Diuris arenaria*, **(c)** *Diuris flavescens*, **(d)** *Diuris tricolor.* (Images: Karen Sommerville, from Offord and Sommerville 2014. The original article can be found at doi:10.17660/ActaHortic.2014.1039.28)

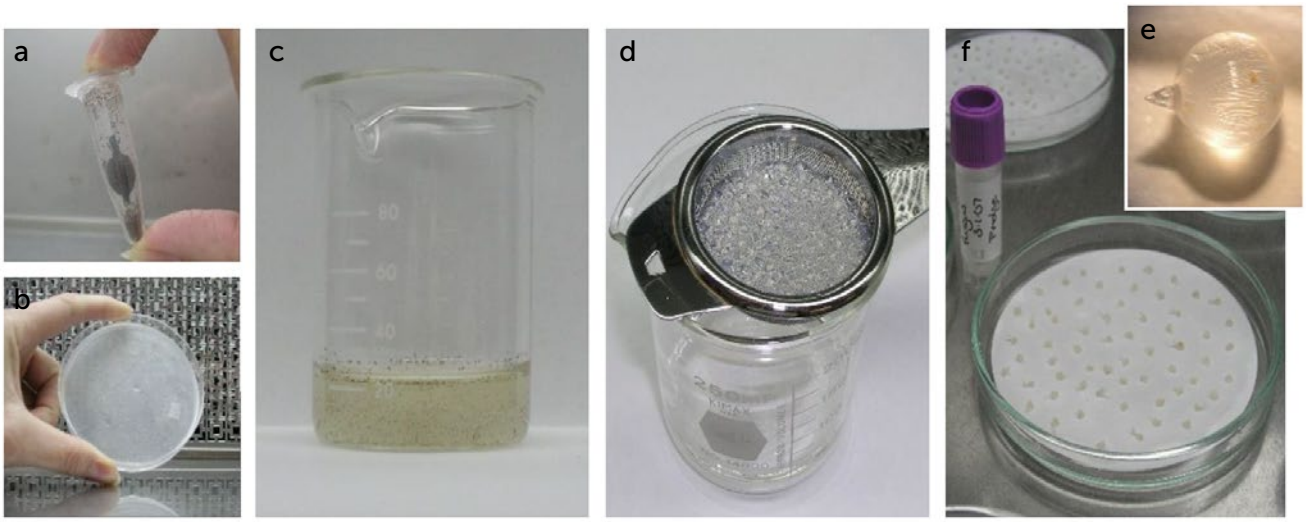

Figure 12.6: The encapsulation-dehydration process entails combining seeds **(a)** and fungal hyphae **(b)** into a solution of sodium alginate **(c)** that is pipetted drop by drop into a solution of calcium chloride to form small beads **(d, e)** that are then pre-treated in a solution of sucrose and dehydrated **(f)**. (Images: Karen Sommerville, from Offord and Sommerville 2014. The original article can be found at doi:10.17660/ActaHortic.2014.1039.28)

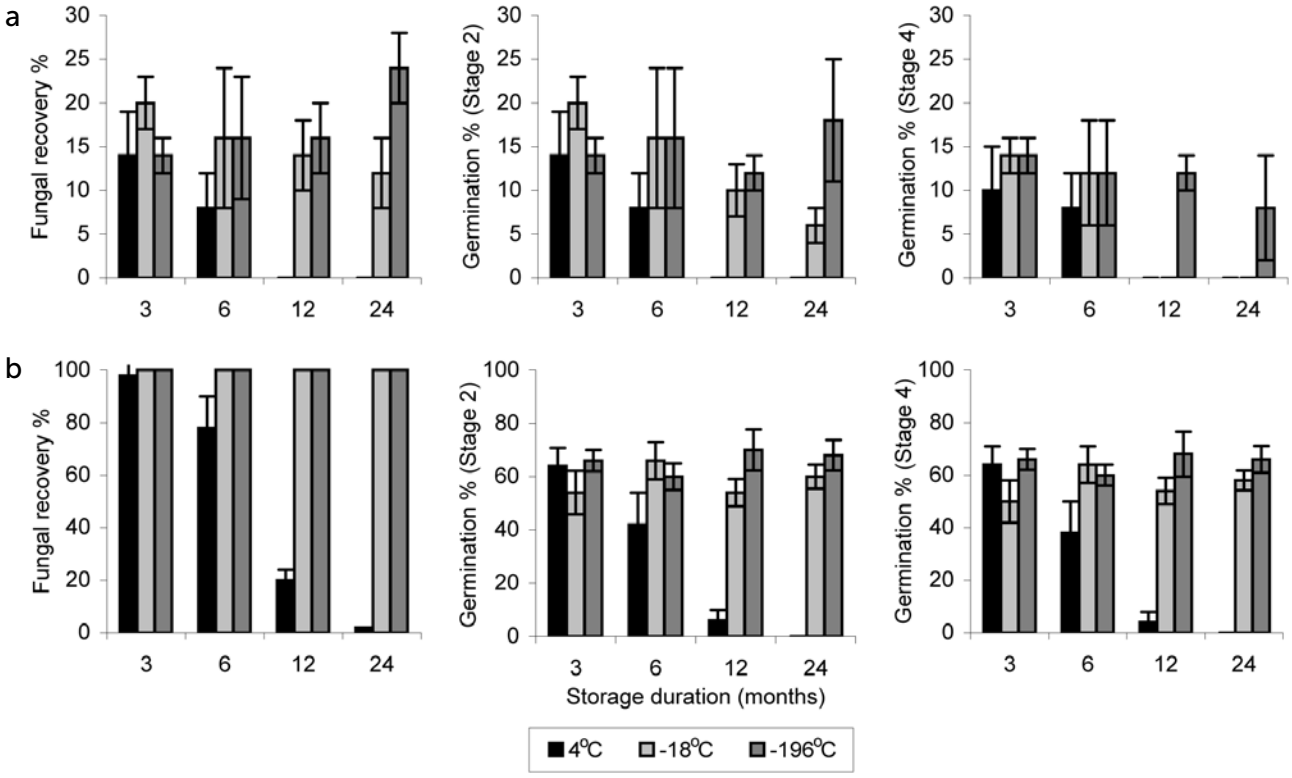

Figure 12.7: The effect of storage temperature and duration on the growth of fungi and germination of seeds encapsulated in sodium alginate beads for: **(a)** *Diuris arenaria* and **(b)** *Pterostylis saxicola*. Values are means ± SE (n = 5). Germination Stage 2 refers to the production of protocorms; Stage 4 refers to the production of a shoot. (Figure from Offord and Sommerville 2014. The original article can be found at doi:10.17660/ActaHortic.2014.1039.28)

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12.3 Utilising rhizobia for conservation outcomes of Fabaceae

12.3.1 Considerations when sampling nodules

Environmental conditions play an important role in the abundance and effectiveness of rhizobial associations and therefore need to be considered before sampling. Nodule abundance within the rhizosphere of a legume species can change given the soil depth, root type, and the size/age of the plant (Howesion *et al.* 2016; Dinnage *et al.* 2019). Typically, nodules are abundant amongst lateral or surface roots of the host but may differ when sampling from previously unstudied groups of legumes. The geographic range of a host species may also influence rhizobial specificity (e.g. Thrall *et al.* 2000).

If sampling from a novel host species, nodules from multiple plants across different populations should be sampled to capture the greatest representation of diversity. Capturing the greatest diversity of rhizobial strains will aid experiments on determining optimal rhizobial strains for plant growth in ex situ populations. For sampling, select for the most vigorous plants in the population, as these may indicate greater overall **fitness** and healthy microbial communities (Howesion *et al.* 2016). Perennial legumes should always be tagged in the field for re-identification purposes. Time, resources, and the rarity of plant material at sites may also influence the sampling process. When sampling in the field, collect both nodules and adjacent soil samples, which can be used as a secondary source of capturing rhizobial diversity via rhizobia trapping (see 12.3.2 nodule collection, isolation, and storage). This alternative collection measure is useful when sampling from difficult to access areas, or from limited plant material or nodules at a given site.

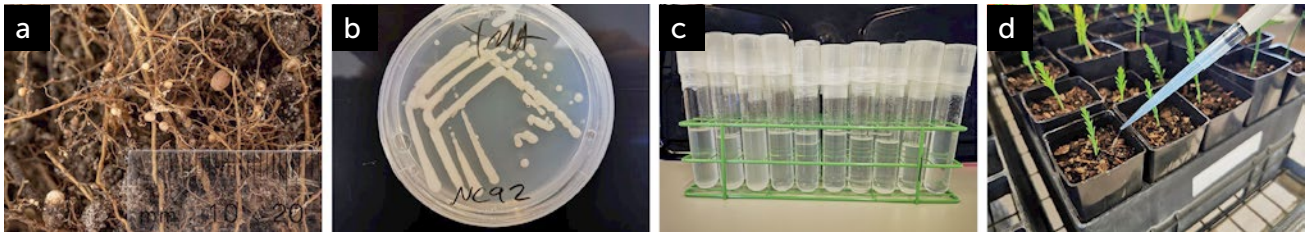

Figure 12.8: (a) Root nodules (b) *Rhizobia* plate cultures (c) *Rhizobia* liquid cultures (d) Inoculating *Sphaerolobium* with *Rhizobia*. (Images: Richard Dimon)

12.3.2 Nodule collection, isolation and storage

Rhizobia can be obtained by directly collecting nodules in situ, or by inoculating ex situ plants with soil samples (also known as rhizobia trapping). A version of rhizobia trapping can also include seed baiting of the target legume species in situ. Further details regarding nodule collection, inoculation, and storage for each of the mentioned techniques can be found in Howieson *et al.* (2016).

When ready to isolate rhizobia from nodules, ensure a small segment of the root is still attached to the nodule when excised. This avoids mishandling the nodule, which can easily rupture, causing contamination and/or sterilant to enter the nodule. Nodules must first be surface sterilised before the internal symbionts can be freed into liquid suspension and plated onto growth media. A common practice involves soaking nodules in 70 % (v/v) ethanol, followed by sodium hypochlorite with a wetting agent. However, the optimal concentration and duration of ethanol/bleach can vary depending on the host species (Hungria *et al.* 2016a). The use of hydrogen peroxide or calcium hypochlorite instead of commercially available sodium hypochlorite may be a suitable alternative to surface sterilisation of sensitive nodules (e.g., Hungria *et al.* 2016a and Wang *et al.* 2018). After surface sterilising nodules, all further steps should be completed in a laminar flow cabinet using aseptic technique. Details regarding nodule isolation, media preparation, and identifying/culturing pure rhizobial isolates can be found in Hungria *et al.* (2016a).

12.3.3 Storage

After repeated subculture and/or prolonged storage at ambient conditions, rhizobial strains have the potential to lose desirable properties (Hungria *et al.* 2016b). There are several methods for storing rhizobial cultures which vary in the longevity and success of re-culture. For short-term research (daily or weekly use), pure agar cultures can be refrigerated for approximately two months. Ensure samples do not freeze during this time, so as not to crystallise water inside the

cells. To store cultures for long-term research or conservation, samples can be frozen for several years with the use of cryoprotectants (e.g., glycerol) and/or a standard rate cell freezing container. Long-term storage using freeze-drying techniques (i.e., **lyophilisation** ampoules) can maintain the viability of rhizobial cultures over several decades (see Hungria *et al.* 2016b).

12.3.4 Inoculation and evaluating effective strains

Once pure rhizobia are isolated, symbiotic strains can be confirmed by inoculating seeds or seedlings and quantifying the effectiveness of different strains in developing nodules. The most common form of inoculation involves watering seeds/seedlings with a pure rhizobial, liquid culture, or a scraped agar culture suspended in sterile water (Yates *et al.* 2016a) (Figure 12.8). Ensure seedlings inoculated with different strains are kept isolated from one another and adequate control treatments are included for comparison (i.e., seedlings with no inoculation and/or fertiliser). After inoculation, allow a pre-determined period of plant growth for nodulation to occur before sampling the roots to assess the results.

The method for evaluating the growth response of the host with the aid of different rhizobial strains depends on the aims of the experiment. From a conservation viewpoint, the overall aim of rhizobial isolations is to discover ideal symbiotic strains that benefit plant growth and vigour for ex situ insurance populations, as well as to obtain a diverse rhizobial collection to aid in the conservation translocation of Fabaceae species. Collecting dry plant biomass is a commonly used technique to compare strain success but is not ideal when working with threatened species due to the destruction of rare plant material. Depending on the health of the plant, semi-destructive techniques may be useful, such as randomly selecting nodules to measure the dry nodule weight between different strains. Non-destructive scoring of nodule abundance using published charts (e.g., Yates *et al.* 2016b p.162) confirms the presence of symbiotic rhizobia associating with the host species, but is not a reliable indicator of strain effectiveness when used by itself (Yates *et al.* 2016a). Depending on the host species, other non-destructive measures may be useful (when used in conjunction with scoring nodulation); for instance, scoring plant height/branching, seed yield, root biomass and plant fresh weight (e.g., Huang and Erickson 2007).

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Chapter 13

Special collections and under-represented taxa in Australasian ex situ conservation programs

Tom North*, Caroline Chong, Adam Cross, Karin van der Walt, Daniel Ballesteros*

13.1 Introduction

Some plants have 'special' types of **germplasm** (spores, **gametophytes**, shoot tips and **gemmae**), while others have 'special' life history stages or growing requirements. Collectively these groups are underrepresented in collections but through an understanding of the physiology of the tissues selected for **preservation**, or the special requirements of the plants, ex situ collections of these species are achievable. This means that a wider range of species are made available for use in **rehabilitation**, **reintroduction** and **ecological restoration**, as well as in horticulture and living collections.

The subject of special collections **conservation** is relatively new in Australia. Most of the work done to date on the conservation of these types of collections have come from institutions in Europe and the U.S.A. Interest in the more obscure plant **taxa** and their germplasm is more recent and is driven by recognition of our need to include all biota in the restoration of landscapes. A growing number of these taxa are likely to be recognised as threatened as knowledge of them improves and environmental pressures increase.

In this chapter we will first cover germplasm from land plants (i.e., **embryophytes** – vascular plants and **bryophytes**); algae (defined broadly) are not included (Figure 13.1). Fungi and lichen are also excluded. We will introduce the **ex situ conservation** options available for non-seed bearing land plants, which encompass bryophytes (i.e., liverworts, hornworts and mosses) and **pteridophytes** (i.e., ferns and fern allies) (see Figure 13.1). Germplasm types for these plants include spores, gametophytes, shoot tips (see also Chapter 9, **Tissue Culture**), and gemmae. The next section will cover pollen collection, curation and storage from **seed** bearing plants.

In the final section, ex situ conservation of land plants such as parasitic plants and carnivorous plants, currently underrepresented in collections, is also discussed. Even though seed collection and banking for these groups of plants is conventional, information on their ex situ conservation in the Australian context has not been collated previously (though see Clarke *et al*. 2018a and

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Cross *et al*. 2020). Ex situ strategies for germplasm conservation should be based on a thorough understanding of the physiology of the tissues selected for preservation and the interaction of the key factors affecting their **longevity** (Custodio *et al*. 2020). For this reason, we will emphasise the basic physiology of the diverse germplasm covered, and how the physiology and intended use of the germplasm inform the selection of storage conditions.

13.1.1 Non-seed bearing plants

The Australian pteridophyte flora (ferns and **lycophytes**) comprises 2 classes, 14 orders, 32 families, 134 genera and 528 species and subspecies (Field 2020). Overall, 208 species are **endemic** to Australia, with Queensland having the highest species diversity and endemism by jurisdiction, and Lord Howe Island having the highest concentration of species and endemics per unit area (Field 2020). In Australia, there has been a long history of fern propagation largely as a consequence of the 'fern fever' of the 19th century, especially in Victoria, and the laws introduced to protect the tree ferns around Melbourne are some of the oldest conservation laws in existence (Bonyhady 2000). There are large commercial nurseries with the knowledge to successfully propagate ferns from spore. There has been less emphasis, however, on the banking of pteridophyte spores for long-term protection of taxa in Australia.

For bryophytes, there is an ongoing effort to update the National Species List[62], but based on the current extent of information, we consider that the Australian bryophyte flora comprises 1,007 species of mosses, 841 liverworts and 30 hornworts, there being 224 species of mosses endemic to Australia (McCarthy 2006; Niels Klazenga and Matt Renner, pers. comm.). Whilst there are very few nationally listed threatened bryophytes and pteridophytes, it reflects the issue that this group of plants has been less well surveyed and documented. They are threatened by the same processes as seed-bearing plants. The ex situ conservation of bryophyte germplasm is rare in Australia (Scott *et al*. 1997).

13.1.2 Pollen

Pollen cannot be used to regenerate a full plant (as possible using seeds, spores, or other vegetative tissues). However, pollen has a vital role in the conservation of crop and wild species. In the ex situ conservation context, pollen is most frequently used in breeding programs, primarily for horticulture, forestry and agriculture. It has a key role to play in the recovery of species where male and female inflorescences are present at different times or separated by significant distance. Preserved pollen may also be useful for taxonomic, **phylogenetic** or ecological studies, but in these cases, pollen is not necessarily needed alive (e.g., pollen microscopy slides for taxonomy and phylogeny[63]).

13.1.3 Under-represented taxa

The interest in parasitic and carnivorous plants and other under-represented taxa is driven by a better understanding of their role in our landscapes. They are found in every environment across Australia and play a significant role as key providers, especially as a food resource. They are under-represented on national threatened species lists. The collection of their seeds is standard according to the protocols developed for more common taxa (Chapters 4 and 5). However, due to the abundance of relatively large, oily seeds (e.g., Santalaceae, Loranthaceae) or very small seeds (e.g., Droseraceae) – seed types known to present storage difficulties – more work is required to understand their longevity during long-term storage, and to optimise their propagation and end-uses.

62 https://biodiversity.org.au/nsl/services/
63 https://www.kew.org/science/collections-and-resources/collections/microscope-slide-collection

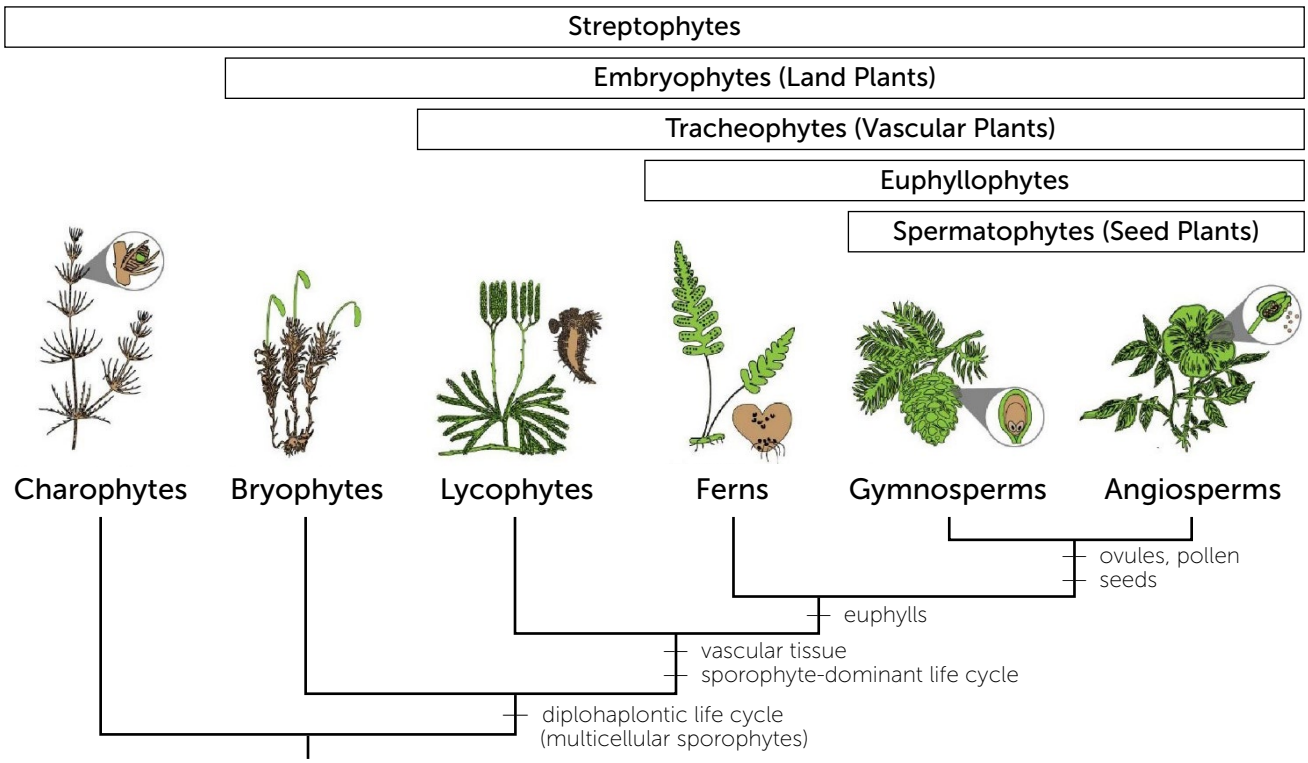

Figure 13.1: Simplified **phylogenetic** tree showing major **clades** of plants in this chapter, in relation to the **charophyte** algae (the closest relative to land plants). Gametophyte phases (brown) and **sporophyte** phases (green) are illustrated using examples from each lineage. All embryophytes (land plants) have multicellular gametophytes and multicellular sporophytes. Source: Sigel EM, Schuettpelz E, Pryer KM, Der JP (2018) Overlapping patterns of gene expression between gametophyte and sporophyte phases in the fern *Polypodium amorphum* (Polypodiales). *Frontiers in Plant Science* **9**, 1450. doi:10.3389/fpls.2018.01450. Reproduced under terms of the Creative Commons Attribution License (CC BY 4.0).

13.2 Non-seed bearing plants

Non-seed bearing land plants include the bryophytes and pteridophytes (Figure 13.1). In this section, collection, curation and storage methods will be outlined, as well as propagation protocols. We will highlight some aspects of the basic physiology of the germplasm covered (e.g., **desiccation tolerance** and longevity), and how this and the final use of the germplasm determine the required storage conditions.

13.2.1 Collection of germplasm

13.2.1.1 What types of germplasm can we use for non-seed bearing plants?

Diverse types of germplasm can be collected to conserve and propagate non-seed bearing land plants. In the case of pteridophytes (Figure 13.2), the preferable germplasm source is the spores. Spores can be considered analogous to seeds in that (1) significant **genetic diversity** can be preserved in a small space, (2) the technical needs for their collection and long-term storage are achievable in seed banks, and (3) spores are relatively easy to propagate (Section 13.2.5.1 and Case Study 13.1). Mature spores can be collected from the **sporophytes**, the most obvious and common form of the pteridophyte life cycle found in the field (Figure 13.2). However, mature spores may not always be found in the field or, if harvested, they may not be of optimal quality and viability (Ballesteros and Pence 2018). In that case, gametophytic tissues or shoot tips from young sporophytes of the target species can be stored ex situ using **cryopreservation** and propagated **in vitro** (Figure 13.3; Section 13.2.5.2).

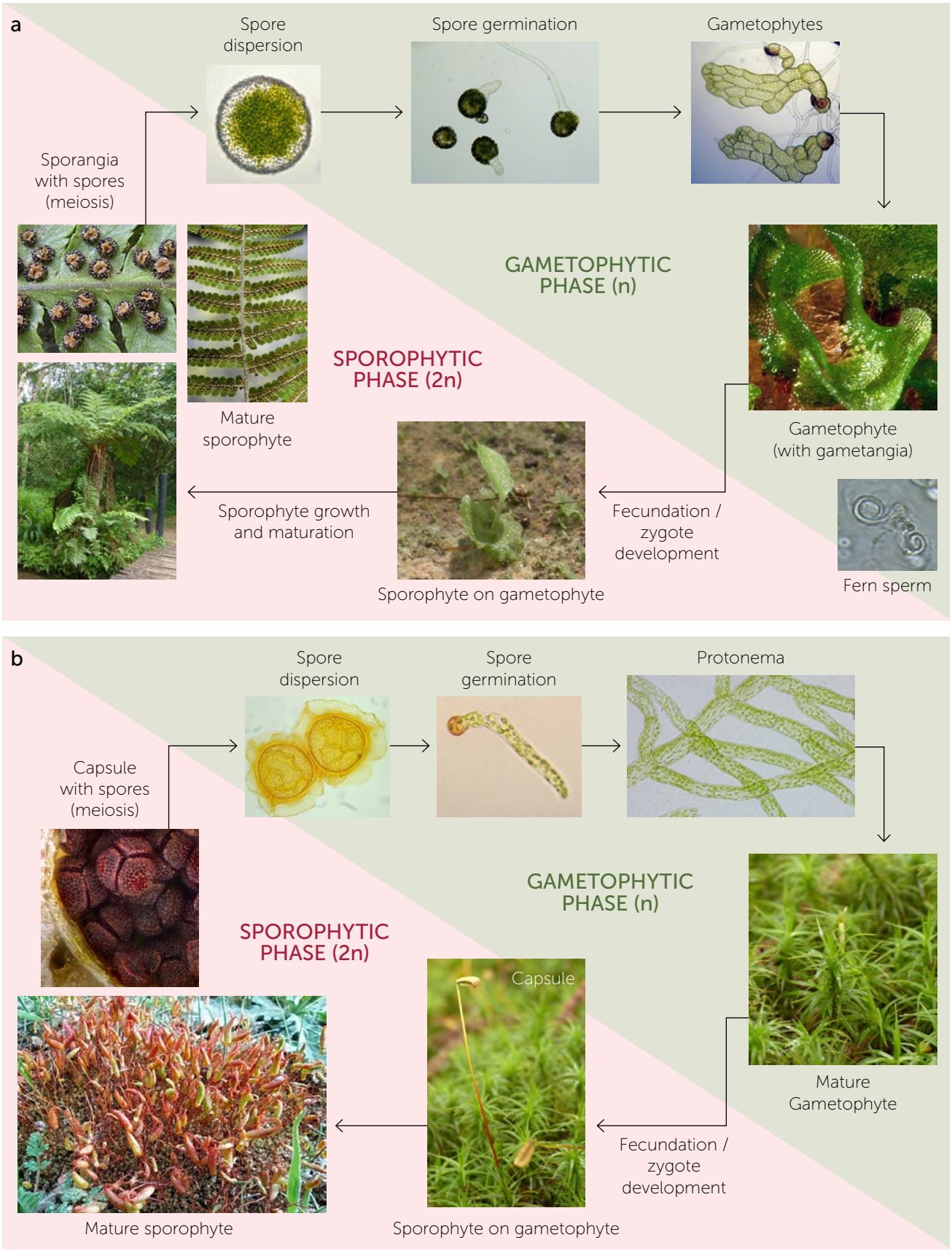

Figure 13.2: Life cycles of **(a)** pteridophytes and **(b)** bryophytes. (Images: (a) Daniel Ballesteros; (b) Capsules with spores from Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Riccia_sorocarpa_spores.jpg), Spore dispersion from Chris Cargill at ANBG (https://www.anbg.gov.au/bryophyte/photos-captions/asterella-drummondii-146.html), Spore germination from Chris Cargill at ANBG (https://www.anbg.gov.au/bryophyte/photos-captions/funaria-168.html), Protonema from Anja Martin of Reski Lab, University of Freiburg (https://es.wikipedia.org/wiki/Protonema#/media/Archivo:Physcomitrella_Protonema.jpg). Images from Chris Cargill used under Creative Commons license © 2012 Australian National Botanic Gardens and Australian National Herbarium, Canberra. Remaining images: Daniel Ballesteros.)

In the case of bryophytes, the gametophyte phase of the life cycle is the most accessible and commonly found in the field (Figure 13.2). It is these gametophytic tissues that are typically harvested and cryopreserved in bryophyte ex situ conservation programs. Alternatives to the gametophyte germplasm include spores and gemmae due to their high desiccation tolerance, and the relative simplicity of the storage methods needed, but conservation methods for these **propagules** are not standardised.

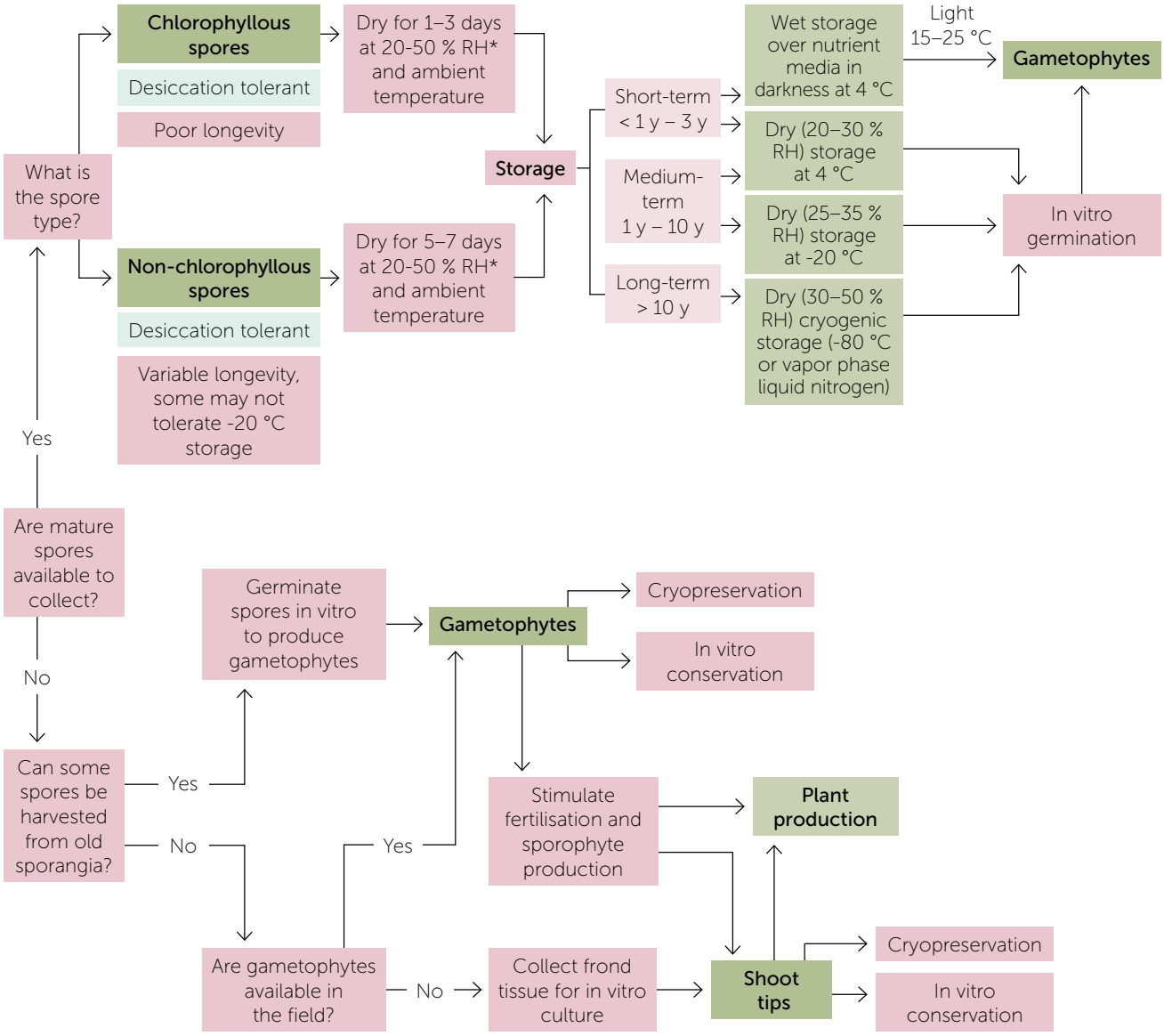

Figure 13.3: Decision tree for the collection and conservation of pteridophyte germplasm based in their field availability, their physiological properties (desiccation tolerance and longevity) and the length of the storage time required (based on Ballesteros and Pence 2018; Nebot *et al*. 2021). * indicates 'depending on final storage temperature'. This tree can also be used as flowchart for pteridophyte plant production, depending on the germplasm source available for harvest.

Case Study 13.1: Ex situ conservation of a critically endangered fern

Caroline Chong, Amelia Stevens, Alasdair Grigg, Tom North, Joe McAuliffe, Lydia K. Guja

Pneumatopteris truncata is listed as critically endangered under the *Environment Protection and Biodiversity Conservation Act* 1999 (EPBC Act). Although *P. truncata* occurs across Indonesia and South East Asia, the Australian regional **population** is confined to Christmas Island, a geographically isolated island in the north-east Indian Ocean (off Western Australia). Here, the **taxon** is known as the Dales Waterfall Fern and occurs as three subpopulations across two locations, including The Dales Ramsar site (Butcher and Hale 2010; Figure 13.4a). The large, erect fern has fronds to 120 cm and occurs only within closed forest where springs create permanently wet limestone **habitat** (Figure 13.4b).

Ex situ conservation was prioritised as a critical response to insure against loss of the wild population. Spore from wild subpopulations was collected and **germination** and propagation techniques were trialled. This work has established ex situ spore and plant collections (more than 100 plants), developed protocols for germination and propagation of this species and identified priority future research for threatened fern species (Chong *et al*. 2021).

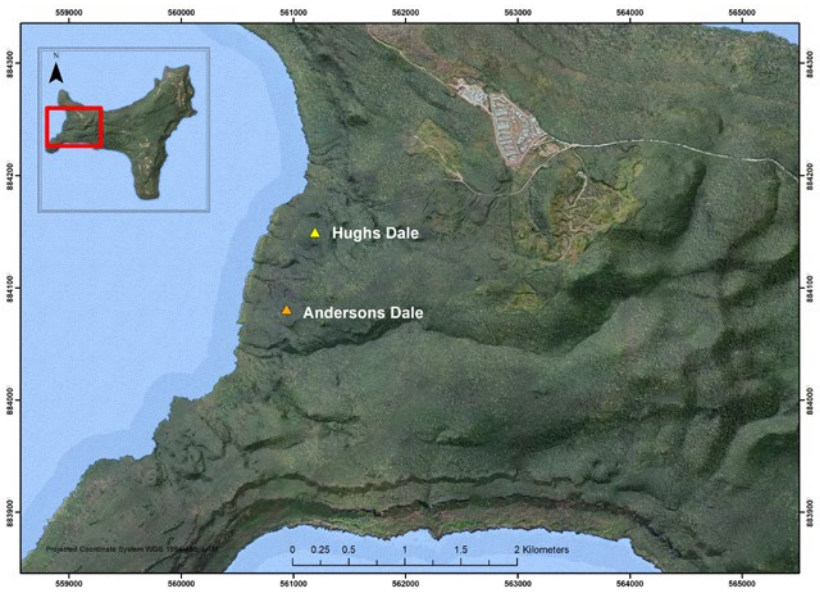

Figure 13.4a: Location of the Hughs Dale and Andersons Dale subpopulations of *Pneumatopteris truncata* on Christmas Island, Indian Ocean.

Figure 13.4b: *Pneumatopteris truncata* (foreground) in forest habitat. (Image: Alasdair Grigg)

Fewer than 50 mature individuals of *Pneumatopteris truncata* are known and the small population size provided the basis for listing the species as Critically Endangered in 2004 (Threatened Species Scientific Committee 2004; Butcher and Hale 2010). Surveys conducted throughout the late 1980s, 2002 and 2003 recorded only 45 mature individuals at two subpopulations (Holmes and Holmes 2002). Since 2010, Parks Australia has conducted annual subpopulation monitoring at Hughs Dale. Monitoring at this site has identified extreme fluctuations in the number of individuals present (juvenile and mature), ranging between 0 to 500 individuals across years with the proportion of juvenile plants typically exceeding 80 % (A. Grigg, pers. comm. 2020). A comparison of the number of individuals per year with annual precipitation indicates that larger numbers of individuals may be positively associated with above-average rainfall and declines associated with dry years (Figure 13.5). Disturbances such as cyclone damage to canopy cover negatively impact the population.

Pneumatopteris truncata was prioritised for ex situ conservation on the basis of **threats**, observed population fluctuations and cost-benefit ranking (Di Fonzo *et al*. 2017).

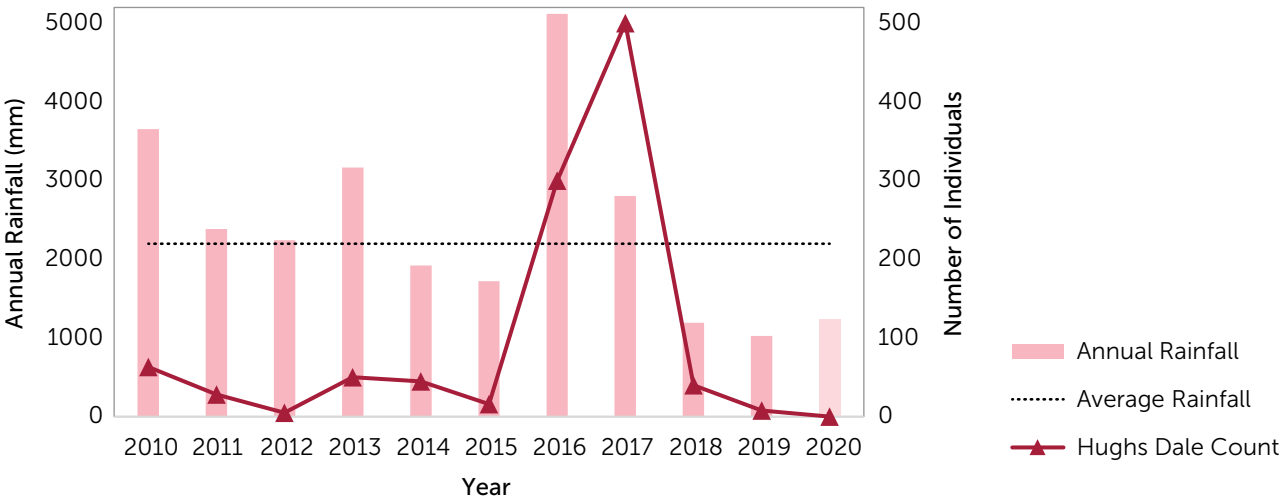

Figure 13.5: Number of individuals of *Pneumatopteris truncata* recorded at the Hughs Dale subpopulation from 2010 to 2020 (green line), annual rainfall (mm) (blue solid bars); average rainfall (mm) (blue dashed line).

Methods

Spore collection

Spore collection occurred in March 2018, closely following the monsoon season. Collecting aimed to maximise genetic representativeness without placing stress on the population. Whole fronds greater than 1 m bearing mature sporangia were collected from 16 individuals and placed into large paper bags. Collected fronds were kept at ambient temperature for 2–3 days until spore release. Spores were then transferred into paper envelopes and kept separate as **maternal lines**. To facilitate spore drying, spore collections were maintained at ambient temperature and approximately 50 % **relative humidity** (RH) for approximately 3 weeks before transport and storage in the dark at 5 °C.

Germination trial

A germination trial was conducted eight months after spore collection at the Australian National Botanic Gardens (ANBG). Four germination media were tested:

1. Milled *Sphagnum* moss (saturated with purified water);
2. 70:30 mix of peat moss and sand (saturated with purified water);
3. 0.7 % water agar (pH 6–7), 4) alkaline 0.7 % water agar (pH 8.0, 1 M HEPES biological buffer).

All media (150 mL per replicate) and containers (90 mm diameter plastic lidded containers) were sterilised in an **autoclave** and water adjusted to establish a thin film of water on the media surface. Four replicates of 15 maternal lines on 4 media types were trialled with 2 mg of spore hand sown in each container and sealed with parafilm. Containers were stored in incubators at 25 °C and a 12/12 hr light/dark photoperiod with light intensity maintained by LED strip lights at 970 lumens per metre.

Containers were monitored fortnightly for germination and contamination. Spore germination was recorded by placing a 1 cm^2 grid across each container and counting the number of grid cells containing green prothalli and/or sporophyte plants. The percentage of grid cells containing germinated spores at 10 weeks was arcsine transformed and analysed by ANOVA in Genstat (VSN International 2020).

Propagation and cultivation

Germinants on agar were transferred to sphagnum moss either 74 or 112 days after the germination trial began. Once all germinants were transferred to sphagnum moss, the plants were moved to a propagation house at approximately 20 °C, 95 % RH under a hood and on capillary matting. Container lids were incrementally removed, and holes pierced in the bottom of containers to introduce gas exchange and water flow gradually over two weeks to prevent shock. The sporophytes showed signs of nutrient deficiency (chlorosis), which was addressed with fortnightly applications of Hortico All Purpose Soluble fertiliser at a rate of 0.5 grams per 500 ml of water (boiled and cooled). Between seven and nine months after the germination trial began, sporophytes were potted into 70 mm pots containing plugger 666 growing medium (Australian Growing Supplies) avoiding root disturbance. At this stage a single application of Multicrop Plant Starter at 24 mL per 9 litres of water was supplied.

Results

Germination trial

Germination was successful from eight-month-old spore from 15 maternal lines from two subpopulations (Figure 13.6a, 13.6b). Of the four media types, germination was only observed on water agar and sphagnum. Time to first observed germination (prothalli) was four weeks after sowing on 0.7 % water agar, while germination on sphagnum moss was first observed at six weeks. Germination media significantly affected both the total germination ($P < 0.001$) and the rate of spore germination ($P < 0.001$; Table 1). Total spore germination varied with maternal line ($P = 0.003$). Contamination in some agar containers after six weeks killed some germinants, which resulted in reduced total germination (Figure 13.6a).

Propagation and cultivation

Although germination was generally five times higher on agar than sphagnum moss, subsequent transfer shock associated with movement to sphagnum growing medium caused many germinants to die. Germinants from both agar and sphagnum dishes were transferred to pots and grown at approximately 20 °C and 95 % RH where they grew equally as vigorously regardless of germination medium.

Propagation and cultivation efforts resulted in 52 plants from 3 maternal lines from the Hughs Dale subpopulation and 51 plants from 5 maternal lines from the Anderson Dale subpopulation, totalling 104 plants established and maintained at the Australian National Botanic Gardens (Figure 13.6c).

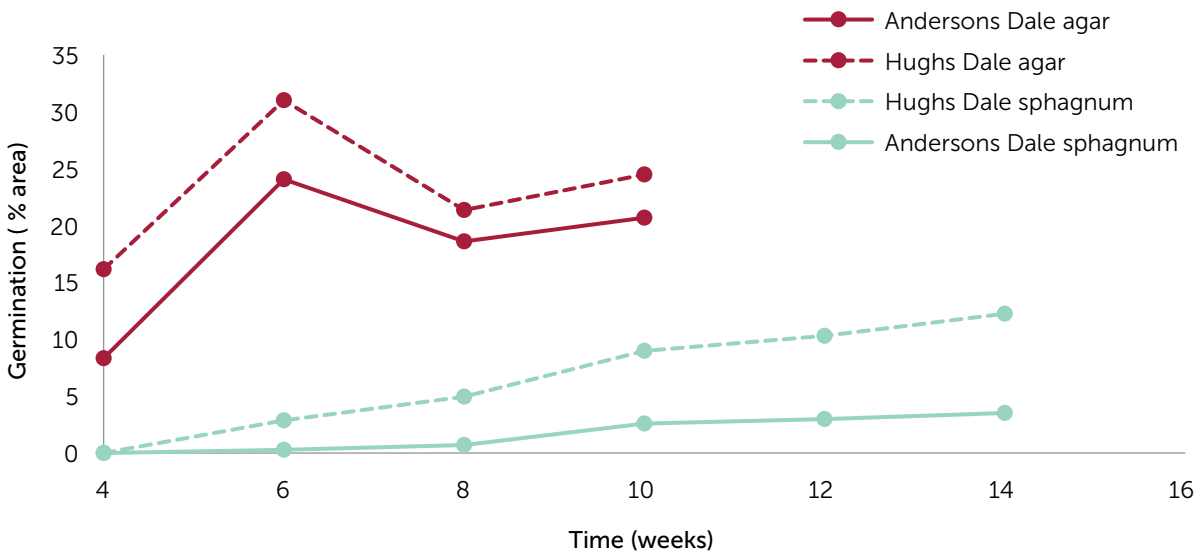

Figure 13.6a: Observed germination of *Pneumatopteris truncata* from two subpopulations (Andersons Dale, Hughs Dale) on water agar and sphagnum moss. X-axis values are time (number of weeks) since spore sowing. Germinants on agar were transferred to sphagnum from 6 weeks following observed germination.

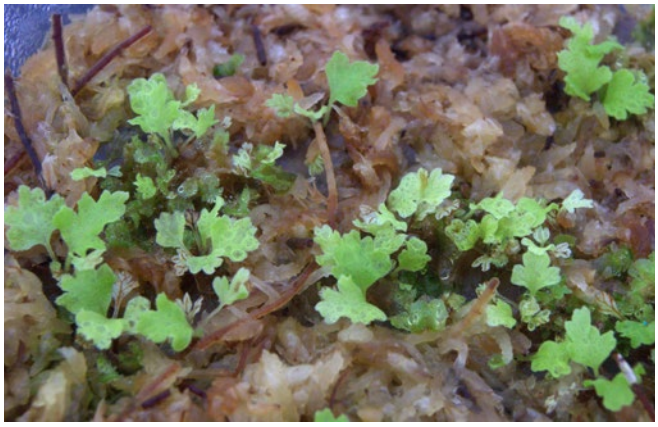

Figure 13.6b: Successful growth of sporophyte plants at 11 weeks following transfer to sphagnum moss from agar and c. 5 months from observed germination. (Image: Fanny Karouta-Manasse)

Figure 13.6c: Potted ferns of *Pneumatopteris truncata* growing at the Australian National Botanic Gardens nursery 9 months after spore germination. (Image: Fanny Karouta-Manasse)

Table 13.1: Summary analysis of variance of *Pneumatopteris truncata* spore germination. Significant sources of variation in bold

	Final germination (%)			Days to first germination		
Source of variation	d.f.	v.r.	F pr.	d.f.	v.r.	F pr.
germination media	3	87.48	**< 0.001**	1	321.30	**< 0.001**
maternal line	15	4.62	**0.003**	15	2.21	0.012

Discussion

We developed protocols for short-term storage, germination and propagation to establish an ex situ population and provide information to guide conservation and research efforts for *Pneumatopteris truncata*. Such ex situ techniques may be suitable to conserve other fern species. We found that *P. truncata* spore, if collected when mature and dried appropriately, can be stored at 5 °C at less than 70 % RH for at least eight months. Germination was greatest in spore sown on water agar, but relative survival was greatest from spore sown on sphagnum moss, potentially due to minimal root disturbance during transfer. Plants grow well in standard potting media and benefit from consistent water and nutrient availability. Cultivation conditions with temperatures between 20 °C to 30 °C and RH greater than 80 % produced healthy growing plants.

The living conservation collection of *P. truncata* plants at the ANBG may represent greater than double the number of individuals in Australia once they reach reproductive maturity. These plants and spores secured at conservation facilities are useful resources to evaluate, under controlled conditions, the environmental requirements for growth and survival to inform potential future augmentation of wild populations.

Future trials at the Christmas Island nursery will aim to understand the environmental thresholds for plant growth and survival. Laboratory trials will aim to understand spore longevity and requirements for effective long term ex situ spore conservation, including through assessing spore desiccation tolerance and chilling sensitivity.

Acknowledgements

Roslan Sani, Leonard Stapp, Ian Shepherd and Heather Sweet for invaluable field assistance; Sally Hawkins, Fanny Karouta and Julie Percival for germination and propagation trial assistance; Sue Fyfe, David Taylor, Judy West and Ashley Field for their support throughout this project. Plant material was collected under the Christmas Island Plan of Management and spore germplasm imported to the mainland under the Department of Agriculture, Water and the Environment Biosecurity Import Conditions case "Permitted Seed for Sowing". Funding was provided by the Parks Australia executive as part of the "Flora Prioritisation" project.

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Di Fonzo MM, Nicol S, Possingham HP, Flakus S, West JG, Failing L, Long G, Walshe T (2017) Cost-effective resource allocator: a decision support tool for threatened species management. *Parks* **23**, 101-113.

Holmes G, Holmes J (2002) '*Conservation Status of the Flora of Christmas Island, Indian Ocean*.' (Environment Australia/Parks Australia North: Atherton)

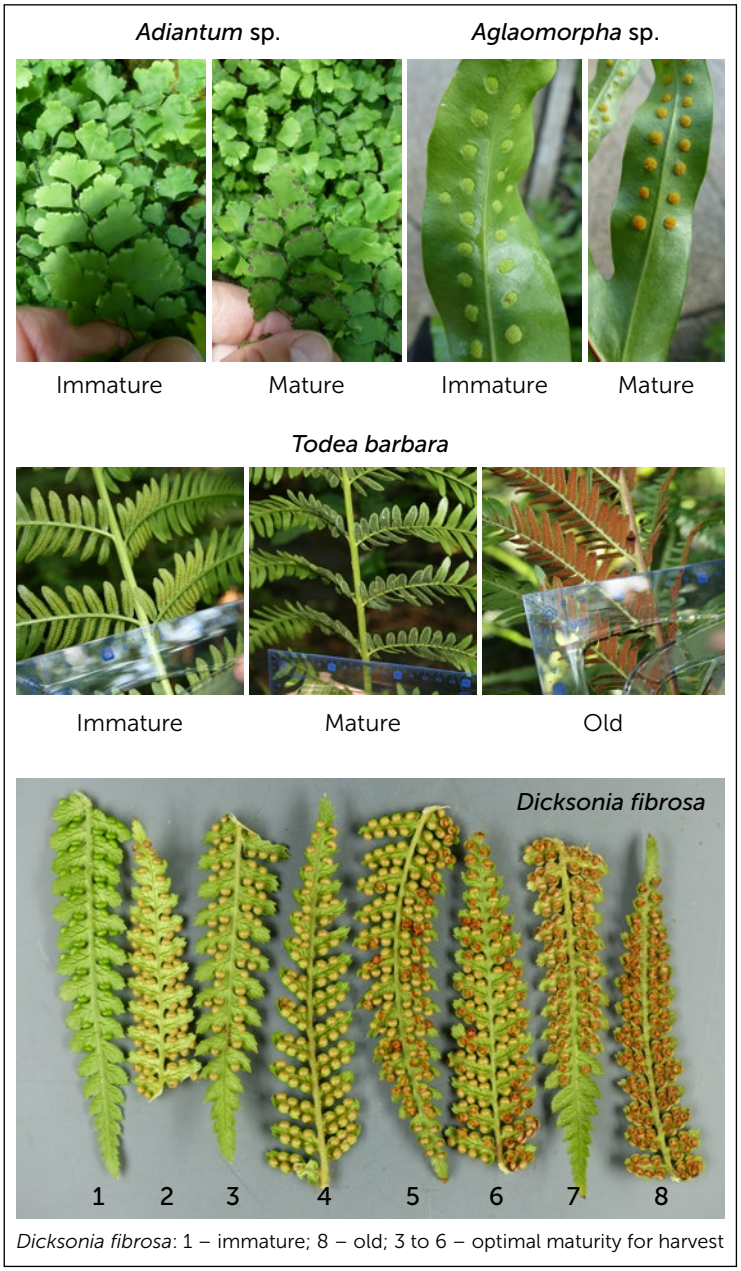
Threatened Species Scientific Committee (2004) 'Commonwealth Listing Advice on *Pneumatopteris truncata* (fern).' (Australian Government Department of Agriculture, Water and the Environment) Available at http://www.environment.gov.au/biodiversity/threatened/species/p-truncata.html [Verified 3 June 2021]

13.2.1.2 Spores

Spores from bryophytes and pteridophytes must be collected at maturity (Figure 13.7) to ensure their maximum germination capacity and longevity. Collecting immature spores or leftovers of spores in open sporangia (likely from the previous season) will result in low germination and aged spores. The use of these spores will affect the propagation of the species (e.g., low production of gametophytes, increased chance of cross contamination with non-targeted species, and high chance of fungal contamination) and will reduce the longevity of the spores during storage (Ballesteros and Pence 2018).

For pteridophytes, spores are typically harvested by drying the frond on a piece of paper, like an herbarium sheet. Relative Humidity (RH) for frond drying is not as strict as that needed for spore moisture adjustment (see following sections). Room/laboratory RH (typically between 50–70 %) is sufficient, but RH between 30 and 50 % is recommended. Very high RH (>85 %) and places with strong air currents (that could blow away all spores) should be avoided. After the frond has dried, sporangia will open, and spores will fall onto the paper (Nebot *et al.* 2021) so they can be collected and cleaned (see next section).

The spores of bryophytes need to be harvested from capsules (sporophyte). Whole closed capsules are collected in the field. Typically, mature spores are found in closed capsules ranging from yellowish or orange to brown colours (Duckett and Pressel 2017). Open capsules may contain mature spores, but as with pteridophytes, they may also contain old spores from the previous fruiting season. Drying conditions for bryophyte spores are the same as for pteridophyte spores. Bryophyte spores can be harvested on paper, but due to the small quantities produced, it may be better to harvest them inside tubes (e.g., Eppendorf tubes) along with the remains of the capsules. Unfortunately, collection of bryophyte spores for ex situ conservation is not standardised and no protocols are available, but some references on spore collection for germination or other experiments can be found in research papers (e.g., Van Zanten 1978a,b; Maciel-Silva *et al*. 2014).



Dicksonia fibrosa: 1 – immature; 8 – old; 3 to 6 – optimal maturity for harvest

Figure 13.7: Pteridophyte spore collection. Spores must be harvested at maturity, avoiding immature spores and spores contained in old sporangia from previous seasons. (Images: D. Ballesteros)

13.2.1.3 Gametophytes

After harvesting spores in the field, spores can be germinated in vitro to produce gametophytes – the sexual phase of the fern's life cycle. These gametophytes can then be conserved ex situ (Figure 13.3; Ballesteros and Pence 2018). In addition, gametophytes may be harvested from the field when this is the only form of the life cycle available (e.g., some species in the Hymenophyllaceae, Vittariaceae and Grammitidaceae may live mostly as gametophytes and sporophytes are rarely found; Stone 1965; Farrar 1985, 1990; Rumsey *et al*. 1998; Ebihara *et al*. 2009; Duffy *et al*. 2015; Park *et al*. 2020).

In the case of bryophytes, gametophytic tissue is directly collected from wild populations, and these tissues are used to establish in vitro tissue cultures that will be used in cryopreservation procedures (Rowntree and Ramsay 2009; Rowntree *et al*. 2011).

13.2.1.4 Shoot tips

Shoot tips of pteridophytes (Figure 13.3) are usually harvested from young sporophytes growing in vitro (i.e., in tissue culture). These in vitro cultures may be initiated through the germination of spores under **aseptic** conditions and the propagation of the resulting gametophytes, or from pieces of sporophytes harvested in the field that are sterilised for initiation (Ballesteros and Pence 2018). In addition to shoot tips, other **meristematic** tissues, such as **green globular bodies**, can be collected from tissue culture for long-term conservation purposes through cryopreservation (Ballesteros and Pence 2018; see Chapter 10).

13.2.1.5 Gemmae

Many bryophytes produce gemmae. These are small, largely undifferentiated vegetative propagules, mostly produced simply as outgrowths from some part of the gametophyte attached or held within **thalli**, stems, leaves, or rhizoids, depending on species. Gemmae are termed either exogenous or endogenous depending on where they form. Endogenous gemmae are released on the breakdown of surrounding cell walls and exogenous gemmae are detached as they mature, allowing them to be broken off fairly easily. Released or detached gemmae can be harvested for ex situ conservation purposes (e.g., Wu *et al*. 2015). Some pteridophytes also produce gemmae from the gametophytic tissue (e.g., Hymenophyllaceae; Stone 1965) and may be used to produce gametophytes in vitro for ex situ conservation purposes (Ballesteros and Pence 2018).

13.2.2 Cleaning, moisture adjustment and packaging

13.2.2.1 Spores

Once mature and good quality spores have been collected, remnants of frond, sporangia and debris that may have dropped on to the paper sheet along with the spores must be removed. Laboratory suppliers can provide stainless steel wire mesh sieves with mesh apertures ranging from 0.075 to 0.150 mm that are very useful and can be cleaned and sterilised easily. The spore will pass through the sieve mesh while most debris and other plant tissues will be retained. Most ferns are homosporous and spores are of one shape and size, usually between 20 to 75 µm in length or diameter (e.g., Makgomol 2006; Zenkteler 2012). Larger mesh sizes are available that could be needed to clean spores of heterosporous ferns (e.g., *Isoetaceae* or *Marsileaceae*, see Table 13.3) or some Australian native homosporous species in which spores reach more than 100 µm (e.g., *Ceratopteris thalictroides*; Makgomol 2006).

The **moisture content** of fern spores must be adjusted before packaging and storage. This can be easily done in controlled environment chambers or in small containers over saturated salt solutions (Ballesteros and Walters 2007; Nebot *et al*. 2021), as described for **orthodox** seeds. Maximum fern spore longevity is reached when stored at a Relative Humidity (RH) between 10 and 25 % (see below). Recommended drying conditions vary slightly depending on the final storage temperature (Table 13.2).

Table 13.2. Recommended drying conditions for fern spores and pollen depends on the final storage temperature.

Final storage temperature	Drying conditions	References
Room (25 °C) to fridge (5 °C)	15 % RH and 15 °C 20–25 % RH and 5 °C	Nebot *et al*. (2021) Ballesteros *et al*. (2017)
Freezer (-20 °C)	30 % RH and 18–20 °C 24–32 % RH and 5 °C 35 % RH and 15–25 °C	Nebot *et al*. (2021) Ballesteros *et al*. (2021)
Ultralow or cryogenic (<-70 °C)	30 % RH and 18–20 °C 24–32 % RH and 5 °C 35 % RH and 15–25 °C	Nebot *et al*. (2021) Ballesteros et al. (2021)

Drying time must be minimised to avoid degradation prior to storage. For non-chlorophyllous spores, moisture is typically adjusted for between 5 and 7 days (Figure 13.3), and spores should not be dried for more than 2 weeks. Chlorophyllous spores are short-lived and drying times are recommended to not exceed 5 days, although it is better if moisture is adjusted for just 2 or 3 days (Figure 13.3). After drying, spores can be stored in different containers, such as small glass vials, centrifuge tubes, **cryovials** or foil bags. It is important that vials and tubes are at least 70 % filled to prevent a large volume of air in the vial that could affect the final moisture of the sample during storage. When spore samples are small (e.g., those occupying less than half of a vial), prepare small aluminium foil or paper packets to be inserted into the vials to minimise air volume.

Detailed protocols for cleaning, moisture adjustment and packaging of fern spores can be found in Ballesteros and Pence (2018) and Nebot *et al*. (2021). These protocols align with seed conservation standards as those described by FAO[64] (FAO 2014), the Millennium Seed Bank Partnership[65] (MSBP 2015) and the Center for Plant Conservation[66] (CPC 2019).

Protocols described above for fern spores could potentially be used for spores of bryophytes.

13.2.2.2 Gametophytes and shoot tips

Gametophytes of bryophytes and pteridophytes, as well as shoot tips or other sporophyte meristematic tissues of pteridophytes, are preserved either in vitro (see Chapter 9 Tissue Culture) or liquid nitrogen (LN) (e.g., Rowntree *et al*. 2011; Barnicoat *et al*. 2011). Species-specific protocols must be followed that are described in research papers and book chapters (e.g., Segreto *et al*. 2010; Pence 2008; Rowntree and Ramsay 2009 for bryophytes; Pence 2008, 2015; Ballesteros and Pence 2018; Barnicoat *et al*. 2011 for pteridophytes).

64 http://www.fao.org/3/a-i3704e.pdf
65 http://brahmsonline.kew.org/msbp/Training/Resources
66 https://saveplants.org/wp-content/uploads/2020/12/CPC-Best-Practices-5.22.2019.pdf

13.2.2.3 Gemmae

After being collected directly from gemmae-producing gametophytes, gemmae can be placed in small paper envelopes for drying in boxes containing silica gel (Wu *et al*. 2015). These authors determined that 3 hours was the drying time that provided the highest viability of *Marchantia polymorpha* following cryogenic storage (Wu *et al*. 2015). Dried gemmae within the paper envelopes can be placed in 10 mL cryovials which will be cooled by direct immersion in liquid nitrogen (Wu *et al*. 2015).

13.2.3 Desiccation tolerance and chilling sensitivity

13.2.3.1 Spores

Spores from most (if not all) bryophytes and pteridophytes are desiccation tolerant and cope well with storage below 0 °C, like **orthodox** seeds (see Chapter 5). However, there are exceptions. For example, some fern spores may present limited desiccation tolerance and age very rapidly at any storage temperature (e.g., chlorophyllous spores), as happens with the green short-lived seeds of *Salix* and *Populus* ssp. (willows and poplars). In addition, some non-chlorophyllous fern spores age faster than expected at -20 °C, as occurs with some oily tropical and subtropical seeds categorised as '**intermediate**' (see Chapter 6). More detailed information on these characteristics can be found in the following references: Proctor *et al*. (2007); Ballesteros (2011); Ballesteros *et al*. (2017, 2019, 2020); Lopez-Pozo *et al*. (2018, 2019).

13.2.3.2 Gametophytes and sporophytes

Gametophytes and shoot tips are not typically desiccation tolerant, and they behave like '**recalcitrant**' seeds (see Chapter 6). This means that gametophytes and shoot tips are sensitive to desiccation and cannot be dried and stored at -20 °C following the standard seed bank procedures. Specific protocols for their preservation must be followed that typically use partial desiccation and fast cooling to liquid nitrogen temperatures (often requiring hormonal and temperature pre-treatments and cryoprotection). Preservation of shoot tips from **gymnosperms** and angiosperms are detailed in Chapter 10 (Section 10.6.4), so only shoot tips from ferns will be briefly discussed here. More information on desiccation tolerance of gametophytes and shoot tips can be found in Lopez-Pozo *et al*. (2018).

13.2.3.3 Gemmae

Gemmae from bryophytes are more tolerant to wider ranges of temperature and humidity than those of gametophytes, and this physiological response has allowed relatively easy dry preservation of bryophytic gemmae (Wu *et al*. 2015). Furthermore, gemmae and other vegetative propagules (e.g., **tubers**, **brood cells**) from many species have been determined to be fully desiccation tolerant (Proctor *et al*. 2007) which would open dry preservation opportunities for many species, including rare and endangered bryophyte species (Wu *et al*. 2015).

13.2.4 Longevity of germplasm from non-seed bearing plants

13.2.4.1 Spores

Due to the high degree of desiccation tolerance of fern spores, they should be dried before storage. Fern spore longevity is higher when stored between 10 % and 25 % RH, independent of the fern spore type (i.e., chlorophyllous or non-chlorophyllous, Ballesteros *et al*. 2017;

see Section 13.2.2.1). In addition, longevity of dry fern spore typically increases when temperature decreases (Ballesteros *et al*. 2017, 2019). Consequently, the drying and storage conditions typically used for orthodox seeds in the seed bank (see Chapter 5) may also be used for fern spores.

Longevity of fern spores under room conditions varies from months to years depending on the species, with chlorophyllous spores aging faster than other spore types (days to months) (Lloyd and Klekowsky 1970). However, specific conditions will minimise loss of viability and quality before and during storage:

- Fern spores (both chlorophyllous and non-chlorophyllous) age rapidly when dried at <10 % RH (Ballesteros *et al*. 2017). To avoid over-drying fern spores before storage and thus reducing the initial germination capacity, do not dry over silica gel (RH can be <15 %), and use drying methods that can ensure a RH between 15-25 % (see Chapter 4 or Nebot *et al*. 2021).
- Drying at room temperature and low RH (e.g., 15 % RH), may induce over-drying in spores when they are subsequently packaged and stored at LN temperatures (Vertucci *et al*. 1994), and reduce their longevity (detailed explanation in Nebot *et al*. 2021). To avoid this type of over-drying damage, fern spores should be equilibrated at room temperature and RH between 25 and 60 % before storage in LN (Nebot *et al*. 2021).
- Chlorophyllous spores age rapidly, even at optimal moisture conditions (Ballesteros *et al*. 2011, 2017, 2019). To avoid viability and quality loss before storage, the time lag between collection and storage should be short, ideally 1 week, and no more than 2 weeks.
- Dry chlorophyllous spores will maintain viability during storage at -20 °C. However, due to the rapid ageing of chlorophyllous spores, lower storage temperatures (<-70 °C) are recommended (Ballesteros and Pence 2018).
- Non-chlorophyllous spores have a high lipid content that may be responsible for a lower than expected longevity when stored at -20 °C (reviewed in Ballesteros 2011, see also Ballesteros *et al*. 2019). Of relevance are freeze/thaw cycles of the fern spores stored at -20 °C, which have been shown to accelerate viability loss (Ballesteros *et al*. 2012). To avoid these problems, storage at -80 °C or LN temperatures has been recommended for long term conservation (Ballesteros and Pence 2018). However, if storage at only -20 °C is available, it is recommended that spores are stored in subsamples for single use to avoid freeze/thaw cycles of the bulk collection (Ballesteros *et al*. 2012; Nebot *et al*. 2021).

Dry storage at low temperatures increases the longevity of chlorophyllous and non-chlorophyllous fern spores. Some Australian fern spores stored in ambient conditions (herbarium storage) have longevity of 15 years (Paul *et al*. 2014). The chlorophyllous spores of *Equisetum hyemale* and *Matteuccia struthiopteris* have p_{50} (time needed to lose 50 % of initial viability) of 12 days and 6 months, respectively, when stored at 20–25 °C, which increases to a p_{50} of 3 and 30 months, respectively, when stored in the fridge (e.g., 4–5 °C) (Ballesteros et al. 2011, 2012, 2017, 2019).

Storage at -20 °C can maintain spore viability for 5 or 10 years for most chlorophyllous and non-chlorophyllous spores, respectively. However, as indicated above, some non-chlorophyllous spores may present challenges at this temperature due to the crystallisation of their storage lipids (Ballesteros 2011; Ballesteros *et al*. 2019). Longest lifespan of fern spores has been observed at -80 °C or -196 °C (LN), with examples of viable spores producing healthy sporophytes after 25 years of storage (Ballesteros and Pence 2018). Predictions indicate that the high viability of both chlorophyllous and non-chlorophyllous spores could be maintained for several decades or even centuries (Ballesteros *et al*. 2011, 2019).

Spores from bryophytes are also tolerant to desiccation, and it is likely that their response to drying and low storage temperatures are like those described for fern spores (Proctor *et al*. 2007;

Pence 2008; Segreto *et al*. 2010). While more research is needed to understand the variation in longevity of bryophyte spores as a function of the moisture content and the temperature during storage, their cryogenic storage could be undertaken relatively easily. For example, bryophyte spores of *Haplocladium microphyllum* have been successfully stored dry in LN for 180 days (Wu *et al*. 2015).

13.2.4.2 Gametophytes, shoot tips and gemmae

Gametophytes of bryophytes and pteridophytes can be preserved for years in tissue culture (e.g., Rowntree *et al*. 2011; Barnicoat *et al*. 2011), however they are typically preserved for the long term in LN. Species-specific cryopreservation protocols must be followed that are described in research papers and book chapters. Some protocols use the natural desiccation tolerance of the gametophyte (e.g., some bryophytes, Segreto *et al*. 2010), but most protocols involve specific preculture and cryoprotection techniques. More information can be found in Segreto *et al*. (2010), Pence (2008), and Rowntree and Ramsay (2009) for bryophytes, or in Pence (2008, 2015), Ballesteros and Pence (2018), and Barnicoat *et al*. (2011) for pteridophytes.

Cryopreserved gametophytes can remain alive for over two decades (Ballesteros and Pence 2018). Gametophyte cryopreservation is the most used method for long-term bryophyte conservation (Pence 2008); however, it is typically used for pteridophytes when spore collection is not feasible or an alternative germplasm source is desired due to the short lifespan of the spore (Makowski *et al*. 2016; Ballesteros and Pence 2018).

Shoot tips are also a source of germplasm from non-seed bearing plants such as pteridophytes. They are processed following the typical methods used for shoot tips of seed plants (see Chapters 8 and 9). Specific protocols are described in the literature (e.g., Pence 2001, 2015; reviewed in Pence 2008 and Ballesteros and Pence 2018) and cryopreserved fern shoot tips can remain viable for years (Ballesteros and Pence 2018). While there may be fewer situations that require banking of shoot tips from fern sporophytes, it can be important for some threatened fern species, when plants are few and difficult to access and/or produce few spores (Ballesteros and Pence 2018).

To our knowledge, cryopreservation of gemmae from bryophytes has been attempted in just one species, *Marchantia polymorpha* (Wu *et al*. 2015). In this paper, gemmae were dried over silica gel for 3 hours and preserved in LN with minor viability changes for at least 75 days, suggesting that dry gemmae could be stored cryogenically for the long-term. Cryopreserved gemmae were thawed by immersing the cryovials containing the gemmae in running water (15– 20 °C) for 15 minutes (Wu *et al*. 2015).

13.2.5 Propagation of non-seed germplasm

Propagation is not restricted to just germination. For example, in the case of the fern spore, it will benefit a restoration strategy to know how to grow the gametophyte and promote sporophyte development, not just spore germination (see Figure 13.2).

Propagation of gametophytes and sporophytes using mechanical fragmentation has been considered a suitable method for mass production of ferns and bryophytes (Hugonnot and Celle 2012; Jang *et al*. 2019, 2020), however, for conservation purposes, gametophytes are generally produced through in vitro spore germination.

13.2.5.1 Spore propagation

Germination of spores requires a substrate that can be sterilised. Recommended substrates include agar-based substrates, finely chopped tree fern fibre, peat moss or sphagnum moss that is sterilised prior to sowing. A sterile substrate will prevent the germination of residual non-target spore from fungi and other plants, which may crowd out the developing gametophyte of the target species (Harvey 1993). When selecting agar-based substrates to germinate pteridophyte spores, one must consider the species (see Table 13.3). While spores from most pteridophyte species can be germinated on water agar, some species require specific mineral solutions (e.g., for Psilotaceae, Lycopodiaceae, Ophioglossaceae, see Whittier 1981, 1990, 1998). In addition, for correct gametophyte development, culture medium or soil is generally required soon after germination. Some basic recipes for fern spore germination and gametophyte development can be found in the literature (e.g., Dyer's Recipe (1979) modified by Quintanilla *et al*. 2000). Bryophyte spores are typically germinated in agar-based culture, using, for example Knop's basal medium (e.g., see Sabovljevic *et al*. 2003).

The spore should be sown in containers that can be sealed, allowing some air space for growth. As a germination test, it is important to develop a procedure to distribute the spore on to the substrate media (see Case Study 13.2) in order to accurately score the resultant gametophyte.

Germination should take place in a controlled environment at 20° C in indirect light, or in complete darkness in the case of some species reliant on **mycorrhizae** (e.g., Psilotaceae, Lycopodiaceae, Ophioglossaceae, see Table 13.3). There has been extensive work examining the intensity and spectrum of light required for germination, which can be found in reviews such as Suo *et al*. (2015). Non-chlorophyllous spores take two to six weeks to germinate, while chlorophyllous spores will be fully germinated in less than a week.

Young gametophytes will appear as a mossy growth after spore germination. When gametophytes are well-formed, they may be pricked off into punnets containing finely sifted soil mixture. Punnets should be covered with glass or plastic until fronds (sporophytes) appear and should not be exposed to direct sunlight. Alternatively, gametophytes in the laminar phase from water-agar cultures can be moved to soil by scraping the surface of the agar medium, washing the gametophytes into a small beaker, and subsequently distributing the gametophytes onto a 1:1 mix of soilless potting mix: pine mulch fines in small plastic boxes with lids (Ballesteros and Pence 2018). When growing on soil, most of the gametophytes will develop into the heart-shaped stage after a few weeks. They can be sprayed with purified water occasionally to maintain high moisture and aid in fertilisation. Sporophytes (fronds) usually appear spontaneously after 3–12 months, depending on the species (Ballesteros and Pence 2018).

13.2.5.2 Stem propagation

Stem propagation is appropriate for a group of plants referred to as Tassel Ferns or Clubmosses, from the family Lycopodiaceae. Propagation is achieved by using a modified cutting/layering technique (see Chapter 8). Apical sections of 5–15 cm long are removed from the stock plant and laid horizontally on top of a typical propagation media. Both ends of the cutting are covered with media, leaving the middle of the stem exposed. These are kept moist, humid and warm (some growers use aquariums or terrariums). After a period of 6–15 months the apical growth emerges from the cutting mix (McAuliffe 2015).

Young plants are potted into a suitable epiphytic mix when they reach approx. 5 cm high or more, and once potted should produce numerous basal shoots. Plants should be allowed to establish at each repotting stage before any further disturbance is undertaken (McAuliffe 2015).

For other ferns, **rhizome** propagation or division may be suitable (see Chapter 8 for techniques).

13.2.5.3 Gemmae propagation after cryogenic storage

Gemmae, after thawing, should be disinfected for 1 min with 0.05 % solution of sodium hypochlorite (NaClO) and washed 6–8 times in distilled water, for a few minutes each. Disinfected gemmae need to be transferred onto Knop's medium and maintained under the following growth conditions for gametophyte development: 16/8 h of light / darkness, 25/18 °C, light at 2,200–3,200 lx supplied by cool white fluorescent tubes (Wu *et al*. 2015).

Table 13.3: Relative spore longevity, and specific storage and germination requirements for species within the families of ferns and fern allies in Australia (Field *et al*. 2020).

Division (phylum)	Family	Spore type[a]	Longevity (room conditions) / Storage requirements	Germination requirements[b]
Psilotophyta	Psilotaceae	NC	–	Special culture medium (elimination of nitrate nitrogen from the nutrient medium), dark
Lycopodiophyta	Lycopodiaceae	NC	–	Special culture medium (elimination of nitrate nitrogen from the nutrient medium), dark
	Isoetaceae	NC; M	–	Can be germinated in water, light
	Selaginellaceae	NC; M	–	Can be germinated in water, light
Equisetophyta	Equisetaceae	C	Very short (days) / fast processing, cryogenic storage	
Polypodiophyta	Ophioglossaceae	NC	–	Special culture medium (elimination of nitrate nitrogen from the nutrient medium), dark
	Marattiaceae	NC	–	
	Osmundaceae	C	Very short (days to weeks) / fast processing, cryogenic storage	

Division (phylum)	Family	Spore type[a]	Longevity (room conditions) / Storage requirements	Germination requirements[b]
Polypodiophyta (cont.)	Hymenophyllaceae	C	Very short (days) / fast processing, cryogenic storage	
	Gleicheniaceae	NC	Short, about 6 months / cryogenic storage	
	Platyzomataceae	NC	–	
	Dipteridaceae	NC?	Could be short (few days) / fast processing, cryogenic storage	
	Marsileaceae	NC; M	Can be very long (decades)	Can be germinated in water, light
	Salviniaceae	NC; M	–	Can be germinated in water, light
	Azollaceae	NC; M	–	Can be germinated in water, light
	Schizaeaceae	NC	1–14 yrs	
	Lygodiaceae	NC	About 1 yr	
	Dicksoniaceae	NC	1–22 yrs, most 1–3 yrs. Some with short longevity (few weeks) (*Culcita macrocarpa*) / cryogenic storage	
	Cyatheaceae	NC	1–3 yrs	
	Dennstaedtiaceae	NC	About 1 yr	
	Lindsaeaceae	NC	–	
	Pteridaceae	Mostly NC, but some C	From weeks (C) to months (some NC) / C require fast processing and cryogenic storage. Some genera (*Pellaea*, *Cheilanthes*) may have very long-lived spores (decades)	
	Parkeriaceae	NC	–	
	Vittariaceae	NC	–	
	Aspleniaceae	NC	2–48 yrs	
	Thelypteridaceae	NC	A few months to 2 yrs	
	Blechnaceae	Mostly NC, but some C	From 3 months to 2 yrs / C require fast processing and cryogenic storage	
	Dryopteridaceae	NC	6 months to 2 yrs	
	Athyriaceae	NC	6 months to 2 yrs	

Division (phylum)	Family	Spore type[a]	Longevity (room conditions) / Storage requirements	Germination requirements[b]
Polypodiophyta (cont.)	Lomariopsidaceae	Mostly NC, but some C	From weeks (C) to months (some NC) / C require fast processing and cryogenic storage	
	Davalliaceae	NC	–	
	Polypodiaceae	Mostly NC, but some C (e.g., grammitids)	From weeks (C) to decades (some NC) / C require fast processing; C and some NC require cryogenic storage	

[a] C: Chlorophyllous; NC: Non-chlorophyllous; M: produce both macrospores and microspores

[b] Most fern spores can be germinated either on water agar or on culture medium (e.g., Dyer's Recipe (Dyer 1979), Knop's basal medium, etc) as well as on different substrates (see Case Study 13.2 and https://www.anbg.gov.au/ferns/fern.spore.prop.html). However, for optimal gametophyte development, culture media or soil is generally required. Light is often required for spore germination. Exceptions to these standard germination conditions are the ones indicated in the table (e.g., Whittier 1981, 1990, 1998).

13.3 Pollen

13.3.1 Role of pollen in plant propagation and ex situ conservation in Australasia

Sexual reproduction and seed production in higher plants are dependent on the transfer of pollen (Cruzatty *et al*. 2020). Pollen is the mature microspore of angiosperms and gymnosperms and is the carrier of genetic information as the male haploid gamete. Pollen is produced by the anther and, once it forms a contact with the stigma, will germinate to produce a pollen tube through which fertilisation of the ovule can take place.

Declining populations and habitat fragmentation can impact, or in some cases, completely cease, pollen transfer between individuals. For species with self-incompatible breeding systems (where flowers from the same plant can't produce **viable seeds**), as well as **dioecious** species (separate male and female plants), this often results in complete reproduction failure. Short and long-term storage of pollen allows for cross **pollination** between individuals that flower at different times, or between individuals that are geographically isolated (Cruzatty *et al*. 2020). To overcome the barriers of pollen availability, pollen banks are commonly used in the cultivation of economically important species such as Coconut (Karun *et al*. 2014), Palm (Araújo de Oliveira *et al*. 2021), Pecan nuts (Wang *et al*. 2021) as well as forestry species such as Pine (Kormuťák *et al*. 2019) and *Eucalyptus* (Wheeler and McComb 2006).

Pollen conservation can be a complementary strategy for ex situ conservation for some species as it is an efficient means of improving genetic diversity in existing populations (Pence *et al*. 2020). Although less commonly used in native species conservation, hand-pollination as well as pollen storage, has been used as part of an integrated conservation strategy for endangered species such as *Lapageria rosea* in Chile (Cruzatty *et al*. 2020), *Luisia macranatha* in India (Ajeeshkumar and Decruse 2013) and *Encephalartos* species in Africa (Nadarajan *et al*. 2018). Pollen storage in Australasia has also largely focussed on commercial species including *Kunzea pomifera,* a native shrub valued for its edible berries (Page *et al*. 2006) and various *Eucalyptus* species

(Wheeler and McComb 2006). Integrated conservation strategies for Myrtaceae in New Zealand have investigated pollen viability and storage of *Metrosideros excelsa* (Nadarajan *et al*. 2021; Schmidt-Adam *et al*. 2000).

Case Study 13.2: Banking on pollen to prevent extinction of the critically endangered tree *Metrosideros bartlettii*

Karin van der Walt

Introduction

The wild population of *Metrosideros bartlettii* (Bartlett's rata, rata moehau, Figure 13.8a) has declined to 14 individual trees representing five **genotypes**, making it one of the most threatened tree species in New Zealand. Coupled with the small population size, long term recruitment failure has caused an age structure which is likely to result in catastrophic decline in the future (de Lange *et al*. 2018). Despite its rarity in the wild, *M. bartlettii* is common in private and public gardens throughout New Zealand (Lehnebach and van der Walt 2018). However, trees in cultivation lack genetic diversity with more than 98 % of genetically sequenced trees originating from the same genotype (pers. comm de Lange 2020). Studies conducted to date have confirmed that individual *M. bartlettii* trees are self-incompatible; so an unrelated pollen donor is required for the production of viable seeds (Nadarajan *et al*. 2021). The ability to assess pollen viability and effective pollen storage are therefore essential components of an integrated conservation strategy for *M. bartlettii.* Pollen grains are shown in Figure 13.8b.

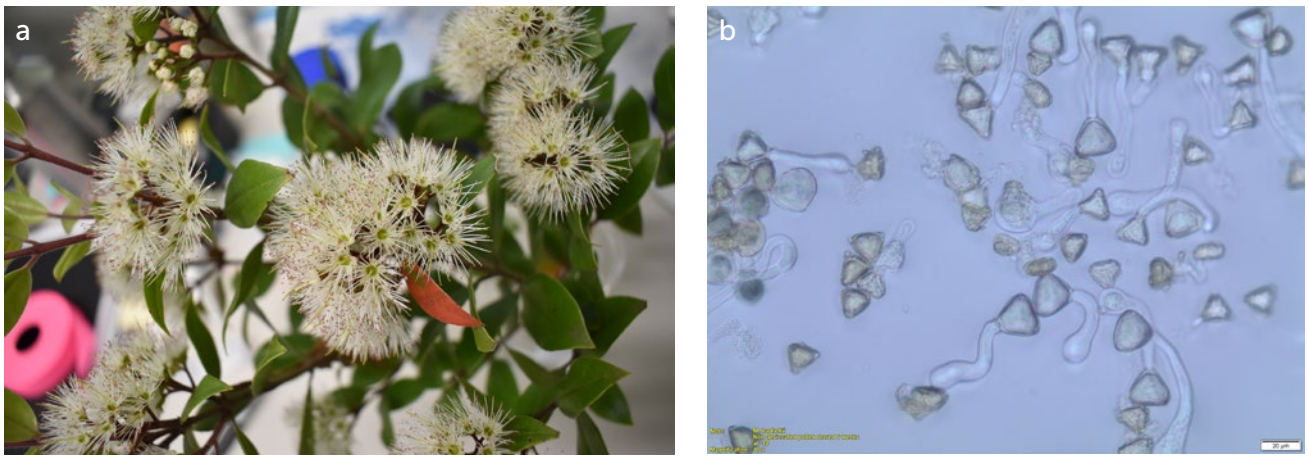


Figure 13.8: **(a)** *Metrosideros bartlettii* in flower (left); **(b)** germinating pollen grains (right). (Images: K. van der Walt)

Materials and methods

The study was conducted on cultivated trees in Wellington, using four trees of known **provenance**, representing two different genotypes ('Kohuronaki' and "Radar Bush'). Pollen viability was assessed through in vitro germination on semi-solid BK pollen medium supplemented with 20 % sucrose (Brewbaker and Kwack 1963). To screen for **desiccation sensitivity**, pollen was desiccated to 15 % **equilibrium relative humidity** (eRH) using lithium chloride, and viability compared to fresh pollen. The impact of desiccation and storage temperature was assessed by storing non-desiccated- and desiccated pollen at 20 °C, 5 °C, -18 °C and -196 °C. Desiccated and frozen pollen was

rehydrated slowly in pre-humidified conditions for four hours at 25 °C before in vitro germination for 72 h at 25 °C (Figure 13.8b). All treatments consisted of five replicates containing a minimum of 100 pollen grains.

Results

There was a significant difference in fresh pollen viability between individual trees, with fresh viability ranging from 50.8 % to 91.4 % (Figure 13.9a). Pollen stored at 20 °C (room temperature) or 5 °C rapidly lost viability, while pollen stored at -18 °C retained the highest viability, irrespective of desiccation treatment (Figure 13.9b).

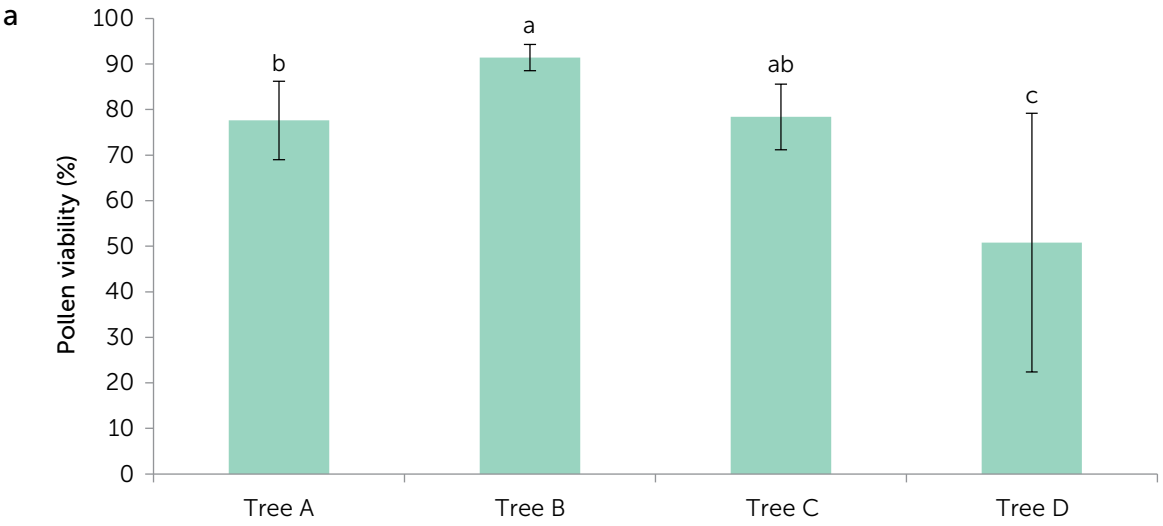


Figure 13.9a: Fresh pollen viability for individual trees of *Metrosideros bartlettii*.

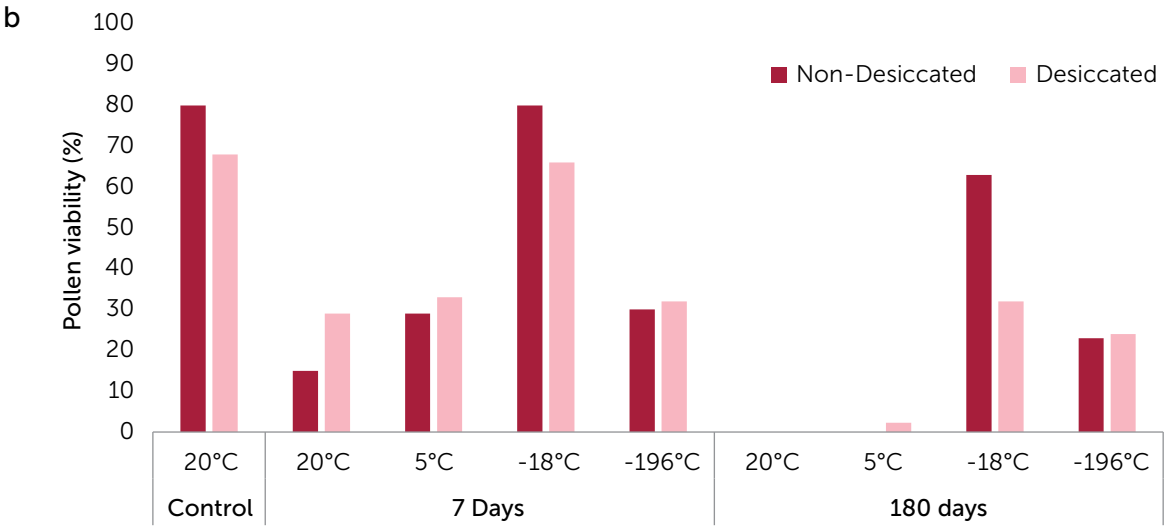


Figure 13.9b: *Metrosideros bartlettii* pollen viability after 0 (control), 7 and 180 days in storage at 20 °C, 5 °C, -18 °C and -196 °C for non-desiccated pollen and pollen desiccated to 15 % eRH.

Conclusion

Pollen storage can provide opportunities to overcome reproductive barriers, but in order for pollen banks to be useful for plant conservation, species-specific pollen storage protocols have to be developed (Kormutak *et al*. 2019). Our study found that *M. bartlettii* pollen can be stored without desiccation at -18 °C, although viability loss slowly continues over time. Pollen stored at -196 °C initially lost viability but remained stable over time, and it is likely that cryopreservation is the most effective long term storage option for *M. bartlettii* pollen. It might also be possible to reduce the initial viability loss associated with cryopreserved pollen by optimising moisture content and desiccation rate. To ensure maximum viability is maintained, it is recommended that freshly collected pollen is transported and stored at 5 °C, then used within 10 days for pollination.

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13.3.2 Collection of pollen

The pollen harvest in maturing flowers must be timely to ensure optimal pollen viability. Pollen should be collected on the same day as anthesis (flowering), preferably in the morning, and used on the same day. This will ensure freshness and viability. Shelf life is short for pollen collected from immature, aged, or weather-damaged anthers.

A sampling strategy for pollen collection should aim to preserve the **alleles** of an individual or population. The number of individuals sampled from a population will depend on the genetic diversity and life-history traits of the species (see Chapter 3 for appropriate sampling) (Volk 2011). Handling of pollen following collection depends on the purpose of the collection: in the field it is often better to process all pollen from a population together, as it is more efficient to collect larger quantities to ensure long-term pollen availability for conservation programs. However, if pollen is collected for a particular breeding program, then processing samples from individual plants to preserve individual alleles and provide male gametes is necessary ('paternal lines' similar to conserving maternal lines of seed, see Chapter 3 Box 3.2).

It is usually more practical to collect anthers or whole inflorescences in the field and then separate the pollen grains from the anthers in a laboratory environment soon after collection (analogous to the collection of fern spores). For wind dispersed pollen (*Araucaria*, *Allocasuarina*, *Callitris* species)

the pollen is released easily when the flower dries. Use of glassine paper pockets or paper envelopes for storing pollen facilitates handling and later cleaning.

As with seed collection, recording pollen collection data is very important, and should include date, time of pollen collection, plant name, location and number of flowers collected from. In the field the pollen should be stored in a cooler bag until appropriate long-term storage is secured (Parsons 2006).

13.3.3 Cleaning, moisture adjustment, packaging and storage characteristics of pollen

13.3.3.1 Pollen cleaning

After collection, all pollen must be processed immediately (within hours) to ensure maximum quality and potential longevity (Volk 2011). As indicated for spores (see Section 13.3.2), pollen can be cleaned from debris from the anthers and flowers using sieves. This method works well for those plants producing large amount of non-sticky pollen, such as trees producing wind-dispersed pollen. Entomophilous (insect distributed) pollen (e.g., *Eucalyptus*) tends to be sticky, is produced in small quantities, and does not always separate from the anthers easily. When pollen does not fall easily from the flowers, cleaning is not recommended to avoid physical damage of the pollen and compromise its quality and longevity. In this case, pollen will remain mixed with anther tissues during storage.

13.3.3.2 Desiccation tolerance and moisture adjustment of pollen

Moisture adjustment and storage temperatures for pollen samples will depend on their desiccation tolerance (DT). Many plant species (e.g., 85 % of trees from temperate regions – Ballesteros *et al*. 2020) produce desiccation tolerant (DT) pollen. DT pollen can be dried to 5–7 % water content and stored at 5 °C for short-term or below zero (ideally LN) for long-term storage (Volk 2011). Species producing desiccation sensitive (DS) pollen are less common but include important groups such as Poaceae species, including *Avena, Pennisetum, Saccharum, Secale, Triticum, Triticosecale* and *Zea*. DS pollen has a very short lifespan at room or fridge temperatures and needs partial fast drying and fast cooling and storage in LN (Volk 2011; Nebot *et al*. 2021).

The moisture content of DT pollen can be adjusted in controlled RH cabinets or chambers as indicated for fern spores. The targeted water contents for pollen storage can be reached after overnight drying at 30 % RH and 20 °C. Drying for no longer than 72 hours is recommended due to the short life span of pollen (Volk 2011; Nebot *et al*. 2021). For desiccation sensitive pollen, partial desiccation (e.g., to water contents between 0.19 and 0.33 g H_2O/g dry weight depending on species and cultivars) is recommended in pollen "flash driers". Details about protocols and flash driers can be found in Nebot *et al*. (2021).

13.3.3.3 Storage temperature and longevity of pollen

Desiccation tolerant pollen can be stored at diverse temperatures depending on use. For short-term storage (days to a few months), a fridge (5 °C) can be used. A freezer (-20 °C) can be used for medium-term storage (months to a few years). Long-term storage (many years) of DT pollen is recommended at LN temperatures (<-130 °C). As indicated above, pollen longevity is generally short but depends on species. Lowering the storage temperature will increase pollen longevity. Although LN is recommended for long-term storage, significant ageing has been detected in pollen stored for a few (5–10) years (e.g., Ren *et al*. 2019).

Nonetheless, LN stored pollen is a great source of germplasm and has been used to restore genotypes lost to disease (e.g., American Horse Chestnut, *Castanea dentata*) after more than 20 years of storage (Pence *et al*. 2020).

13.3.4 Pollen viability testing and use of pollen for seed production

Pollen viability can be measured via several approaches depending on time and resources. Viability in pollen is observed as a pollen grain's ability to germinate on the stigma (Firmage and Amots 2001).

Pollen development is sensitive to changes in temperature and is negatively affected by heat. The timing and method of measuring viability should be carefully considered before harvesting and using the pollen for seed production. Methods of assessing pollen viability include staining, in vitro, in vivo and automatic viability analysers. Pollinating flowers artificially by hand using fresh harvested or stored pollen, and then assessing seed set is one means of ascertaining viability, although slow, and possibly wasteful of time and effort if the pollen is not viable.

Staining techniques involve different methods to determine whether tissue is live – Aniline Blue for the detection of the callose in the pollen walls and pollen tubes, and iodine to determine starch content.

In vitro testing involves germination on semi-solid medium supplemented with sucrose such as BK pollen medium (Brewbaker and Kwack 1963). The percentage of sucrose in the medium needs to be adjusted depending on species. For pollen grown on a medium containing sucrose, boric acid and calcium nitrate have been shown to be very important nutrients for pollen germination in many different species.

In vivo testing involves taking pollen from the flower and growing on the stigma. The stigma is then removed after pollination and placed on a microscope slide and incubated at ambient temperatures with high humidity for 40 minutes. The style is then stained with aniline blue in a potassium phosphate buffer for 2–3 minutes.

Refer to Firmage and Amoti (2001) and Bengtsson (2006) for more detail and methodology on all the above techniques.

Automatic viability analysis[67] can quickly test pollen viability, pollen count and pollen ploidy and development stages.

13.4 Consideration of under-represented taxa in ex situ conservation programs

There are a few orders of taxa that have been largely overlooked in ex situ conservation programs for plants in Australia. The previous sections of this chapter have dealt largely with conservation of germplasm from non-seed bearing plants and pollen. The following sections deal with seed-bearing plants that are currently under-represented in conservation programs.

67 For example: https://amphasys.com/

13.4.1 Conservation of parasitic plants

13.4.1.1 Introduction

Parasitic plants should be considered as part of ecological restoration programs due to the role they play in landscapes across Australia (Table 13.4). Parasitic plants are components of many habitats and have pronounced effects on animal diversity, shaping distributions, influencing movement patterns and boosting species richness (Watson *et al*. 2009). They are a cosmopolitan group of taxa, found in most environments across the continent, except that mistletoes are not found in Tasmania.

For the purposes of conserving germplasm and promoting its use in ecological restoration, observing the habit and physiology of the various parasitic groups will give a better understanding of their storage and germination requirements. Parasitism in **angiosperms** has been classified into three different categories relating to the way in which the taxa form their association with their host (reviewed in Baskin and Baskin 2014). These are: **holoparasites**, **hemiparasites** and **mycoheterotrophic** species (excluding orchids). All parasitic plants produce a structure (the **haustorium**) that allows them to extract water or nutrients from their host. Because of the diversity and possible phylogenetic implications of the **morphology** of the haustorium, researching the diverse and intricate connections between parasitic plants and their hosts could assist their reintroduction.

Table 13.4: Classification of types of parasitism found in plants[68]

Type of plant parasitism	Definition
Obligate	An obligate parasite cannot complete its life cycle without a host.
Facultative	A facultative parasite that can complete its life cycle independent of a host.
Stem	A stem parasite attaches to the host stem.
Root	A root parasite attaches to the host root.
Hemiparasitic	A hemiparasitic plant lives as a parasite under natural conditions but remains photosynthetic to some degree. Hemiparasites may obtain only water and mineral nutrients from the host plant, or many also obtain a part of their organic nutrients from the host.
Holoparasitic	A holoparasitic plant derives all of its fixed carbon from the host plant. Commonly lacking chlorophyll, holoparasites are often colors other than green.
Mycoheterotrophic	Myco heterotrophic describes the symbiotic relationship between certain kinds of plants and fungi, in which the plant gets all or part of its food from parasitism upon fungi rather than photosynthesis.

13.4.1.2 Major groups of parasitic plants

Holoparasites in Australia include the genus *Cuscuta* and depend on their host plant for photosynthates for nutrition and survival.

Hemiparasites that produce roots can be separated into a number of categories:

- those that do not require a host to initiate germination (e.g., *Cassytha*) but whose roots die soon after haustorial initiation with the host;

68 Adapted from https://en.wikipedia.org/wiki/Parasitic_plant

- those that do require a host to germinate (e.g., *Alectra*);
- those taxa that do not need a host to germinate and produce roots which are retained throughout their life (notably taxa in Santalaceae). Taxa within Santalaceae are commonly trees or shrubs that are self-supporting but seek out the roots of other plants to obtain extra nourishment.

Common hemiparasitic species from genera such as *Exocarpos, Santalum* and *Thesium* have desiccation and freezing tolerant seeds (orthodox storage behaviour). They generally have larger seed sizes than other groups of parasitic plants, with larger nutrient reserves from the **endosperm** and a thicker pericarp, which gives them the ability to survive for longer periods without attaching to a host plant. They will eventually die unless the haustoria attaches to a host. In both groups of holoparasites and hemiparasites, propagation studies are still required, in conjunction with their hosts, to determine efficient use of germplasm material. There are a number of papers on the propagation of both *Exocarpos* and *Santalum* (Sedgley 1984; Loveys and Jusaitis 1994).

Hemiparasites that do not produce roots are known as the mistletoes, and in Australia, include the families Viscaceae and Loranthaceae. The family Viscaceae are restricted mainly to the wet tropical areas of north-east Australia. Some have no visible leaves, and all have very small flowers. Host species are varied but can include members of the other mistletoe family, Loranthaceae (known as secondary parasitism). The family Loranthaceae is widespread across Australia but does not occur in Tasmania. Loranthaceae includes the two charismatic monotypic genera *Nuytsia* and *Atkinsonia,* which are endemic terrestrial species to Australia. The haustoria of Loranthaceae primarily tap into the xylem of their hosts (Vidal-Russell and Nickrent 2008), which may be a factor to consider in the propagation of taxa from this family.

There has been significant work on mistletoes and their roles in landscapes, both beneficial and detrimental to landscape function, but little has been done on the conservation of mistletoe germplasm. Mistletoe seed is classified as a pseudoberry, and the seeds have underdeveloped, differentiated **embryos**. The seed is not protected by a seed coat but is instead enveloped in a translucent pulp known as viscin held within the **fruit** wall. The viscin acts to keep the seed protected and forms a barrier to aid in the retention of moisture. Once the fruit wall barrier has been broken, then germination is initiated quickly. This should be considered when processing the fruit ready for storage, as removal of the fruit pulp will initiate germination. Fruit can be dried, with the pulp intact to maintain the viscin around the seed, at 15 % RH and 15 °C (Baskin and Baskin 2014).

Most mistletoes are bird dispersed. Both families have morphological **dormancy** (MD; see Chapter 7) with a rapid rate of germination once the fruit wall has broken down. There are also instances of morphophysiological dormancy (MPD, see Chapter 7) having been recorded in mistletoes where cold **stratification** was required even after MD was broken. Literature covers some of the mistletoes that have been assessed for dormancy, but more work is required. Further work on propagation of mistletoes is also required – a number of reports detail success with germination but longevity of seedlings is short.

Mycoheterotrophic (MH) plants derive some or all of their nutrition from fungi. Several studies have shown that seeds of MH plants germinate in response to their specific fungal hosts. Hynson and Bruns (2010) demonstrated that MH species will also germinate in response to closely associated symbiotic fungi and whilst seedlings will grow in the absence of symbiotic fungi, no mature plants will persist. It is estimated that 10 % of vascular plants have a juvenile and subterranean phase of growth either as spores or seeds, during which they are dependent on fungi, perhaps the

best-known being orchids (Chapter 12). Most grow out of this phase to develop green leaves and become partially or fully **autotrophic**.

Mycoheterotrophy occurs in a number of families: Ericaceae, Gentianaceae and Polygalaceae, all of which occupy significant niches within Australasian **ecosystems**. It occurs in these families, and these families are widespread in Australia, but there is a dearth of research on Australian species and as a result their propagation and therefore ex situ conservation remains a challenge. Common characteristics of MH species are that they generally produce thousands of seeds in a single fruit; the seeds may be dust like and rely on wind, animals and water as vectors for **dispersal**; embryos are not differentiated and hypocotyl, epicotyl and cotyledons may only exist as a few cells; and seeds have a limited amount of endosperm. Plants of MH species frequently grow in shaded areas with lots of leaf litter, an ideal environment for associated fungi. Some, for example, *Rhizanthella* and *Thismia*, totally lack chlorophyll and may have a completely subterranean existence (see Chapter 12 section 12.2.5.1 for details of mycoheterotrophic orchid species). There is an excellent review of mycoheterotrophy in a 'virtual special issue' of *New Phytologist* **185**(3) from 2010.

13.4.2 Conservation of carnivorous plants

13.4.2.1 Introduction

Carnivorous plants in Australia are commonly inconspicuous or small herbs occurring in a wide variety of habitats, often in seasonally-wet areas. Approximately 245 species of carnivorous plant species, from five families – Droseraceae, Nepenthaceae, Byblidaceae, Lentibulariaceae and Cephalotaceae – are currently described from Australia (Western Australia alone harbours almost a quarter of all known carnivorous plant species; Clarke *et al.* 2018a), making Australia the centre of diversity for several genera. Their prevalence is attributed to the extremely nutrient-impoverished sandy soils typical of many Australian ecosystems, coupled with long evolutionary isolation and the geological and climatic stability characterising **biodiversity hotspots** such as southwestern Western Australia (Cross 2019). They have special features to attract and capture small animals, usually insects, from which they obtain mineral nutrition using a variety of digestive enzymes (see Adamec and Pavlović 2018; Matušíková *et al.* 2018).

Although some species of carnivorous plants, including several Australian *Drosera* species, have been described as ruderal and are colonisers of recently-disturbed habitats, they are still highly ecologically specialised and are extremely sensitive to disturbance; more than a quarter of all species globally, and at least 30 Australian species, are at risk of **extinction** (Cross *et al.* 2020). Yet, there are few examples of carnivorous plants being included in ecological restoration projects (see, for example, Koch 2007), and these species should feature more prominently in conservation and restoration programs. Alongside improving the understanding of ex situ methods for their conservation (below), further study is required to improve the success of returning propagated individuals to restored environments given their particular ecological requirements (e.g., Brewer 2005).

The seed dormancy and germination requirements for most Australian carnivorous plants are reasonably well understood. Most carnivorous plants produce small to minute seeds (<5 mg per seed), and the seeds of species from all genera producing seeds with fully developed embryos (in Australia, including *Aldrovanda, Byblis, Drosera,* and *Nepenthes*) appear to possess physiological dormancy (PD; Cross *et al.* 2018a). Some species of *Drosera* (predominantly tropical and sub-tropical species) produce seeds that are non-dormant or very weakly dormant at maturity, while the seeds of *Cephalotus follicularis* (Just *et al.* 2019) and many species of *Utricularia* (Cross *et al.* 2018a) have underdeveloped embryos requiring a period of maturation prior to

germination and thus have morphological dormancy (MD). Morphophysiological dormancy (MPD), and rarely PD, have also been reported for species of *Utricularia* (Cross *et al.* 2018a).

The seeds of most Australian carnivorous plants display orthodox seed storage behaviour and are relatively long-lived in both **in situ** and ex situ seed banks (Cross *et al.* 2018a). For example, ongoing studies show little or no decrease in germination for species of *Drosera* and *Utricularia* after 14 to 34 years of ex situ storage, with both conventional seed storage conditions (drying to 15 % RH at 15 °C before storage at -20 °C) and dry cryogenic storage (drying to 5–10 % seed moisture content before storage in LN) suitable for long-term ex situ seed storage in numerous species (Ballesteros D, Davies RM, Peach JL and Pence VC, unpubl.). Similar results have been reported for species of *Byblis* (Cross *et al.* 2013, 2018b). However, further studies of a wider range of Australian species are required to better understand the conditions facilitating successful long-term seed storage supporting conservation goals. Species of *Drosera* from Section *Bryastrum* also produce gemmae, and these may be amenable to cryopreservation as described in Section 13.2.4.2.

13.4.2.2 Seed germination requirements and storage traits of Australian carnivorous plant genera

Aldrovanda (Droseraceae, waterwheel plant) contains the monotypic *A. vesiculosa*, which is a free-floating aquatic plant rarely more than 15 cm long. It's whorled leaves terminate in a 2-lobed snapping trap, and the species occurs almost exclusively in shallow, acidic, oligotrophic wetlands where competition from other macrophytes is low (Cross 2012; Cross *et al.* 2015). Although *A. vesiculosa* is primarily reliant on vegetative (clonal) reproduction throughout its global range, the few seeds produced possess PD which is alleviated by short periods of cold stratification (Cross 2012; Cross *et al.* 2016). Germination is photophilous (light-requiring), occurs at an optimal temperature of 25 °C, and is markedly improved by exposure to **ethylene** gas (a natural by-product of microbial activity in wetland sediments; Cross *et al.* 2014, 2016). While the seeds of *A. vesiculosa* are tolerant of desiccation and anecdotal evidence suggests they may persist for multiple years in sediment seed banks, they display non-orthodox storage behaviour, with seeds losing viability and failing to germinate within 12 months of storage at 15 °C and 15 % RH, or within 3 months of dry storage at -18 °C (Cross *et al.* 2016). These results suggest that *A. vesiculosa* may have an intermediate storage behaviour similar to that found in some *Cuphea* species (Crane *et al*. 2003), and further research is required to understand the cause of rapid viability loss of dry-stored seeds; this could include the presence of active chloroplasts or a high lipid content with particular thermal properties (Ballesteros *et al*. 2020). Preliminary research indicates the cryopreservation of vegetative tissues or **zygotic embryos** for *A. vesiculosa* is challenging (Cross AT, Bunn E, unpubl.), and future research should develop protocols for cryopreservation and seed cryostorage to facilitate long-term ex situ conservation.

Byblis (Byblidaceae, rainbow plants) produce linear leaves covered in two distinctive types of prey-capturing glands – longer sticky glands and shorter digestive glands – with six annual species and two perennial species currently described, all but one endemic to Australia (Lowrie 2014; Cross *et al.* 2018c). Germination in most *Byblis* is smoke-responsive, the two perennial Mediterranean species being described as post-fire **ephemerals**, and significant recruitment events typically occur following fires (Cross *et al*. 2013, 2018b). All *Byblis* produce PD seeds with linear, fully-developed embryos, with dormancy alleviated by a period of either after-ripening (subtropical species) or warm stratification (Mediterranean species); germination is markedly improved by exposure to smoke-derived chemicals for most species, or ethylene in populations of subtropical species from wetter habitats (Cross *et al.* 2018b). *Byblis* seeds display orthodox seed storage behaviour (Cross *et al.* 2013).

Cephalotus (Cephalotaceae, Albany pitcher plant) contains the monotypic *C. follicularis,* a perennial, rhizomatous species producing both non-carnivorous and insect-trapping leaves endemic to peaty swamps and sandy swampland in a small area of southwestern Australia (Cross *et al.* 2019). While mature individuals resprout rapidly after low-intensity wildfire, recruitment is not linked to fire and intense or high-frequency fires can devastate *C. follicularis* populations (Bradshaw *et al.* 2018; Just *et al.* 2019). The species produces MD seeds with an underdeveloped embryo, and germination occurs between 15–20 °C after 8 to 16 weeks of post-dispersal embryo maturation and growth (Just *et al.* 2019). Although freshly-collected seeds show sensitivity to desiccation, with germination and viability declining after drying for seven days at both 50 and 20 % RH (Just *et al.* 2019), seeds have been successfully germinated after months or years of dry storage and after storage at -18 °C, indicating that ex situ storage success likely depends upon the state of embryo development prior to drying seeds (Cross AT, Turner SR, unpubl.).

Nepenthes (Nepenthaceae, tropical pitcher plants) are rosetted or climbing perennial herbs producing highly-modified insect-trapping pitchers. Three Australian representatives are known from lowland swamps in the tropical Cape York region of northern Queensland (Clarke *et al.* 2018b). Most *Nepenthes* produce lightweight, filiform, wind-dispersed seeds with a small but fully developed embryo, and although few studies of *Nepenthes* seed germination biology have been undertaken it appears most species produce photophilous, short-lived non dormant (ND) or PD seeds (Cross *et al.* 2018a).

Drosera (Droseraceae, sundews) are small annual or perennial herbs generally occurring on nutrient-poor, acidic, exposed substrates, which in Australia commonly include Kwongan shrubland, swampland, and the moss swards and skeletal soils of granite outcrops (Fleischmann *et al.* 2018). Seed morphology in *Drosera* is highly variable, including seed size and mass, but all species produce PD seeds (rarely non dormant, ND) with a fully-developed embryo that display orthodox storage behaviour (Cross *et al.* 2018a; Royal Botanic Gardens Kew 2021). Seed dormancy is alleviated in most Australian *Drosera* by a period of **after-ripening** (subtropical and Mediterranean species), warm stratification (Mediterranean species), or cold stratification (temperate species), and germination is cued for at least some species (particularly those from fire-prone habits) by exposure to smoke-derived chemicals. Most Australian species develop persistent, long-lived soil seed banks and are stored readily for decades under suitable conditions (Cross *et al.* 2018a); for example, the seeds of numerous tested *Drosera* remained viable and germinated readily after 25 years of ex situ storage (Ballesteros D, Davies RM, Peach JL and Pence VC, pers. comm.).

Utricularia (Lentibulariaceae, bladderworts) are small to minute annual or perennial herbs producing a network of branching stolons bearing subterranean suction traps. *Utricularia* include free-floating aquatic, affixed aquatic, and terrestrial species, but always occur in substrate that remains waterlogged or moist for the duration of the growing season. *Utricularia* produce MD or MPD seeds (occasionally apparently PD) with an undifferentiated dwarf embryo requiring a period of maturation prior to germination, and like other carnivorous plants occurring in mesic habitats, the germination of many *Utricularia* is cued or improved by exposure to ethylene gas (Cross *et al.* 2018a). Many Australian *Utricularia* produce extensive persistent soil seed banks, and seeds are orthodox in their storage behaviour (Cross *et al.* 2018a; Royal Botanic Gardens Kew 2021). Indeed, seeds have been readily germinated after 18 years of storage under standard storage conditions (Ballesteros D, Davies RM, Peach JL and Pence VC, pers. comm.).

13.4.2.3 Propagation

Details on the seed dormancy of all carnivorous plant genera, and the requirements for its alleviation and the conditions required for seed germination, can be found in Cross *et al.* (2018a). Broadly speaking, seed dormancy is alleviated in species with PD or MPD either by a period of warm dry after-ripening (1–3 months of dry storage at 30–35 °C), warm stratification (8–12 weeks at 30–35 °C on a moist substrate), or cold stratification (8–12 weeks at 5 °C on a moist substrate). Once dormancy has been alleviated, germination is commonly cued by smoke-derived chemicals (i.e., **karrikinolide**, which can be delivered as **smoke water**) or ethylene gas (which can reportedly be readily delivered by sealing seeds in a plastic bag containing ripe banana peel; Baskin *et al.* 2003). Optimal temperatures reported for seed germination vary between climatic regions, ranging from 10–15 °C for species from temperate and Mediterranean regions (Cross et al. 2013, 2016; Just et al. 2019) to 20–30 °C for subtropical species from Northern Australia (Cross et al. 2018b). It is recommended that germination conditions replicate as far as possible the environmental conditions from which the species originated. Indications for the propagation and growth of carnivorous plants can be found in numerous publications (e.g., D'amato 2013), or in the websites and online documents of the *International Carnivorous Plants Society*[69] or the *Australasian Carnivorous Plant Society*[70].

13.5 Conclusion

The purpose of this chapter has been to encourage better use of applied techniques in ex situ conservation to diversify the range of germplasm in ecological restoration, **translocation**, horticulture and living collections. As this chapter is an initial review of the work on special collections and under-represented taxa in an Australasian context, it is obvious that there are gaps in our understanding of how our taxa would perform when international curation methods and procedures are applied. There is significant opportunity for further research associated with the taxa and germplasm discussed throughout this chapter. New collection, curation, storage and propagation protocols are required to better conserve and use under-represented taxa extend of current protocols to taxa lacking research and data. For this to happen, collaboration and partnerships between plant conservation institutions and land managers is required to expand their conservation and use. The required technology and facilities are already available in Australia (Chapter 2) and it is now a matter of conducting research on each species to enable their conservation using those ex situ techniques. Simple steps can be taken to facilitate this work, and ensuring that there are sufficient collections in ex situ conservation for research purposes would be a good start.

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69 https://www.carnivorousplants.org/
70 http://www.auscps.com/

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Chapter 14
Risk management and preparing for crises

Graeme Errington, R.O. Makinson, Tony D. Auld, Damian Wrigley

14.1 Introduction

This chapter covers the key points relating to the identification and management of risks to stored **conservation germplasm** collections and guidelines for responding to critical events that may threaten the viability of stored collections and the associated data.

Risk factors and remedies that relate to aspects of collection acquisition, management and use that fall under the headings of other chapters of these Guidelines are, for the most part, dealt with in those chapters. This chapter discusses some of the overarching issues of risk assessment, avoidance, and response.

14.2 Planning to manage risk

The germplasm held in conservation collections is inherently valuable at the level of species, **genotypic** lineages, **genes**, or **alleles**. In some cases, the germplasm may be irreplaceable, now or in the future.

Risk management strategies accordingly need to be robust, have a long-term perspective, and be multilayered, recognising different levels and likelihoods of risk, different timescales along which risk processes may operate, and different types of risk (e.g., biological problems as distinct from those arising from human systems and human error, or natural disaster).

It is likely that the increasing impact of climate change with regards to severity of storm events, exposure to temperature extremes and potential sea level rise will have an impact on the design and potential location of germplasm storage facilities and their long term resilience. As factors contributing to climate change become a priority, it is likely that the environmental footprint of germplasm conservation facilities will need to be addressed but there are likely to be direct benefits. For example, a low energy use facility supported by local/onsite renewable energy generation and battery backup will reduce the need for reliance on external power supply.

14.2.1 Risk management methodology

There are various ways in which the spectrum of risks can be categorised and addressed, but in general, any risk management approach should involve:

- identification of **threats** to the biological integrity, **longevity**, and revivability of the conserved material (e.g., physical disasters; building and power faults and failures; contaminants or pathogens; gradual loss of viability, germinability, or post-germination **fitness**);
- identification of threats to the systemic capacity to maintain the material for the widest possible range of end-use options, subject to collection permit restrictions (e.g., handling or records-system failures resulting in data corruption or lack of consistency in records or loss of control of individual **seed** lots or lineages, or location and identity of planted material; essential elements of collection management being under control of management lines or personnel not familiar with or fully committed to the program; funding cuts or loss of key expertise);
- identification of threat of loss of supply of material from wild **populations** to maintain collections (including clearing of **habitat**, disease, weeds, feral animals and fire and subsequent need for potential re-prioritisation of use of stored material);
- evaluation of the likelihood and significance (magnitude of consequence and reversibility) of risks across the above areas;
- development of risk avoidance and minimisation strategies and practices;
- harm minimisation and recovery strategies and practices should risk factors come to pass.

14.2.2 Factors for consideration

A comprehensive risk management strategy should have wide-ranging analysis and planning including consideration of factors:

- that are within the reasonable internal control and expertise capabilities of the project or institution personnel;
- where new resources, robust external expertise, or collaborative emergency response agreements should be sought (e.g., upgraded power backup systems; better collection housing or security; emergency access to external freezers, cool rooms, or shade houses);
- of a type or potential magnitude not fully amenable to either avoidance or rapid reaction and recovery (e.g., earthquake/tsunami/bushfire risk; serious civil unrest or war) and for which particular pre-event mitigation strategies should be employed (typically including duplication of collections at two or more sites or adding to collections before threats, such as diseases, eliminate these options).

Depending on the size and importance of the collection, and the complexity of prevention/avoidance/recovery revealed by the risk analysis process, a Risk Management Plan or Framework may be developed as a single integrated document, or as a set of documents and protocols dealing with different aspects.

14.3 Managing risk to collections: design and operation

In terms of *infrastructure* for germplasm collections, the identification, selection, design and/or construction or adaptation of an appropriate storage repository suitable for maintaining the long-term viability of collections is critical (Linington 2003). This aspect requires an intensive risk analysis, initially to identify key factors in the selection of storage type, and an understanding of asset maintenance and management in the long-term. Decisions made at this point may have outcomes that are fixed for the short to medium term depending on the availability of asset funding. Capital funding is often allocated case-by-case and dependant on supporting documentation such as a business case. Maintenance funds will generally be needed on a year-by-year basis over the life of the equipment.

A second key area of risk management is the *operational* aspect of an **ex situ conservation** program. This will include specific procedures to ensure the appropriate processing and timely storage of germplasm collections, the identification, testing and use of suitable equipment (storage containers, labels, database etc.); identification of (and ongoing training and succession planning for) the specific skills and technical expertise required by staff to manage the processing and maintenance of germplasm collections, and Workplace Health and Safety protocols.

The risks and vulnerabilities associated with the *storage techniques* to be used now or in the future should be closely considered in relation to the biological and ecological characteristics of the target plant types, the likely end uses, and the available resources. There is an extensive literature on storage techniques, cited in other chapters of these Guidelines. For a useful condensed summary of the risks associated with the various storage modes, see Havens *et al*. (2004) and Chapter 2.

14.4 Managing risk to data

The management of collection data is fundamental to day-to-day business as well as operating through, or recovering from, a crisis. Understanding the collections your seed bank holds, including how to store (Chapters 5–13) and use them (Chapter 7, 8, 11 and 15), will assist with responding post-crisis. The potential threat to the long-term viability and use of collections due to data loss can be substantial, particularly when considering the investment made over many years of field work, managing collections and delivering research.

The loss of data, such as the results of **germination** or storage tests, may limit a seed bank's ability to reuse collections without repeating germination trials – this can waste both time and individual seeds. The loss of initial germination trial results can also limit a seed bank's ability to compare the effects of specific storage methods on long-term collection viability. Furthermore, the loss of data associated with the original seed collection such as its **provenance** data may limit its utilisation in future research or **restoration** projects that would rely on this specific provenance data.

Seed banks should therefore seek to implement a suitable data management system or database that enables users to establish suitable data fields (considering data standards), efficiently curate data and subsequently export data in a usable format (Chapter 15 section 15.2.1). Individual seed banks will likely be limited by the resources available for purchasing a data management system or database, however the type of system does not reduce the importance of the data it holds.

Initially, a simple electronic spreadsheet is likely to suffice (see also Grose *et al*. 2021), however, as the collection and the associated data expands, a more sophisticated data management system or database should be considered where resources will allow. Furthermore, seed banks should implement backups or failsafe protocols to ensure data is secure from physical or viral damage to computer hardware and software, including external hard drives or remote servers. As part of these measures, seed banks should consider storing multiple copies of their data, including as cloud based/online and offline versions to ensure data remains available during and after a crisis.

An additional complication may arise where the choice, maintenance and replacement of data management systems, and data backups, are not under the control of the collections management unit or institution. Appropriate commitments across management lines to ensure continuity and data integrity during backups and migrations may be needed.

14.5 Planning for major adverse events

In addition to planning for risks and avoidance/mitigation actions associated with the establishment and 'routine' operation of the collections, a Disaster Management Plan (DMP) – also known as an Emergency Response Plan – should be developed, and its procedures rehearsed.

The DMP aims to prepare for the possibility of a disaster by identifying and maintaining emergency resources, including equipment, documentation, emergency contacts, staffing and external collaborators or contractors in readiness for when they might be required. A detailed DMP will identify how to assess the disaster, what is an appropriate response, who to inform and how to manage the situation in both the short and long term. Finally, a DMP is about knowing how to recover from a disaster as soon as possible, through effective organisation and implementation of the recovery operation, knowing which salvage techniques are available and being able to use them, and drawing upon duplicated elements of the collection or its data that have been housed elsewhere.

A Disaster Management Plan should include the following:

1. An introduction that outlines the scope of the plan. This identifies individuals and teams that are responsible for overall management of disaster recovery;
2. Prevention and preparedness. A risk assessment that identifies the key resources that require protection, identifies risks and lists mitigation actions. Pro-active and preparatory actions are identified;
3. Response. A clear hierarchy of responsibility and notification procedures. A clear pathway of response for major events is identified;
4. Immediate post-disaster recovery;
5. Long term restoration and rehabilitation. This includes a review of actions and effectiveness.

14.6 Factors to consider in preparation for management of critical events

14.6.1 Type and intensity of events that may impact your conservation facility

Critical events for consideration will fall into one of two broad categories:

- Environmental events such as bushfire, earthquake, water inundation due to rain or flood water, storm damage from wind, hail or lightning, and temperature extremes.
- Non-environmental events such as loss of utilities (power, water, communication); security issues; police emergencies, property damage and theft, social upheaval including medical and pandemic events which may limit supply lines and access for maintenance contractors and staff; breakdown in supply lines for consumables such as liquid nitrogen; hazardous chemical spills; site security and access management.

14.6.2 Design and construction of facilities in relation to identified threats

The type of event and relative risk will have a significant effect on the location and design of a specialised facility and may impact budget and resource requirements. A comprehensive design and cost analysis will need to consider key points such as: resistance to extreme environmental events and/or weather, building management monitoring systems and procedures, emergency breakdown procedures, backup or stand-by plant and equipment.

For example, emergency backup equipment is not necessarily installed but is identified as accessible in an emergency to replace failed equipment. A system that uses a backup unit will be cheaper initially but there may be a period of non-operation while the backup unit is sourced or installed. The importance of a non-continuous operating system on collection integrity will need to be assessed as part of the decision-making process. Another option may be the installation of 'stand-by plant', that is a duplicate set of equipment e.g., compressor or dehumidifier, that operates in a duty or stand-by function, but has the capacity to manage the load if one fails. A stand-by system will have double initial cost but should last twice as long, as well as having the capacity to operate during a breakdown.

14.6.3 Identification of critical operations

There will be some activities, such as appropriate asset monitoring and maintenance, and ongoing monitoring of collection viability, that will be critical to the long term success of the facility. There will be occasions where a natural disaster or pandemic limits normal operational access and staffing for an extended period. Critical operations and related minimum resource requirements will need to be identified and secured.

Consider factors such as:

- Staffing/skills requirements and management – Identification of key skills for staff (both asset management and technical or operational) for minimum maintenance of collections; succession planning/training; management of mental and physical health during disasters or crisis situations;
- Standardised procedures – Documented procedures encompassing appropriate standards for seed bank operations, packaging and labelling (consideration of longevity and failure, see Chapter 5 section 5.7); collection access (Chapter 2 section 2.6.2 and Chapter 15 section 15.4.2);

germplasm types (see Chapter 2 section 2.4); identification and management of reference, **base** and **active** portions of collections (Chapter 2 section 2.6.1); and material distribution (Chapter 15 section 15.4);

- Assessment of storage longevity and viability retesting (see Chapter 5 section 5.7.2 and Chapter 15 section 15.3.1.2 for discussion);
- Development of organisational and personal networks and associations – Establishing and nurturing personal and professional networks can provide significant benefit when managing through a crisis. Seeking advice, guidance, and collaborative commitments (whether for training, preparedness, or actual emergency/recovery help) from trusted individuals, institutions, and contractors can assist with how your facility is able to respond and support others through a crisis.

14.6.4 Management and maintenance of data

Much of the value of wild source collections lies in having associated data, particularly original source data. The realisation of the value of wild sourced collections comes when land managers and researchers seek to undertake restoration or research using specific **taxa** using seeds from conservation collections. As collections are held in storage for longer periods, the data relating to handling and storage and viability monitoring becomes more important. See Chapter 15 section 15.2.1 for details of data management. The management of data and its availability, long term security and ready access should therefore be considered as part of any Disaster Management Plan. For example, is all data available electronically? Are minimum hard copy records necessary?

14.6.5 Collection management

Activities and management actions that are key to the long term security and maintenance of viability should be identified and appropriate actions planned. Some actions to consider include:

- Duplication of collections – As a risk management strategy for complete or partial loss of **accessions** at the main repository, a duplicate sample of accessions should be distributed and held at a suitable partner storage facility partner storage facility, including a robust collaboration deed or memorandum of understanding. The chosen document should clearly identify expectations of both organisations, including the conditions under which the duplicate collections are stored, who has access to them, and the processes and procedures for the repatriation of those collections should they be required for use by the main repository in the future.
- Accessioning system – Each collection of germplasm is unique in time, location and genetic capture and requires a systematic approach to apply a separate identifier to each collection. A unique collection identifier is valuable way of managing related data. Storage containers will need to have suitable long-term labels, internally and externally (Chapter 5 section 5.3.1).
- Taxonomic changes – Taxonomic changes should be regularly managed at a database level and with labelling updates, if appropriate, depending on how the collection is arranged. Collections that are arranged by accession will not require label updates or rearrangement to be easily located. Taxonomic changes may be due to systematic changes and revisions or may be at the accession level as related herbarium **voucher specimens** are reviewed.
- Monitoring of seed moisture content – Where collections are dried and sealed in containers prior to placement in the storage environment, the moisture content and/or integrity of containers sealing should be monitored (Chapter 5 section 5.4.2).

- Disease management – Generally, collection of disease-affected plant material should be avoided. While the success of vegetative propagation can be severely limited by using sub-optimal material, there is also a significant risk of disease introduction to other living collections either in nursery or planted collections that may ultimately compromise **translocation** actions (see Chapter 11 section 11.8). Some seed collections may inadvertently contain spores that remain undetected during the cleaning process. In some specific cases, there may be no alternative to collecting diseased or potentially diseased material as no disease-free material is available for collection. In these situations, specific management and handling procedures will need to be developed, most likely on a case by case basis. See Case Studies 11.4 and 11.8 for discussion of these issues for species susceptible to Myrtle Rust.
- Pest Management – Although facility design may account for significant exclusion of rodent and insect pests, an appropriate pest monitoring and management system should be considered (Chapter 8 section 8.4). This will depend on types of material being stored, facility location and likely pests.
- Recognise that future wild collection opportunities may change due to ongoing clearing and increasing adverse climate impacts (see Chapter 1 for a brief summary). Planning for re-collection, supplementation of collections and emergency collections will need to factor in such changes.

14.7 Workplace Health and Safety (WHS)

Organisational WHS management procedures should be followed where they exist and activities around germplasm conservation should be incorporated. The relevant risk identification and management framework should cover field work, laboratory and storage facilities and procedures. Remote field work, handling of seeds, plant material and soil samples, using chemicals and hazardous substances, and working in low humidity and temperature environments (drying and storage), with equipment at high temperature and pressure (autoclaves), or with liquid nitrogen (**cryopreservation**) will all require specific attention.

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Chapter 15
Maintenance, utilisation and information storage

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15.1 Introduction

Germplasm conservation aims to collect and store plant genetic resources. Given that it covers a broad range of techniques and materials, there are therefore a number of key issues common to both maintaining and using ex situ collections.

Once germplasm is secured ex situ there are several important considerations in order to maintain those collections over time to ensure they remain useful and usable. In this chapter, we discuss the essential requirements and practices involved in the maintenance of germplasm collections. We include the important role of databases and image collections in enabling and complementing germplasm conservation, highlighting the key features and applications of digital records associated with germplasm collections. This chapter also provides a guide for maintaining germplasm through time, with the continued curation of collections being guided by the intended end use of the collection. For **seeds**, this includes monitoring of storage conditions, checking for, and managing seed viability decline, and bulking seed collections. For other germplasm types, this includes meticulous record keeping, turnover or propagation of plant material (being mindful of genetic impacts and the minimum sample size required to produce a genetically appropriate and healthy **population** for the desired end use), duplication of germplasm at other institutions and considerations for sharing of germplasm with end users. Finally, this chapter summarises the utility of germplasm collections for in situ conservation, research and horticultural purposes, highlighting how the end use will inform its collection and maintenance.

15.2 Managing collection records

Conservation collections are only as valuable as the data that supports them, and data management is a critical issue requiring appropriate consideration, resourcing and ongoing management. The main areas to consider are data and image management.

Historically, records related to conservation collections were kept as hard copy files by individual institutions. While this is still a useful and practical approach, the digitisation of collection records has advanced the ability to store, organise, and interrogate data, allowing it to be more widely available and useable. An example is the ASBP's Australian Seed Bank Online portal accessed

through the Atlas of Living Australia[71]. Creating and utilising databases that serve the functions required for germplasm conservation can be challenging, as the information associated with such collections is diverse and often institution specific. Below we discuss some key principles and examples to aid in maintaining and utilising the virtual data associated with the physical collections.

15.2.1 Data management

Conservation collections have three equally important key components: the germplasm material (seed or vegetative); a correct identification, usually through an herbarium **voucher specimen**; and the collection data. The three components should have a unique character linking them – initially this will usually be a field collection number. When the collection information is databased this number or a second unique identifier (**accession** number which is usually a sequential identifier), becomes the primary identifier linking three components.

After the collection, any data relating to the germplasm propagation treatments, handling, storage, management and distribution of material is critical to record to ensure the original source and future availability of material for use. The correct source material is key to successful **ex situ conservation** and **translocation** activities. Historical data relating to propagation and management of nursery stock will inform future propagation work.

A database can be as simple as a spreadsheet or workbook or as complex as a dedicated data management system (see Section 15.2.2). The extent of the data to be recorded and the need for long-term management of data should be considered when identifying the data storage method. Any conservation data management system should be duplicated and regularly backed up as a risk management strategy.

It can take several years from time of collection to establishing plants in the ground and in some cases, it may be many decades between the original collection of material and the use of that material in translocation actions. The ability to make assessments on the quality and viability of existing ex situ collections and the capacity to realise the **genetic diversity** of those collections through maximising **germination**, may depend on the extent and quality of the data relating to these collections. The data may have been accumulated over several years and potentially from several sources. The capacity to store and manage raw data rather than derived/simplified data should be a consideration. Given the value of the data, there should be procedure- and/or policy-defined limitations on editing rights, access and distribution of data.

71 https://asbp.ala.org.au/

15.2.2 Databasing

Databases containing germplasm information serve two main purposes.

1. Data inputs – offer a structured and uniform way of recording and organising data associated with a species or collection.
2. Data outputs – provide a central set of data that can be drawn upon to inform curation, propagation and research activities.

Accordingly, the two essential questions that should dictate the structure of a living collections/genebank database are:

1. What data will be recorded? (inputs/fields)
2. What questions will be asked about the collections in the future? (outputs/queries)

Consider that question 2 has implications for question 1. Building a database from scratch is often an iterative process but starting by considering how the data can be utilised in the future will save time.

Required fields (question 1) would be information from field data records (Chapter 4), as well as dates when material was received, processed and stored at the genebank (Chapters 5–11). A possible selection of record fields and the nested nature of these fields in a seed bank database, are presented in Figure 15.1 (see below). Basic field data is a common dataset collected for most plant records and is adequately covered by biological data standards like Darwin Core (Darwin Core Task Group 2009) and the Multi Crop Passport Descriptors for agricultural crops (Alercia *et al*. 2015). As such, these fields are generally easily accommodated in popular database solutions. Fields generated by seed processing and quality assessment activities are specialist data sets and data standards are still being developed. Fields could include (but are not limited to): seed weight, seed size, **embryo** type, collection purity, seed **moisture content**, storage behaviour, germination data, and if regenerated seed lots are stored, year and site/location of production should be included. Of these fields, germination data is perhaps the most complicated to deal with. Both replicate data and summary data based on averages will be useful for different users and purposes. Due to the niche nature of seed bank work, commercially available databases do not currently cover processing and germination data very well, so a database to adequately cover such information may need to be purpose built.

As collection numbers grow, outputs or queries (question 2) become increasingly important to understand the number and size of accessions held of each family, genera or species; to group species relevant to particular projects or collection permits; or to collate data and report on the number of collections made in each year. Analysis of germination data can be extremely valuable in dealing with new or small collections (so the best germination treatment can be used to minimise the number of seeds used in testing, see Chapter 7); or to group collections according to whether germination is above a threshold value for assessing germination success or conversely, below a threshold value that can indicate decline in viability (see Chapter 5). In addition, retrieval and summary of **taxon**-specific germination data is also very useful to external groups (e.g. groups making seed requests or nurseries utilising banked seeds), and when undertaking seed regeneration to replenish seed stocks.

Taxonomic Data [1]	
Family	- *Family name, Order*
Genus	- *Genus name*
Taxa	- *Species, Infraspecific_rank, Infraspecific_epiphet, Author, Journal, Endemic*
Threat Status	- *EPBCA, State listing*
Collections Data [1]	
Field Data [2]	- *Accession number, Collector number, Date collected, Date donated, Provenance, Donor type, Distribution policy, Collecting notes, Permit number*
Collector/s Data	- *Collector name/s*
Geographical Data	- *Bioregion, Location details, GPS coordinates, GPS datum, Altitude, Altitude method*
Ecological Data	- *Habitat, Modifying factors, Land form, Land use, Geology, Slope, Aspect, Soil type, Other site notes*
Associated Flora	- *Taxa*[3]
Harvesting Data	- *Material collected, No. plants sampled, No. plants found, % fruiting, Area sampled, Sample notes, Collection weight, Plant condition or Plant health data*
Specimen Data	- *Taxa*[3]*, Plant type, Avg height, Plant description, No. of herbarium specimens*
Processing Data [2]	- *Processing notes*
Storage Fractions[4]	- *Sub-accession number, Stored material, % Debris, Original quantity, Current quantity, 1000 seed weight*
Quantity data	- *Sample size, Remainder weight*
Sample Weights	- *Weights*
Collection Quality	- *Quality type*[5]*, Quality count*
Storage History	- *Date, Storage conditions*
Banking	- *Bank location, Packet location, Packet quantity*
Germination Tests	- *Start date, Pre-storage conditions, % Result, % Viability, % Adj result, Passed?, Accepted?*
Test Treatments	- *Step number, Duration*
Treatments[6]	- *Treatment name, Temperature range, Thermal period, Photoperiod, Mechanical treatment*
Media	- *Components, Quantity*
Test Replicates	- *Replicate no., No. sown*
Scoring	- *Days, No. germinated*
Cut-Test	- *Quality type*[5]*, Quality count*
Dispatches [2]	- *Date sent, Date received, Recipient, Destination, Dispatch type*
Collections	- *Accession number, Material type*

Figure 15.1: Possible data fields and nested hierarchies of tables in a seed bank database (linking ID number fields between various tables and subtables not included for simplicity). Items in italics are suggested data fields. Items not in italics can be either major data divisions or tables. Key: 1) Major data divisions within database; 2) Major tables with data better arranged as nested sub-tables as related data fields can have multiple values; 3) ID number linking to entries in the taxa table; 4) Sub-accessions are a way of dealing with collections that may have multiple fractions i.e., separate maternal samples, collections separated over a collecting season to evaluate maturation, or fractions stored under different conditions; 5) Possibly link to values in a quality table containing descriptors and their quality values; 6) Storing germination test data can be tricky as tests can be very simple (incubation on agar) to very complicated (multiple pre-treatments, scarification and chemical applications) (see Chapter 7). The approach in the model above is to encode each item as a step possibly involving temperature, light, scarification and chemicals. In this model, chemical treatments are captured in the Media table that forms part of the treatment description.

15.2.3 Image management

15.2.3.1 Image type and quality

Along with databases, the evolution of image collections to online and computer platforms has allowed the recording and sharing of photographic data associated with germplasm conservation. Images, along with their associated **metadata**, provide benefits to conservation work by recording details that would otherwise not be captured, such as field collection conditions, plant health at time of collection, laboratory experimental set ups for research trials and morphological characteristics of seeds and **fruit**. Access to such images also provides a valuable tool for science communication.

Two main types of images are useful to germplasm conservation:

1. "General Images" are photographs that document activities associated with germplasm conservation such as images taken in the field of the plants and fruits, seed collection activities, germination trials and laboratory experimental set ups.
2. "Scientific Images" are photographs of the seeds, often under a microscope or x-ray machine. Such images provide morphological seed data and are generally associated with a specific seed collection e.g., South Australian Seed Conservation Centre 2020[72], Erickson *et al.* 2016.

In recent times, the fall in price, and increased quality of digital cameras has made the option to record and archive image data a realistic proposition. Additionally, with the affordability and increasing capacity of hard drives, secure storage is a more easily achieved goal. As the internet has matured, the possibilities of online storage have also become appealing and provide options to share this information more readily with a wider audience (see Case Study 15.1).

The real challenge in archiving images is managing this content: Is what is being archived useful? Can relevant content be identified and retrieved easily? What should be archived? Not all images captured should, or need to be stored, i.e., there's little value in archiving blurry images. Image quality issues are less problematic than they have been in the past, as the resolution on most digital cameras has improved substantially over the last two decades. The broad definition of what you decide to store is obviously a decision to be made by the institution, but criteria should be established to ensure that the images have a set quality standard and be associated with good metadata. Clear guidelines should be established to determine what images are stored.

Examples of criteria that can be applied when deciding which images to keep are:

- Resolution i.e., the detail an image holds. Is it suitable for the intended end use/s of the image?
- Clarity i.e., the image is in focus and the subject of the image is clearly differentiated from background clutter.
- A base standard of metadata e.g., date, location, associated accession number/s.
- Use of a colour checker card to standardise colour assessment.
- Historical value. Situations may exist where a poor-quality image is the only information recorded for a natural value and therefore warrants preservation.

72 https://spapps.environment.sa.gov.au/seedsofsa/

15.2.3.2 Image metadata

The second crucial aspect is recording of appropriate metadata associated with an image. This is critical to manage information loss to staff turnover and fading memory. The use of metadata tagging is a good way of attaching key data. Many image formats allow for the embedding of this data within the image file itself (appropriate software will be required, but competent free options are readily available e.g., ExifTools, QuickImageComment, FastStone Image Viewer). Alternatively, or in addition, image library software can record and manage image metadata tagging. Essentially tagging is the addition of keywords to a document to allow for ready electronic identification and retrieval. This can be in the form of barcodes, Quick Response (QR) codes, or use of keyword tagging. A basic form of keyword tagging is presented below.

> TSCC0022385 JW-646 Australia Tasmania "Central Highlands" "Central Plateau Conservation Area" "Lake Mackenzie" Plantae Asterales Asteraceae Xerochrysum "Xerochrysum alpinum" "alpine everlasting" everlasting flower collecting habitat

The syntax of the line above is important to the function of adding keywords. In the example above a space is given to separate each keyword. To identify keywords composed of more than one word, quotation marks need to be added. If you are unfamiliar with the concept of metadata tagging the Wikipedia article[73] on the subject is a good introduction. For the purposes of storing scientific data it is worth evaluating the section on triple tags as a way of identifying the data types of your keywords. Triple tags take their structure from the Resource Description Framework, which is part of the World Wide Web Consortium specifications. Triple tags were initially devised to handle geotagging for specific social media websites, but the system was later extended to cover taxonomy. The three parts of a triple tag consist of a namespace, a predicate and a value[2]. Below is the same example of some basic collection data given above, but this time containing both tags and specific triple tags for key datasets.

> TSCC0022385 JW-646 "geo:country=Australia" "geo:state=Tasmania" geo:lat=-41.67607 geo:long=146.37853 "Central Highlands" "Central Plateau Conservation Area" "Lake Mackenzie" taxonomy:kingdom=Plantae taxonomy:order=Asterales taxonomy:family=Asteraceae taxonomy:genus=Xerochrysum "taxonomy:binomial=Xerochrysum alpinum" "alpine everlasting" everlasting flower collecting habitat
> (Note the use of quotation marks around triple tags that could or do have more than one word in the value)

The key benefit of triple tagging is that it identifies what each keyword refers to. Thus, we can distinguish between keywords which may have several meanings, for example *taxonomy:genus=Banksia* or *geo:location=Banksia*. It should be noted that the triple tag system is not a globally accepted standard, but this system can obviously be adapted for specific institutional usage. For example, if you wanted to attach collection bioregions you could create the following namespace and predicate combination of "geo:ibra7" followed with the region code value.
The most important aspect here is to develop a system and then to ensure that it is implemented consistently. Generating triple tags for collections can appear to be somewhat time consuming. However, it's worth exploring the possibility of a collections database generating these tags for you (if the bulk of your keywords are fields stored in your database) and/or developing a spreadsheet that will automatically concatenate a pre-defined series of fields and additional image descriptors into a single line of appropriately tagged keywords with the correct syntax. The key tip here is to always add quotations marks to your tag for a data field that can have a space character in it

73 https://en.wikipedia.org/wiki/Tag_(metadata)

(i.e. more than one word). A database/spreadsheet approach also avoids the critical issue of spelling mistakes, which will sabotage a consistent tagging system.

Case study 15.1: Photo sharing on Flickr

James A. Wood

The Royal Tasmanian Botanical Gardens started archiving images on the photo sharing site Flickr.com several years ago. This is by no means a perfect system but delivered much of what the institution required for image management at low cost.

1. First and foremost a searchable repository for images. Prior to this, the gardens image collection was essentially just a random collection of personal folders of photos scattered across the institutions shared drive. Some users would try to rename files with helpful titles to aid location when required, but there was no agreed or consistent nomenclature. This lack of a systematic image databasing meant that trying to locate useful images could take a long time and often came up empty handed. Flickr allows images to have a title, a description, attachment of keywords through their tag system and ability to record geolocation data through their maps system. These features allow for a comprehensive storage of metadata for each image. Additionally, the system allows notes to be attached to smaller sections of an individual image, which can be useful if parts of the image need to be distinguished clearly. Time stamp data is automatically retrieved from digital images but can be amended by hand where incorrect or lacking.
2. Flickr is essentially an image sharing site and this has allowed the gardens to share content with the public. Flickr accounts allow you to set the accessibility from private, to restricted, to fully public and you can also set copyright with nine options. This has permitted the Gardens an additional social media channel. The adding of description to images essentially make this a micro-blogging site, disclosing to the public what we do. It has also permitted the sharing of image selections to journalists or other third parties complete with the metadata explaining what the images are and where/when they were taken.

15.2.3.3 Seed library images

Creating a library of seed images serves to illustrate the extensive diversity of seed **morphology**. Such diversity is largely unexplored yet presents a multitude of opportunities to understand the ecology of plants, the use of seeds in conservation and **restoration**, and inform us about the taxonomy and evolutionary history of plants (Clinton and Guja 2016). Additionally, seed images can be used for seed identification purposes e.g., by quarantine organisations, or for scientific studies. With new technologies and programming, machine learning can be used to interrogate images to identify characteristics, or traits, or interest.

Seed library images are typically taken with a camera which can be operated through a microscope for smaller seeds, or manually for larger seeds. In addition to seed images taken with a camera are seed images taken by an x-ray machine. These types of images provide information on quality of seed collection (**seed fill**, infestation) and internal seed structures (seed coat thickness, embryo type) (see Chapter 5).

Seed image libraries function best when a standard set of image requirements are met.

Basic requirements include:

- Size scale
- Colour scale
- Species name and collection/accession number
- Agreed background properties e.g., consistent colour backdrop or dark field.

Images such as these can be made available for analysis using computer software, enabling the automated generation and collection of seed morphology data such as seed size and shape. This application of seed images is becoming more widely used in agricultural genebanks and in applied research, and provides an efficient way to collect valuable information about seed collections.

Imaging small seeds can present additional challenges. Very small seeds can be successfully photographed using a good quality macro lens, but such images tend to have a very small depth of field. This means that for seeds with minute hairs, bristles or complex surface textures, adequately capturing this detail can be difficult. One solution to this problem is the use of focal stack compositing, but this requires the use of both specialist equipment and software. The process can also be time consuming, so may be an activity handled by a keen volunteer. As with databasing collections, it is important to adequately record information, such as species name and collection number for seed images, and be able to search and access these images in a timely way. If a seed image is from a certain seed or plant collection, all relevant data should be associated with the image.

15.3 Ex situ collection maintenance

Ex situ collections can be maintained for differing periods of time, depending on the conservation goal for the **taxa**, with the aim to produce healthy plants for the intended end use (Chapter 2, Section 2.1). The maintenance requirements for different germplasm collections vary significantly in terms of the resources required and the expected time frame for holding accessions (Table 2.1). The maintenance of seed bank collections is detailed below, leading to a discussion of re-collection and regeneration from seeds. Maintenance common to other germplasm types is provided in relevant chapters on **tissue culture** (Chapter 9), **cryopreservation** (Chapter 10) and living collections (Chapter 11).

15.3.1 Maintenance of seed bank collections

Seed collections that have been appropriately processed, dried, packaged and stored should not require significant maintenance but there are several key areas for consideration including monitoring both the storage environment and seed viability.

15.3.1.1 Monitoring of storage environment

The type of monitoring will depend on the type and size of the storage units, whether this is a standalone unit such as a fridge or freezer or a purpose-built facility with walk-in storage environments. A purpose-built facility may have a computer-based building management system that can give a live feed of conditions and send alerts when defined parameters of operation are exceeded. Smaller, simpler systems will require a regular systematic check of the equipment operations. It is important to have a breakdown/backup plan to enable timely repairs and/or replacement of equipment and potentially the transfer of collections to temporary storage (Chapter 14). Minor or temporary changes to storage conditions are not necessarily lethal to a collection. Whilst these changes may have an impact on the **longevity** of seeds in storage, a realistic understanding of the short-term effect of conditions is essential in making appropriate decisions about asset maintenance and potentially relocating collections during periods of breakdown.

Monitoring of seed moisture content (see also Chapter 5.4, especially 5.4.2)

Equilibirum relative humidity is usually used as an indicator of seed moisture content and can easily, and non-destructively be measured using a **hygrometer** (see Chapter 5). When collections are removed from storage, relative humidity should be checked to see that the required seed moisture content is being maintained. If glass containers are being used for storage, indicating silica gel can be placed into the container. This gel will change colour when the humidity increases giving a visual warning of a problem. Hermetically or vacuum sealed foil containers can be checked for seal breakages during stocktake or prior to use to alert to possible moisture uptake. If a system with a **base** and **active collection** is used (see Chapter 2), periodic checks should be undertaken to ensure that the active collection is representative of the base.

Continued collection curation

- Label integrity. The longevity of the labels will depend on the type of label, ink, adhesive and storage environment. The integrity of the labels will require periodic checks to ascertain whether they require replacing.
- Taxonomic updates. Depending on the system used to arrange and store accessions, taxonomic updates will need to include updates to databased information and possibly changing of labels and rearranging of accessions. Arranging collections by a bank or inventory location (a reference to the collection storage location) will avoid the need to rearrange the collection following taxonomic changes.
- Highly active collections, where accessions are regularly accessed provide an ideal opportunity for checking and replacement of humidity indicators, storage containers, labels, or packaging. Regular stocktakes will ensure accuracy of reporting, timely targeting of species for recollection for exhausted or low quantity accessions and assist with identifying appropriate resourcing and future storage requirements.
- Feedback from distributed material. Seed banks are often a source of material for seed germination, biology and agricultural research (see Section 15.4). While plant distribution agreements often include a requirement for sharing of knowledge derived from the distributed material, it can be challenging to ensure that relevant information returns to the donor institution on a regular basis. Distribution of material should be noted on accession records and efforts made to follow up, particularly where data may be relevant to seed storage longevity, **dormancy** alleviation methods and germination techniques. A user survey distributed with the seed, or an online feedback form can encourage return of user data.

Understanding seed storage longevity (see also Chapter 5.7.2)

While the storage longevity for many species is not known, data is available that can indicate groups of plants that may be short lived even in optimum long-term storage conditions (for example, predictive and real time seed aging data, see Chapter 5; and data on seed storage behaviour, see Chapter 6). Potentially short-lived species should be managed using one or more of the following options:

- Investigate alternative storage protocols such as cryostorage for all, or part of, the collection (see Chapter 10).
- Decrease the re-testing period to ensure viability decline is detected before a significant loss of seed viability occurs.
- Identify species for recollection.

15.3.1.2 Seed viability over time

Viability monitoring

Monitoring seed viability is a critical task for the management of germplasm in long-term storage to ensure that the genetic diversity represented by the collections is being maintained. Genebank managers want to be able to detect a decline in the viability of a collection before it becomes a potential issue. It is assumed that seed deaths in a collection are normally distributed in time (Roberts 1972). This means that viability decline will be low at first as the shorter-lived seeds in the collection die, there will then be a period when the majority of seeds in the collection die, and finally a tailing off as the final, long-lived seeds die (see also Chapter 5, Figure 5.7). What level of viability decline is deemed to be unacceptable is subjective. However, a reduction in viability of 15 % below the initial viability (i.e., 85 % of initial viability) is often used as a trigger to implement a contingency action and known as the regeneration standard (FAO 2014).

To check for a decline in viability, seeds should be periodically tested through time (Chapter 5 and 7). This testing can be costly in terms of time, and in seeds. When seeds are scarce, testing and re-testing will become a trade-off between knowledge about the viability of a collection and efforts to conserve seeds of a species. A discussion of the statistical challenges of detecting viability decline in collections can be found in Guerrant and Fiedler (2004).

Methods of determining seed viability are presented in Chapter 5, with the most common method being a germination test. When a germination test is used, the treatments and conditions applied should be the same as in the initial test, so that any changes can be linked to viability rather than treatment. Where a standard germination method has not been developed, as is often the case for wild species, improved knowledge may lead to improved techniques, resulting in improved germination (Crawford *et al.* 2007). In these instances, it would be prudent to change the treatment to the improved technique whilst also replicating the original treatment for comparative purposes. Decisions on the size of samples to be used for re-testing will need to be made considering factors such as: available seeds; initial seed viability; the magnitude of viability decline you wish to detect; your tolerance of results indicating a false decline; and your tolerance of not detecting a viability decline.

The frequency of re-testing will also be a factor that needs to be considered. A re-test interval of ten years for long-lived species and five years for short-lived species is recommended for agricultural species (FAO 2014) and has been adopted for wild plant species. However, for many wild species it may not be known whether it is likely to be long- or short-lived, though significant progress has been made in estimating the longevity of Australian native species (Chapter 5, Section 5.7.1). Analysis of the viability of seed collections stored under long-term storage conditions from Western Australia has shown that the majority of collections tested have maintained their viability in the medium term (5 to 12 years) (Crawford *et al.* 2007) and more broadly, the majority of seed collections (over 85 %) of wild species stored at the Millennium Seed Bank in the UK have maintained their viability for up to 20 years (Probert 2003). For most Australian species, a re-test period in the order of ten years should be considered; shorter re-test intervals are recommended for species such as orchids (Chapter 12), species from alpine **habitats** (Chapter 5 and Satyanti *et al*. 2018) and rainforest habitats (Chapter 6 and Sommerville *et al*. in prep), or for high value collections where premature viability loss cannot be accepted. In some instances, re-testing may not be feasible due to limited seed numbers. In these cases, a decision about how to deal with the collection in the absence of viability data will need to be considered.

Management of viability decline

Where an unacceptable level of viability decline is detected for a collection or if a collection has not been re-tested within an appropriate time, it should trigger a management response. There are three ways of dealing with viability decline in a collection: accept the decline (and retain the species in the collection with appropriate data recorded to inform end users of the expected germination outcome), recollect the species, or regenerate the collection. A decision framework for deciding upon the appropriate response is presented in Figure 15.2.

If, after consultation with major stakeholders and considering current and future uses, it is decided that the collection is no longer considered important to the conservation of the species or the population then it may be decided that no action will be taken. However, this should be noted in the metadata associated with the collection. In most cases, further action is required, and this action will depend on the status of the species or population in the wild. If collection from the original source population is still possible and the available genetic diversity that can be sampled is as good, or better, than the original collection then re-collection should be considered. Future decisions about the collection will then depend on the success of re-collection, including obtaining seeds of appropriate physical quality and genetic diversity. The final scenario is where the collection in which viability decline has been detected is important to the conservation of the species and re-collection is not possible or practical. In this situation the collection will need to be regenerated to salvage as much of the genetic diversity represented by the collection as possible.

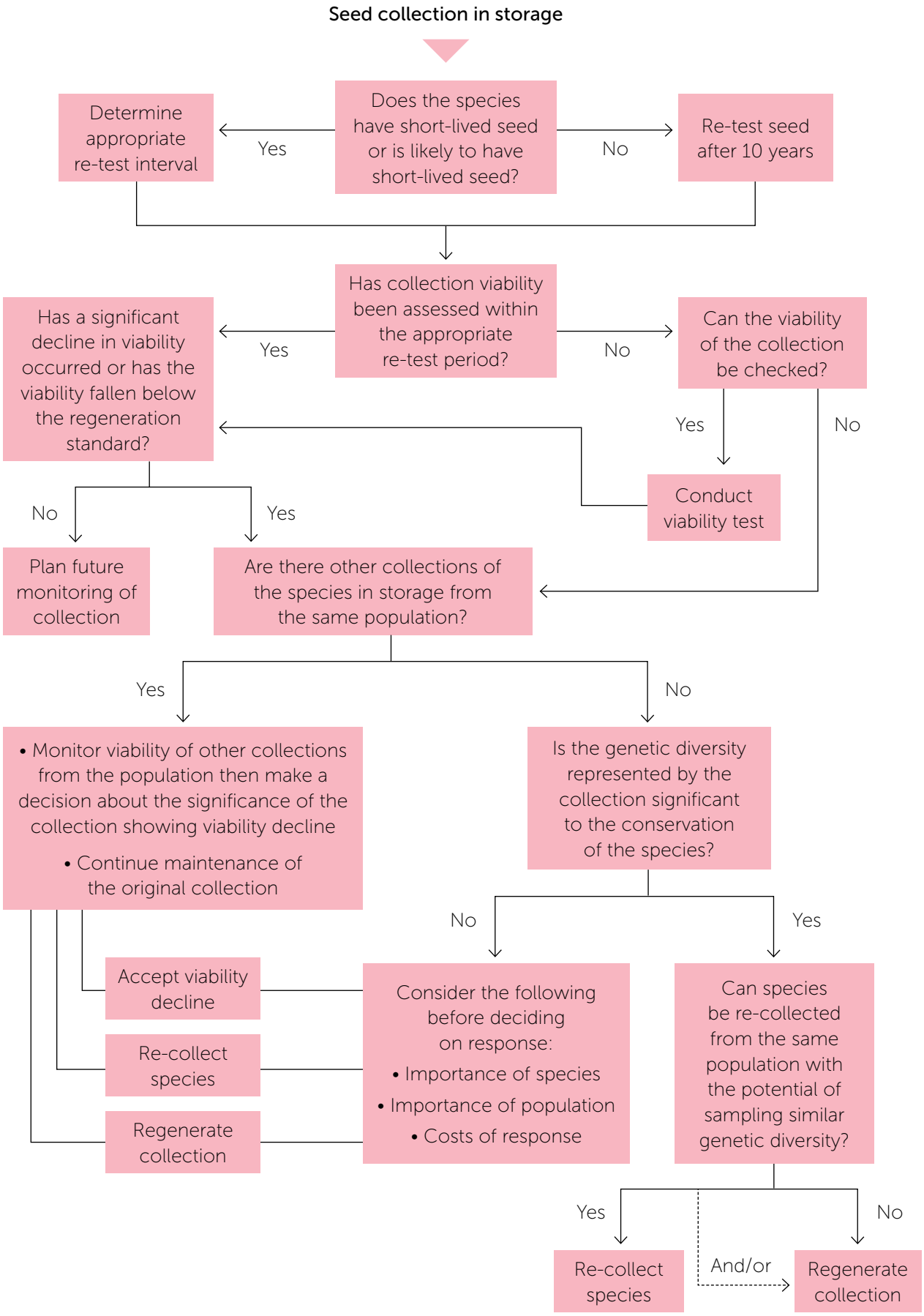

Figure 15.2: Collection management flow diagram, to assist with decision making for viability monitoring intervals, changes in viability and subsequent decisions to recollect a species or regenerate a collection.

15.3.1.3 Regenerating collections from seed banks

As seed collections age over time, they eventually lose their ability to germinate even under the best ex situ storage conditions. To prevent loss of valuable germplasm, genebanks may regenerate accessions to re-establish collections with good quality, quantity and viability for further conservation and utilisation; or they may choose to recollect that species (see Figure 15.2).

The regeneration of a seed collection involves the growing out of plants from the seed sample and then harvesting seeds from the resulting plants. The process of regenerating accessions is dependent on the species that needs to be grown out, their biology including life cycle, access to pollinators, improvement status (wild, cultivated, primitive variety), genetic diversity (i.e., inbred elite released varieties, breeding lines, **landrace** varieties, wild species or special genetic stocks), the ecogeographic localities of their natural environment, and importantly, the end use of the material. The **mating system** is also an important consideration (see Chapter 3). For example, with species that can self, seed collected from these may have less diversity than the original collection as selfing reduces **heterozygosity** and loss of **alleles** may occur. Furthermore, seeds produced by **self-fertilisation** or biparental **inbreeding** in preferential outcrossers can lead to serious problems with inbreeding depression. Active cross-pollination of such species may be necessary and may even increase diversity. Even for annual plant species that complete their life cycle (seed, to mature plant, to seed again) in a single season, the process of regeneration is costly and can be risky from the perspective of preserving genetic diversity with potential losses or shifts in diversity during the process (Espeland *et al*. 2017). For long-lived, perennial species which may be large in size, costs will be significantly higher, and will require far larger areas to grow the plants. This means a much longer timeframe to grow and manage the plants before seeds can be collected.

Failure to consider these critical factors may result in the failure of seed production nurseries, or the production of seeds of low quality and viability or compromised genetic diversity of the resulting daughter seeds compared to the original collection. An overview of regeneration requirements for genebank accessions can be found in Sackville Hamilton and Chorlton (1997). Additional considerations when planning to regenerate genebank accessions are the resources and funding required to undertake such action (see also Chapters 8 and 11).

Regeneration of accessions is the most expensive activity undertaken by genebanks.

Regeneration of a collection in a managed situation for re-collection of seeds can be done through:

- Cultivation in a nursery (see Chapter 8), botanic garden or agricultural land (Case Study 15.2);
- Use of a seed production system (Case Study 15.4) or seed orchard (see Chapter 11; Case Study 3.3 on *Eucalyptus benthamii*; Gibson-Roy *et al*. 2021);
- Translocation of the species back into the wild (see Commander *et al*. 2018a).

As the aim of translocation is to establish a self-sustaining population, collection of seeds to replace material in ex situ storage may not be possible for some time. Risks of the translocation not being successful and seeds not being able to be replaced need to be considered, as well as further loss of scarce seed resources during the process (see Case Study 4.2).

Case Study 15.2: Regenerating conservation seed collections at the Australian Grains Genebank

Sally L. Norton

The Genebank Standards (FAO 2014) recommend regenerating an accession when viability declines to 85 % of the initial viability, or when the seed quantity is less than three sowings of a representative population for that accession. In practice, most agricultural genebanks use a combination of viability and seed quantity to set a threshold for each genus and/or species that triggers the need for regeneration. These thresholds are often variable among species within a genus, and are related to the accession's improvement status, **fecundity**, and viability measured using standard germination tests. Examples of regeneration triggers within the Australian Grains Genebank show the variable nature for species within and across genera (Table 15.1).

Table 15.1: Critical trigger points to identify accessions for regeneration for agricultural crops (Sorghum and Mung Bean) and their wild relatives in the Australian Grains Genebank.

Taxonomy	Improvement Status	Pollination Control[1]	Critical seed quantity (count)	Critical Viability (%)	Grow site[2]	Notes[3]
Sorghum bicolor	Elite variety	O,B	300	85	Field	Direct sow, non-shattering
Sorghum bicolor	Landrace	O,B	300	70	Field	Full excise, direct sow; may shatter
Sorghum macrospermum	wild	O,B	300	60	GH	Full excise, GA_3 controlled germination; shatters
Sorghum angustum	wild	O,B	300	40	GH	Full excise, GA_3 controlled germination; shatters
Sorghum nitidum	Wild	O,B	300	30	GH	Full excise, GA_3 controlled germination; shatters
Vigna radiata	Elite variety	Se,N	200	80	Field	Direct sow, non-shattering
Vigna vexillata	Wild	Se,N	300	50	GH, BGH	Check for hard seed; scarify; may shatter

[1] Pollination control: O,B: outcrossing, bagging required; Se,N = natural self-pollinating, no control required.
[2] Grow site: GH = Glasshouse; BGH = soil bed glasshouse.
[3] The notes field capture any specific protocols for germination, shattering etc., and can be accession-specific.

For many ex situ collections, the number of accessions that require regeneration based upon the above thresholds exceeds the available resources, so a prioritised regeneration system is used. Prioritisation is based on interactions between viability, seed quantity, and end use/importance. If deemed important to maintain over the long term, the highest priority for regeneration are accessions with low (or unknown viability) and low seed quantity, while lowest priority are accessions with a high quantity of seed stocks and reduced viability.

During regeneration, crop and/or species specific protocols are required to ensure the genetic integrity of an accession is maintained, with good amounts of high viability seed produced. Regeneration protocols for some major agricultural crops and wild species are available through online sources such as the Genebank Platform[74] , and through conservation networks. These protocols (Sackville Hamilton and Chorlton 1997; Dulloo *et al*. 2008; FAO 2014) provide clear guidance on how to manage all aspects of seed production for healthy growth, pollen viability and seed production including:

- Parent seed source – the most original seed sample possible;
- Number of seeds sown – a representative sample to ensure rare alleles are represented;
- **Pollination** control (where required) – bags, hot water treatments, isolation cages, physical distancing, insect pollinators to facilitate pollination;
- Environment – closely aligned to the natural conditions of the accession including soil pH, drainage, and nutrient composition, temperature, humidity, light intensity and daylength;
- In crop care – to control plant nutrition, pests, disease, and weeds.

The FAO (2014) recommend sowing at least 100 seeds to regenerate agricultural species. For cultivated and high seed producing species, 100 seeds should be readily available. However, for wild species or accessions that produce small amounts of seed, sowing 100 seeds may not be feasible or possible. Recent research has shown that depending on the genetic diversity of an accession, the number of plants required to maintain genetic integrity can vary considerably within a species and across genera for both self-pollinating and out-crossing species (Allan *et al*. 2020; see also Chapter 3). Guidance on pollination issues in seed production areas can be found in Gibson-Roy *et al.* (2021).

The regeneration protocol for different cultivated crops can be very similar as they have been selected and developed over time for high seed production under comparable environmental conditions. However, non-cultivated wild species within the same genera can have vastly different regeneration requirements, as they are adapted to areas not suited to agricultural production. Additional information can be found in Chapter 8 of these guidelines around vegetative reproduction and nursery production to support ex situ conservation.

As an example of contrasting regeneration requirements, we can look at the *Sorghum* genus that has cultivated and **progenitor** species originating in Africa and Asia, and 17 Australian native crop wild relative (CWR) species that require vastly different environments for seed production. Cultivated Sorghum and its progenitor species can be grown across a range of soil types within sub-tropical environments. They are adapted to heavier, marginal soils and drier conditions compared to other grain crops (Figure 15.3a) and are easily regenerated under field conditions. The 17 Australian CWR Sorghum species are very different to their cultivated counterparts, as they have adapted over millennia to infertile, loose draining soils (Figure 15.3b) and grow poorly using the same soil types, irrigation, and nutrient regimes that suit the cultivated species.

74 https://www.genebanks.org/

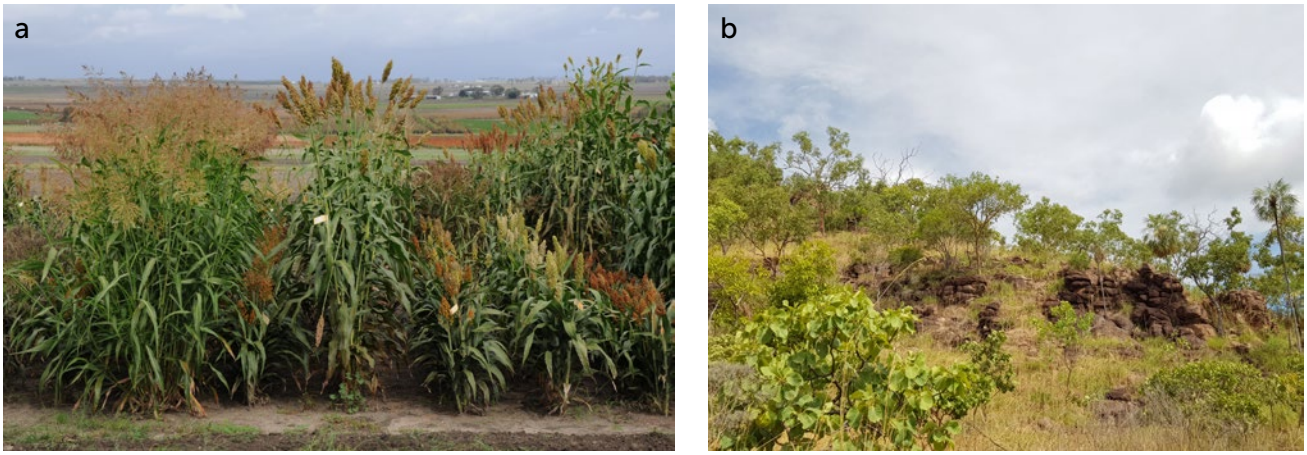

Figure 15.3: Contrasting environments of **(a)** cultivated and progenitor Sorghum species and **(b)** the in situ environment of Australia native CWR Sorghum species. (Images: (a) Kerrie Rubie © The State of Queensland 2021, used under CC BY 4.0; (b) Sally Norton)

The regeneration of Australian CWR Sorghum species should be undertaken in controlled environments such as greenhouses or glasshouses where temperature, daylength, irrigation and nutrition can be customised to suit species requirements (Fig 15.4a). Soil type is a significant factor in successful regeneration of CWR Sorghum as their natural environments stretch across sub-tropical northern Australia, and hence can have vastly different soil requirements with ranges in pH, nutrition, organic matter content and water holding capacity. Where day length cannot be appropriately controlled, germination of CWR Sorghum should be initiated as close to their natural germination and early vegetative growth period as possible, which for most Australian CWR Sorghum is November-January across northern Australia.

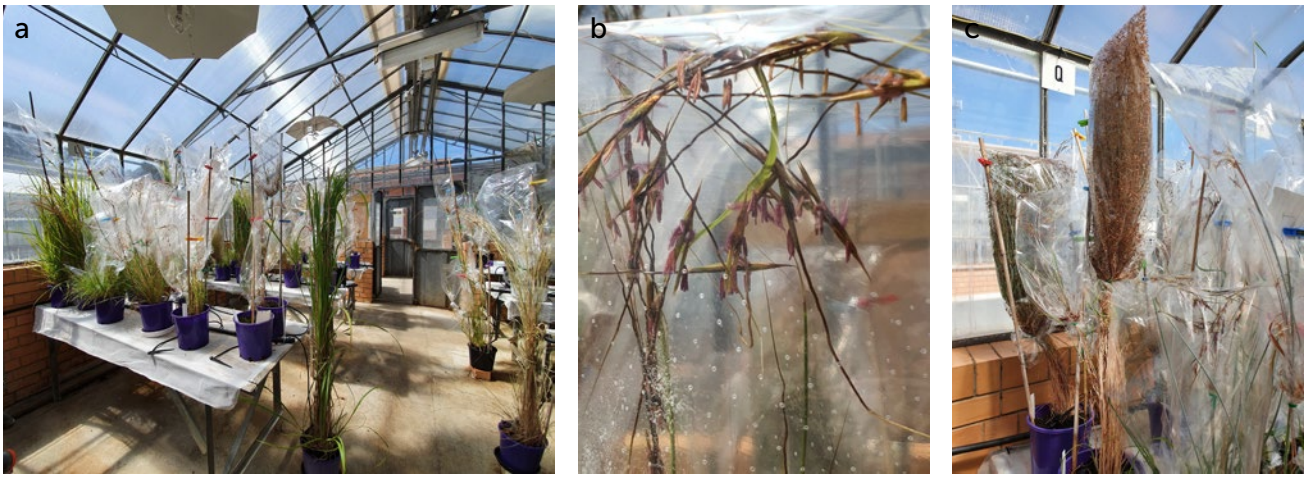

Figure 15.4: Examples of regeneration of Australian native CWR Sorghum species showing **(a)** Glasshouse pot-based growth; **(b)** Panicles showing florets with fully exerted stigma and anthers; **(c)** Multiple panicles from a single plant bagged to prevent cross pollination between accessions. (Images: Sally Norton)

Cultivated Sorghum species germinate readily and have a diversity of panicle types ranging from closed to open (Figure 15.3a). They are generally non-shattering, with an **outcrossing** rate of around 6% that is easily managed by bagging the head for pure seed production, or by removing the top third of a head prior to harvest if pure seed is not required. In contrast, the germination requirements for Australian native CWR Sorghum species are challenging in ex situ conditions (Case Study 7.5): they have loose, open panicles that shatter, with highly exerted stigma and anthers (Figure 15.4b) and hence, are highly outcrossing. These species require bagging to prevent cross pollination between accessions, and to retain seed that shatters at maturity. The heads should be bagged as the panicles fully emerge from the sheath, and prior to becoming fertile. Multiple heads at the same flowering stage from multiple plants within an accession can be grouped into one bag to facilitate pollination and good seed set (Figure 15.4c). The seeds often have large awns, and all species shatter as a mode of seed **dispersal**. As such, the regeneration protocols are highly specialised and labour intensive, and often produce small quantities of seed compared to cultivated Sorghum.

During regeneration, many genebanks collect **phenotypic data** to characterise the accessions. Data that could be collected include emergence, early vigour, plant architecture (plant habit, branching, internode lengths, stem thickness etc.), flower colour, plant height, inflorescence characteristics, pod characteristics and seed attributes (size, shape, colour, 1,000 seed weight), amongst many others (see section 15.2.1). Descriptor lists for some of the major agricultural crops are available from the Bioversity International website[75], which also has a range of other resources and a guide to assist in developing a list of descriptors to suit different needs (Bioversity International 2007). This information can be used for quality assurance purposes within the genebank to validate accession identity, and is highly valued by end users to select germplasm that meets their research or conservation purposes.

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75 https://www.bioversityinternational.org/e-library/publications/categories/descriptors/

15.3.2 Maintenance of tissue culture, cryopreserved germplasm and living collections

Maintenance of germplasm collections where plants are maintained in tissue culture, cryopreservation or as living collections involve processes that are unique to each method. These challenges are outlined in Chapters 8–13. However, the maintenance of germplasm collections through these methods has many elements in common with germplasm maintained in seed banks including the record keeping standards, level of resources required, and the need to monitor and response to declines in the collections.

15.4 Utilisation of collections

There are several considerations for the utilisation of germplasm collections:

- Purpose/end use of germplasm collections
- Level of access to a germplasm collection
- Phytosanitary and biosecurity considerations

15.4.1 Purpose/end use of germplasm collections

When planning and maintaining germplasm collections, it is critically important to consider the anticipated 'end use' or outcome of the collection. Ex situ collections of germplasm are often made as part of a Recovery Plan for a **threatened** species or the re-establishment of key species in threatened communities and may have specific goals that need to be achieved. For agricultural genebanks, ex situ collections are utilised daily for research and plant breeding that underpins food security. When making a germplasm collection it is therefore important to consider the possible ultimate use(s) of the material as this will impact how the collection is made and stored. For example, collections intended for restoration projects will require large amounts of germplasm material which have been sampled from across the range of genetic diversity of the source population or species (see Commander 2021), whereas collections intended for plant breeding often target specific genetic traits such as form, colour, extended flowering period or other desirable physical characteristics of the 'parent' stock. For collections intended for use in research programs, the spatial, **temporal** and genetic diversity of collections must be considered to ensure the full suite of genes can be available for use (Chapter 3). Thus, there are a wide range of end-uses for an ex situ germplasm collection including translocation, **embryo**, research, horticulture, living collections, plant breeding and display.

15.4.1.1 Translocation

Translocations, the deliberate transfer of plants or plant regenerative material to a new location, are used in the management of threatened plant species to augment the numbers of individuals in small and declining populations, **reintroduce** plants to **extinct** populations and introduce plants to new locations that are free from threatening processes (Silcock *et al.* 2018; Commander *et al.* 2018a). The species considered for translocation are generally those that are listed as Threatened, rare species and sometimes distinct populations (e.g., *Banksia brownii*, Coates *et al.* (2018) and Case Study 4.1; *Banksia cuneata*, Case Study 15.3) that are at risk. Broadscale plantings of species for restoration, agricultural or forestry activities are not considered under the definition of translocation in this section as they have their own additional considerations for germplasm sourcing (see Gann *et al.* 2019; Commander 2021) but can offer valuable information.

If the end use of a germplasm collection is for the establishment of a translocated population then the goals and objectives of the translocation must be considered when planning and undertaking the germplasm collection. Primarily the goal for translocations of threatened species is to establish resilient, self-sustaining populations that retain the genetic resources necessary to undergo adaptive evolutionary change (Guerrant 1996). However, there may be goals that are specific to the species or the translocation project that need to be considered as well (Pavlik 1996; Silcock *et al.* 2018). Case Study 3.1 provides an example of using genetic data to plan translocation of *Hibbertia puberula* subsp. *glabrescens*.

The generation of plants for translocation can be done in many ways including through direct seeding; germination, propagation, and planting of seedlings (Case Study 15.3); and propagation of plants through cuttings, grafting or tissue culture. The propagation method will need to be considered when sourcing germplasm as it may impact collection strategy (see Chapter 8; Commander *et al.* 2018b; Swarts *et al.* 2018). For example, if collecting vegetative material for the generation of plants through cuttings, grafting or tissue culture, there will be a need to collect from many source plants and to ensure material from each source plant is kept separate and tracked through the propagation process because plants generated from vegetative material will be exact replicas, or **clones**, of the plant from which it is sourced. It is possible to produce many plants (clones) from vegetative material from the same plant which could result in a translocation with many genetically identical plants if this aspect is not carefully managed (see Chapters 3 and 8; Commander *et al.* 2018b). This is also true for some seed collections, depending on mating system and method of reproduction (selfing, outcrossing, clonality including **apomixis**). This is another reason why it is important to maintain **maternal lines** in all conservation collections (see Chapter 3 Box 3.2) and in particular, those intended for translocation.

Other considerations for the collection strategy for translocation should include how many and which source populations will need to be collected to provide propagation material, and how much seed and/or vegetative material is needed to generate the required number of plants (see Case Study 4.2; Swarts *et al.* 2018). The translocation proposal for the species will provide the information to guide this part of the process (see Nally *et al.* 2018). Throughout the germplasm collection procedure, it will be important to label collections with source populations and sometimes source plant (mother plant) information (Dillon *et al.* 2018, see Chapters 3, 4 and 5; Grose *et al*. (2021) Module 4 – Record keeping). This information will need to be tracked from collection, through propagation and translocation as it is essential to know the origin of the plants that have been established in the translocation site. The decisions made regarding source material for translocation are likely to impact on whether the translocation is successful (Weeks *et al.* 2011; Dalrymple *et al.* 2012; Neale 2012).

Case Study 15.3: Utilisation of *Banksia cuneata* (Matchstick Banksia) seed collections for translocation planting

Leonie Monks

Banksia cuneata is a shrub or small tree from the Proteaceae family. It grows up to four metres in height and produces matchstick-like flower inflorescences between September and December (Figure 15.5). There are only 12 known populations of *B. cuneata* found across a range of just 89 km near the wheatbelt towns of Brookton and Quairading in the south west of Western Australia. The species was listed as Threatened in 1982, with populations declining due to altered fire regimes, grazing, drought, disease and habitat degradation (Department of Environment and Conservation 2008).

Since the species has been listed as Threatened, seed collections have been made from all populations. In the mid 1990's the banked seeds were used to establish experimental translocations in four different localities. There was mixed success from these early translocations, with two of the four populations establishing well and lessons learnt about successful propagation and planting techniques. Further seed collections were made in 2007 and 2014 and these included collections from the two translocated populations.

Figure 15.5: Matchstick Banksia (*Banksia cuneata*) inflorescence. (Image: Leonie Monks, DBCA)

Unfortunately, four of the natural populations continued to decline due to deteriorating habitat quality or lack of recruitment. As a result, in 2016 a decision was made to re-introduce plants into one of these populations. The site chosen had declined due to lack of recruitment, however good quality habitat remained so it was considered a suitable site for the translocation. Seeds that was banked in 2007 and 2014 was drawn upon to grow seedlings, including seeds from the population where the translocation was to occur, from two of the previously successful translocation sites and from two nearby natural populations. Seeds were germinated in the laboratory, so that the germinability of those collections could be assessed as part of the process of obtaining plants. Following germination, seeds were transferred to a nursery and were grown on for six months. In winter 2018, a total of 471 seedlings were planted at the translocation site (Figure 15.6). Seedlings were planted into areas that had been fenced to protect them from herbivores such as kangaroos and rabbits, were tagged with an individual number and irrigated over the dry summer months (November to April) for the first two years. A second planting of 235 seedlings took place in winter 2019.

Each seed accession was tracked through the collection, storage, germination, nursery, and planting stages. Monitoring of the performance of individual plants in the translocation site then provides feedback on whether further plantings of particular seed accessions or source populations are required. It can also inform whether additional seed collections are needed to ensure sufficient seeds in storage for further recovery work.

Survival at the site in 2020 is 38 % of the first planting and 28 % of the second planting. Plants from the first planting have now started to flower and the population is large enough that it meets initial criteria for success for the translocation. Collection of seeds from all the populations before declines occurred were essential in underpinning our ability to undertake the translocations and a critical step in ensuring that this species is on the path to recovery.

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Figure 15.6: Sam Webb planting Matchstick Banksia (*Banksia cuneata*). (Images: Leonie Monks, DBCA)

15.4.1.2 Ecological Restoration

Differences in utilisation of germplasm collections for translocation of a single species, restoration of a threatened community and restoration at the landscape level are very much scale dependent. Collections made for long-term conservation are not necessarily on a scale suited to large scale habitat restoration, although they may be of use in small scale restoration applications such as the repair of Threatened Ecological Communities (see Case Study 15.3) or establishment of a seed production area that can then be used for large scale restoration (see Chapter 11; Gibson-Roy *et al.* 2021). Collections made in partnership with the Millennium Seed Bank (see Case Study 2.6), and associated data, are already being used to **rehabilitate** degraded land in Australia and elsewhere (Liu *et al.* 2018). Seed collections for ecological restoration often are not stored for as long as conservation collections, but where they are stored for long periods understanding seed longevity (Chapter 5) may be beneficial in optimising storage procedures. For further information on collections for restoration see Commander (2021) or Gann *et al.* 2019.

Case Study 15.4: Using seed production areas to restore endangered Cumberland Plain Woodland at The Australian Botanic Garden, Mount Annan

Peter Cuneo and Jordan Scott

The critically endangered Cumberland Plain Woodland (CPW) in the Sydney Basin Bioregion faces multiple threats including urban development and weed invasion. The dramatic expansion of the exotic woody weed African Olive (*Olea europaea* subsp. *cuspidata*) is a Key Threatening Process impeding the survival of remnant vegetation within this ecological community.

The Australian Botanic Garden Mount Annan includes high quality CPW remnants as well as 15-year-old stands of dense African Olive canopy, which limits the growth and recruitment of native understorey grasses and forbs as well as Eucalypt species (Cuneo and Leishman 2015).

A program of work to mechanically clear dense stands of the invasive African Olive in preparation for ecological restoration commenced in 2005, alongside long-term ecological monitoring of remnant CPW vegetation (Benson and von Richter undated). A variety of ecological restoration

techniques was established in an experimental framework, with monitoring of native and exotic species including African Olive emergence for 36 months (Cuneo and Leishman 2015). Direct seeding of CPW understorey native species, particularly grasses, was used as a successful 'bottom up' ecological restoration technique.

Establishment of a native grass seed production area was critical to provide the volume of seeds required for ecological restoration of cleared olive sites (Figure 15.7). Four native grass species *Dichelachne micrantha* (Plume Grass), *Microlaena stipoides* (Weeping Meadow Grass), *Chloris truncata* (Windmill Grass) and *Poa labillardierei* (Tussock Grass) were planted as tubestock into the 1,500 m^2 seed production area, in an area with high public visibility alongside the Australian PlantBank. Seeds for the production area was wild source collected from CPW and grasslands within the botanic garden, which provides a reference vegetation type and condition to guide restoration. Grass seed output from the first 2014/15 summer season resulted in 118 kg of seed material harvested (Figure 15.8), and a total output of over 13 million **viable seeds**. Co-location with the Australian PlantBank allowed for seed quality assessment, processing and seed germination testing, with the final seed product cleaned to 'restoration' grade (e.g., some florets remaining) rather than pure seed.

Direct seeding at the recipient site in densely sown strips, followed site preparation as described in Cuneo *et al*. (2016), with the intention of creating an in situ seed source for these degraded areas, to maintain soil stability, improve ecological resilience and accelerate the establishment of CPW species on these degraded areas. Good germination rates were observed after autumn rainfall, with seedling recruitment densities of up to 608 seedlings m^{-2} recorded from monitoring plots after 10 months. Maximum species diversity was observed after 3 years of establishment, where the average native grass density was 48 plants m^{-2} with 14 native grass species recorded (Figure 15.9, Cuneo *et al*. 2018). Direct seeding of CPW shrub and tree species augmented regeneration of the **soil seedbank** after grass establishment, facilitated by ongoing seed collection and testing of CPW species at the Australian PlantBank.

The ecological restoration of key CPW species, and particularly the establishment of a well-presented seed production area (SPA) attracted interest from visitors to the Australian Botanic Garden, as well as bush regeneration practitioners and scientists visiting the Australian PlantBank (Figure 15.7). It also became the focus of hands-on training on African Olive control and ecological restoration. The lifespan of this SPA was five years; however, this was largely dependent on the longevity of individual species used and the ability to maintain weed control. For example, *Microlaena stipoides* established as a dense sward and was still productive seven years after planting.

Figure 15.7: Seed production areas provide landscape impact and visitor interest at the Australian PlantBank. (Image: Peter Cuneo)

Figure 15.8: **(a)** Harvesting *Microlaena stipoides* seeds using a small mechanical brush harvester. **(b)** High volume seed output from native grasses – hand harvesting *Dichelachne micrantha*. (Images: Peter Cuneo)

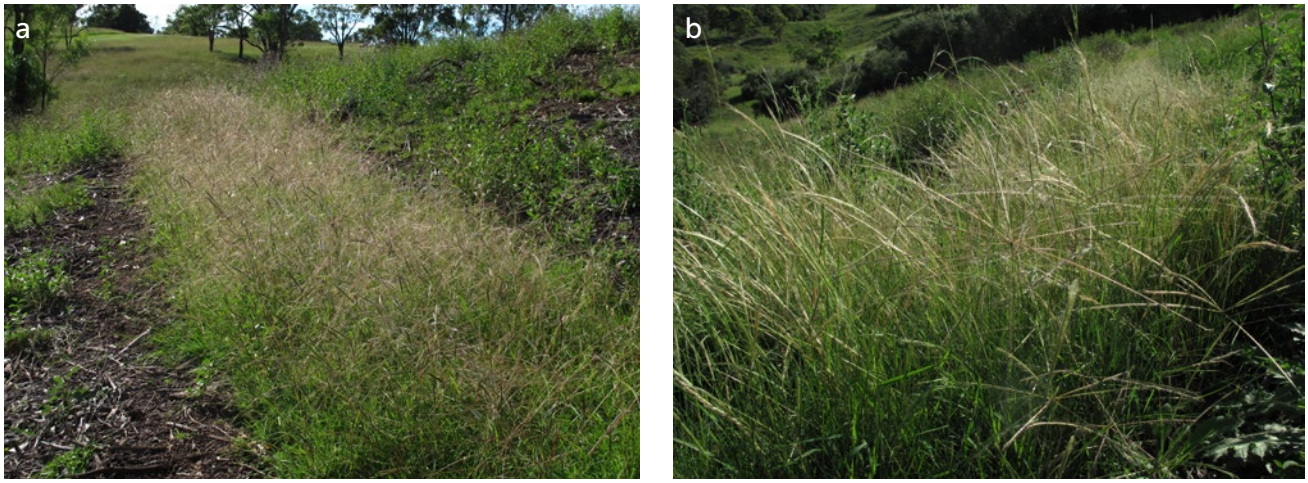

Figure 15.9: **(a)** Strong native grass establishment in direct seeded strip after one year. **(b)** Native grass establishment in direct seeded strips after two years. (Images: Peter Cuneo)

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5.4.1.3 Research

Ex situ germplasm collections also play a valuable role in facilitating and underpinning research into rare and threatened plants, and significant species in ecological restoration. This research can provide vital information on species biology and ecology, assisting with on-ground management (e.g., Yates and Broadhurst 2002; Sommerville *et al.* 2018; Liu *et al.* 2018), helping resolve taxonomic uncertainty (e.g., Shepherd and Crawford 2020), understanding taxonomic relationships between species (e.g., Byrne 2019) and assisting with development of practical storage and germination protocols (e.g., Sommerville *et al.* 2008; Hamilton *et al.* 2012; Sommerville *et al.* 2013; see also Chapters 5, 6 and 7 and references therein). Research on plant response in ex situ collections can also assist in management of threats such as fire, disease susceptibility and grazing (e.g., Shearer *et al*. 2004). Seed bank data from routine germination tests can provide information on species ecology that is valuable for in situ conservation, especially for rare and threatened species where information is often scarce (Clemente *et al.* 2017). Consideration of the likely research uses for a collection can help direct how a germplasm collection is made. For example, if a study of the mating systems of a species is to be undertaken there will be a requirement for a minimum number of plants to be sampled in a population, for the maternal lines to be kept separate, for a minimum number of seeds per maternal line and for these collections to be made from a range of populations (Chapter 3, particularly Box 3.2). In contrast a seeds collection for the development of germination protocols may require a large number of seeds from a single population or collection and prompt testing of fresh seeds rather than stored seeds (Chapter 7).

Agricultural research can be considered as pre-breeding, or breeding. Pre-breeding encompasses a wide range of research including physiology, phenotype, genetics, and evaluation for biotic and biotic stress tolerances. This kind of research is undertaken to identify key characteristics, or traits, that would be beneficial to agriculture. Pre-breeding research is undertaken on cultivated species, their traditional landrace village varieties, their progenitors, and an increasing focus towards use of crop wild relatives. Breeding research involves use of lines that have an identified beneficial trait, making crosses to an adapted cultivated variety, and then assessing the progeny to determine if they are more resilient and productive than their parents. The volume of seeds used for pre-breeding and breeding research can be quite large, and hence the genebanks must ensure that adequate seed stocks for their core sets of germplasm are maintained so they are readily available, with regeneration of large numbers undertaken each year to ensure this.

15.4.1.4 Horticulture/living collections/plant breeding/display

Conservation collections can be a valuable resource for horticulture. It is important to note though, that collections made specifically for targeted horticultural outcomes may not be suitable for species conservation (Chapter 11).

Horticultural use of ex situ collections can include the following:

- Enhancement of horticultural and botanical developments, such as propagation, establishment and cultivation trials. Records created during horticultural maintenance of living collections generates valuable data that can be used to improve management practices for ornamental collections, conservation collections and in planning translocation.
- Public displays such as in botanic gardens, university gardens and arboreta. These displays can establish landscapes that are evocative of the natural world and inspire ornamental horticulture through design and species selection while being underpinned by a conservation message (see Case Study 11.3). Additionally, diverse living collection displays provide accessibility to the visitor for pleasure, artistic outcomes and horticultural therapy generally, enhancing the value of, appreciation for, and human connection to the flora.

- Conservation of species that produce non-viable, few, or no seed or **desiccation sensitive** seed (see Case Study 11.5).
- A valuable resource for public education outcomes. Cultivated horticultural displays are beneficial in providing educational messages about individual species, ecological communities, potential threats, conservation challenges and important research being undertaken (see Case Studies 8.3 and 11.2).
- A resource for formal botanical reference outcomes.
- Plant breeding and utilisation of the flora through commercial avenues such as the horticultural, agricultural and pharmaceutical industries. Plant breeding develops plants for horticultural uses, often focussing on forms with aesthetically pleasing or unusual colours, those with extended flowering times and other attractive attributes. Plant development outcomes are increasingly focussed on providing environmental benefits by developing cultivars that are adaptable to a range of climates and soil types, with lower water and fertiliser requirements.
- Producing material and displays to facilitate conservation and research purposes (see Case Studies 11.2, 11.4 and 11.5). Management details of living collections can provide valuable information and data that can aid in understanding aspects of ongoing conservation.
- Generation of 'stock' plants to be used for future propagation material, to help reduce pressure on natural populations.

15.4.2 Access to a germplasm collection

Where appropriate, and where material can be made available, germplasm may be shared with interested parties. Germplasm should always be distributed in accordance with permit conditions; federal, state and territory laws; and relevant international treaties and conventions e.g., The Convention on Biological Diversity, the International Treaty for Plant Genetic Resources for Food and Agriculture, and the Nagoya Protocol[76], with a relevant agreement (e.g., Material Transfer Agreement). The Material Transfer Agreement outlines its permitted end use, data sharing requirements and ownership of any material propagated from the original collection. A germplasm collection should be accompanied with all relevant documents required by the recipient. Recipients of germplasm are legally required to respect any intellectual property (e.g. Plant Breeders Rights) and comply with requirements for use of the germplasm. They may also be required to provide information about the use of the germplasm collection back to the facility that supplied the germplasm (Section 15.3.1.1).

15.4.3 Phytosanitary and biosecurity considerations

Phytosanitary guidelines must be considered when moving germplasm material between facilities, prior to translocation to the wild, and to germplasm requestors within and outside of Australia as the material needs to be disease and pest free. A phytosanitary certificate is likely to be required when moving material between jurisdictions and states and is often required for movements between countries. Even material originating in one location may need a phytosanitary certificate if it is being repatriated from a duplicate collection location. Information on the necessary steps and documentation is available from federal and state departments of Agriculture or equivalent, or from Biosecurity Australia[77].

76 http://www.fao.org/home/en/ref
77 https://micor.agriculture.gov.au/Plants/Pages/default.aspx

15.5 Germplasm collections are valuable resources

Germplasm collections are a valuable and sometimes irreplaceable resource. Every collection made in some way impacts on the source population and so it is important to ensure that the resource is utilised effectively. Key to ensuring the maximum value for a germplasm collection is to consider the likely end-use when planning and making the collection, and throughout the storage period.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABSA	access and benefit sharing agreement
AIATSIS	Australian Institute of Aboriginal and Torres Strait Islander Studies
ANBG	Australian National Botanic Gardens
ANOVA	analysis of variance
ANPC	Australian Network for Plant Conservation
ANZECC	Australian and New Zealand Environment and Conservation Council
ASBP	Australian Seed Bank Partnership (formerly AuSCaR, Australian Seed Conservation and Research Network)
BA or BAP	6-benzylaminopurine
BGANZ	Botanic Gardens Australia and New Zealand
BGCI	Botanic Gardens Conservation International
CBD	Convention on Biological Diversity
CGIAR	Consortium of International Agricultural Research Centres
CHABG	Council of Heads of Australian Botanic Gardens
CPA	cryoprotective agents
CPC	Center for Plant Conservation
CPW	Cumberland Plain Woodland
CREW	Center for Conservation and Research of Endangered Wildlife
CSIRO	Commonwealth Scientific and Industrial Research Organisation, Australia's national science agency
CWR	crop wild relative
DAWE	Department of Agriculture, Water and the Environment
DBCA	Department of Biodiversity, Conservation and Attractions
DEST	Department of the Environment, Sport and Territories
DMP	disaster management plan
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSC	differential scanning calorimeter
EA	embryonic axis (pl. embryonic axes)
eRH	equilibrium relative humidity
FAO	Food and Agriculture Organisation of the United Nations
FDA	fluorescein diacetate

Fst	proportion of total genetic variance in a subpopulation relative to total genetic variance
Fis	inbreeding coefficient
FIM	fungal isolating media
GA_3	gibberellic acid
GBS	genotyping-by-sequencing
GGBs	green globular bodies
GLM	Generalised Linear Model
GLMM	Generalised Linear Mixed Model
GPS	Global Positioning System
GSPC	Global Strategy for Plant Conservation
H_2SO_4	sulphuric acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IPBES	Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services
IPGRI	International Plant Genetic Resources Institute
ISTA	International Seed Testing Association
IUCN/SSC	International Union for Conservation of Nature/Species Survival Commission
KAR_1	Karrikinolide (the butenolide, 3-methyl-2H-furo[2,3-c]pyran2-one), a seed germination stimulant present in plant-derived smoke
KNO_3	potassium nitrate
LN	liquid nitrogen
MC	moisture content
MD	morphological dormancy
MPD	morphophysiological dormancy
MS medium	Murashige and Skoog medium
MSBP	Millennium Seed Bank Partnership (formerly MSBP, Millennium Seed Bank Project)
MTG	mean time to germination
NAA	1-naphthaleneacetic acid
NGO	non-government organisation
NZ	New Zealand
OMF	orchid mycorrhizal fungi
OTU	operational taxonomic unit
p_{50}	time taken in storage for viability to fall by 50%
PD	physiological dormancy
PDA	potato dextrose agar
PGR	plant growth regulator

PLB	protocorm-like body
PPE	personal protective equipment
PVS	plant vitrification solution
PVS2	plant vitrification solution two
PVS3	plant vitrification solution three
PY	physical dormancy
QR code	Quick Response code
QTL	quantitative trait locus
RBGV	Royal Botanic Gardens Victoria
RH	relative humidity
RO	reverse osmosis
RRS	reduced-representation sequencing
RTBG	Royal Tasmanian Botanical Gardens
SE	somatic embryo
SID	Seed Information Database of the Royal Botanic Gardens Kew[78]
SNP	single nucleotide polymorphism
SPA	seed production area
T_{50}	time to 50% germination
TDZ	thiodiazuron
TSCC	Tasmanian Seed Conservation Centre
TZ or TTC	2,3,5-triphenyltetrazolium chloride
VIV	vacuum infiltration vitrification
WHS	Workplace Health and Safety
WPM	Woody Plant Medium

78 https://data.kew.org/sid/

Glossary

abiotic	of non-living components of the natural world such as geology, soils, and climate.
accession	in the context of ex situ collections, usually refers to material collected from a single population on a particular date. An accession number is a unique identifying code relating to an accession.
active collection	the portion of an ex situ collection for general use (cf. **base collection**, which is used only in defined circumstances).
adaptation	the process by which a species or population acquires novel traits better suited to new environments or new environmental pressures.
after-ripening	the progressive loss of dormancy in mature, dry seeds; controlled by factors such as temperature and seed moisture content.
agamospermy	asexual production of seeds or embryos in the absence of fertilisation, a form of apomixis.
alleles	the possible forms of a gene or other region of the genome.
angiosperm	a flowering plant; a division of Spermatophyta that produce ovules and seeds in closed megasporophylls (carpels) cf. **gymnosperm**.
anthropogenic	resulting from or produced by human activity.
apomixis	a process of asexual reproduction in which seeds are produced without fertilisation or the involvement of meiosis.
arcsine square root transformation	a combination of the arcsine and square root transformation functions, that takes the form of asin(sqrt(x)) where x is a real number from 0 to 1. The square root transformation helps to deal with probabilities, percentages and proportions that are close to either one or zero. Also known as the angular transformation.
aseptic	a technique or conditions free from pathogenic microorganisms.
aspirator	a machine designed to separate seeds from other plant parts using a constant flow of air; used for the purpose of obtaining clean seed batches.
autoclave	a vessel for carrying out sterilisation at high temperature and pressure.
autotrophic	a type of nutrition in which organisms synthesise the organic material they require from inorganic sources.

auxins a group of naturally occurring hormones that play a key role in regulating plant growth throughout the life cycle; in tissue culture, natural and synthetic auxins are used to elicit various types of growth responses, including root induction.

axenic free of foreign organisms.

axillary bud a bud that grows from the axil of a leaf or node and has the potential to form stems and branches with leaves or reproductive shoots with flowers.

base collection the portion of an ex situ collection kept separately from the active collection, and only used in defined circumstances. (cf. **active collection**).

Bayesian approach statistical methods based on Bayes theorem which can be used to derive probability distributions for a parameter. This method can incorporate prior information to modify the probability distribution.

binomial distribution can be used to calculate probabilities for different numbers of successes out of 'n' trials, given a known probability of success on any individual trial. An important error distribution for modelling variables with binary outcomes (e.g., germination vs no germination) using logistic regression.

biodiversity the variety of life forms, the genes they contain and the ecosystems they form.

biodiversity hotspot a biogeographic area with an unusually high diversity of species that is threatened by human activities.

bioinformatics the science of collecting and analysing large complex biological data sets.

bottleneck a sudden restriction in population size for one or more generations; is often followed by a genetic bottleneck, where gene diversity is also lost as a consequence of increased inbreeding.

bradysporous species that store mature seeds in the plant canopy; syn. **serotinous** (preferred); cf **geosporous**

brood cells (of **bryophytes**) specialised spherical cells that grow on the protonema of some mosses, that can detach allowing vegetative reproduction. Sometimes brood cells can grow together on the protonema forming 'brood bodies' that can grow into a new plant.

bryophytes a taxonomic division of non-seed-bearing non- vascular plants which produce spores; mosses, hornworts and liverworts.

caespitose of tussocking habit.

callus protective tissue, consisting of parenchyma cells. that develops over a cut or damaged plant surface.

callus culture	a type of tissue culture consisting of a mass of parenchymatous cells from which new organs or whole plants might be induced to form.
canopy seed bank	the store of seeds held in the canopy of serotinous (bradysporous) plants; many such plants shed their seeds after fire.
charophyte	a member of a group of relatively complex algae with whorled stems and leaves; they are considered to be the algae that are closest relatives to land plants.
chi-squared test	a statistical test used to compare observed and theoretical (expected) frequencies in categories.
chromosome	the structure within the cell nucleus that carries genetic information.
circa situm	a conservation setting within an altered agricultural landscape, outside the natural habitat but within a species' native geographic range.
clade	a sub-group of organisms from among a larger group, sharing common ancestry.
cleistogamy	self-fertilisation that occurs within a permanently closed flower.
clonal collection	a collection of plants derived from one or more clones.
clone	an individual or group of plants produced asexually from a single parent, (in horticulture often known as a cultivar); members of a clone are both phenotypically and genetically identical, although minor variation may occur (see somaclonal variation); the process of a propagating a clone is called cloning.
cohort	a group of individuals of the same age, recruited into a population at the same time.
congeneric	of plant or animal species, belonging to the same genus.
conservation (plant)	protection, care, management and maintenance actions that allow the taxon to follow an evolutionary path in its natural environment cf. preservation. More broadly, conservation refers to actions that maintain ecosystems, habitats, species and populations, within or outside of their natural environments, in order to ensure their long-term existence.
cross-fertilisation	the union of male and female gametes derived from different individuals to form a zygote.
cryobiotechnology	the use of modern technologies to understand the response of biological systems to low temperature environments, whether natural or imposed, leading to the production of knowledge, goods and services, including the cryopreservation of organisms, cells and tissues for use by industry, agriculture, medicine and conservation (Pritchard 2018).
cryopreservation	preservation of germplasm through storage at very low temperatures in or over liquid nitrogen (usually <-130 °C).

cryoprotectant	a substance that prevents tissues from freezing or prevents damage to cells during freezing.
cryovial	a vial used to store material for cryopreservation.
cut test	a destructive sampling technique used to determine the viability of a seed; when cut (usually in half), a viable seed should have firm, usually white flesh, and, in many species, a visible embryo.
cytokinin	a class of plant growth regulators (hormones), produced by the roots and travelling upward through the xylem, that promotes tissue growth and budding; commonly used in tissue culture to promote cell division.
dehiscent fruit	any fruit in which the fruit wall splits open or develops apertures at maturity, releasing seeds.
depauperate	of a flora, fauna or ecosystem, lacking in numbers or variety of species (of low biodiversity).
desiccation sensitive species	species that have seeds that are sensitive to desiccation to the levels tolerated by 'orthodox' species, and are either killed or have poor storage ability when dried. Also known as recalcitrant species.
desiccation tolerant species	(of a **seed**) see orthodox species.
dewar	a vacuum flask used to keep liquids at a particular temperature; commonly used to hold liquid nitrogen for cryopreservation (named after J. Dewar 1842–1913).
diaspore	the unit of reproductive dispersal in plants (typically whole fruits or individual seeds or spores, but also sometimes specialised vegetative units).
dioecious	a condition in which male and female flowers are produced on separate plants.
disjunct	distinct from one another; of populations that are remote or outlying from the core of the organism's distribution.
dispersal	the process by which seeds are spread from a plant, e.g., through gravity, wind, water or other vectors such as animals.
dispersal mechanism	the re-distribution of seeds following release from the parent plant; can be explosive (ballistic), or via wind, water, or animals (including insects). Can also refer to traits; see **geosporous**, **serotinous**.
dispersal unit	see **diaspore**.
divergence	(in evolutionary biology) an evolutionary process where differences accumulate between closely related species.
DNA	the genetic material of most living organisms; deoxyribose nucleic acid.

dormancy	the property of a seed that prevents its germination even under environmental conditions that normally favour germination. Dormancy usually operates to prevent the seed germinating at a time that is not conducive to good seedling growth and establishment, or to maximise the chances of successful recruitment by spacing germination over a succession of years or seasons. Dormancy classification is based on seed coat permeability to water, embryo morphology and physiological responses of seeds to temperature or temperature sequences.
ecological restoration	the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed. See also **restoration**.
ecosystem	assemblage of biotic and abiotic components in water bodies and on land in which the components interact to form complex food webs, nutrient cycles and energy flows.
ecotype	a group within a species, having unique characteristics genetically adapted to particular environmental conditions.
ecotypic variation	variation occurring due to a species adapting genetically to its local habitat or a particular environmental variable.
edaphic	of the soil, substrate or topography.
elaiosome	a lipid and protein-rich structure attached to a seed, derived from various seed or fruit tissues. Often attractive to ants.
'embling'	analogous to a seedling, refers to a somatic embryo induced to develop and 'germinate' like a zygotic embryo.
embryo	the result of fertilisation, it is the young plant contained within the seed coat generally consisting of a shoot apex (plumule) and root axis (radicle); from the centre of the axis, grow one or more seed leaves (cotyledons).
embryophyte	members of the subkingdom Embryophyta (i.e., most land plants), that develop from an embryo. Includes gymnosperms, angiosperms, hornworts, liverworts, mosses, ferns and their allies but excludes algae.
embryo culture	1. a tissue culture using an embryo as the explant; 2. tissue cultures in which embryos are induced to form from somatic tissue i.e., somatic embryogenesis.
endemic/endemism	(of a species) native to a specific geographic area (often with a limited distribution); a high level of endemism often occurs in floras that have been isolated by continental or landscape events.
endocarp	the innermost layer of the **pericarp** (wall of ovary at fruiting stage); often hard, bony or papery e.g., the woody wall of a nut like an almond.
endosperm	the nutritional storage tissue in the seeds of most angiosperms.

ephemeral	an organism with a short emergent life cycle, and often a long or indefinite phase as a dormant seed or spore.
epigenetic	relating to or arising from non-genetic influences on gene expression.
equilibrium moisture content	the moisture content of seeds at equilibrium with the air within the drying or storage conditions (see equilibrium relative humidity).
equilibrium relative humidity (eRH)	the percentage relative humidity at which a given equilibrium moisture content is expressed (at a given temperature).
ethylene	a colourless gas, C_2H_4, that occurs naturally in plants and acts as a plant hormone with a variety of physiological roles.
exflasking	a procedure by which explants can be removed from tissue culture and potted into propagation mix, also known as deflasking.
ex situ conservation	conservation of an organism away from its original habitat; the maintenance of living plant material away from the wild.
exceptional species	plant species that cannot be effectively and efficiently conserved long term ex situ under the conditions of conventional seed banking, requiring alternative conservation approaches. Includes species with few or no seeds available for banking; species intolerant of desiccation and freezing, or seeds that tolerate drying but not freezing; or species that tolerate storage at -20 °C for less than 10 years.
exocarp	the outermost layer of some fruits e.g., the 'skin' or 'peel' of an orange. See also **pericarp**.
explant	the excised portion of a plant used to initiate and perpetuate a tissue culture.
extant	existing or living at the present time.
extinct	of taxa, known or believed to no longer be extant (conventional definitions include taxa not located in the wild during the past 50 years, or not found in recent years despite thorough searching of all likely habitat).
fecundity	the rate of reproduction, productivity, fruitfulness, abundance; low fecundity results in a low number of seed produced.
fitness	reproductive and establishment/survival capability of an organism and its genetic contribution to the gene pool.
free radicals	atoms or groups of atoms that are extremely reactive due to an unpaired electron, commonly resulting in oxidation reactions.
fruit	structure formed from the ripened ovary and sometimes, surrounding tissue, that encloses the seed or seeds.

gametophyte	the generation in a plant life cycle that bears gamete-producing sex organs (in angiosperms, the pollen grain and embryo sac); the dominant phase in the life cycle of mosses and liverworts.
gemma (plural: gemmae)	small clumps of undifferentiated cells developing on the surface of plants such as mosses and liverworts and capable of acting as a diaspore; gemmation is a type of vegetative reproduction.
gene	the main functional unit of heredity; the part of the DNA molecule that encodes a single enzyme or structural protein unit.
gene flow	the dispersal of genetic material in space and time from one individual to another or one population to another. For plants this is achieved by dispersal of seed and pollen.
generalised linear model	a model in which the variable of interest (y) is described by a combination of a series of parameters (regression slope and intercept); generalised model fitting uses maximum likelihood estimation to accommodate distributions other than normal distribution and relationships between the mean and variance.
genet	genetically distinct individual (cf. **ramet**).
genetic diversity	the array of variable genetic characteristics or genotypes present within a population or species. Often measured with molecular markers. Genetic diversity arises through evolutionary processes, including mutation, genetic drift, natural selection, and migration (e.g., gene flow).
genetic drift	in small populations, some alleles or genotypes may become reduced in frequency or disappear by random chance if they are not passed on to further generations. Differences in phenotype or genotype due to genetic drift do not reflect adaptive differences.
genetic variability	variation in the genetic composition between individuals, populations or taxa.
genome	all the genes contained in a single set of chromosomes. Genomics is the branch of genetics concerned with the study of genomes.
genotype	the genetic constitution of an individual organism, fixed except under certain conditions (mutation).
genotyping	using an appropriate set of molecular markers to obtain a profile of the genetic constitution of an individual.
geosporous	species that release seed into the soil seedbank; cf **bradysporous**, **serotinous**.
germination	emergence of the radicle (or sometimes shoot) from the seed coat, occurs between imbibition and emergence from the soil/substrate.

germplasm 1. living tissue from which new plants can be grown; 2. the genetic material that carries the heritable characteristics of an organism e.g. cells, seed, plants.

gibberellic acid a plant hormone. A family of compounds that can regulate seed germination and other aspects of plant growth; often used as a germination promoter of seeds with physiological dormancy.

glass in the seed storage context, a highly viscous solid state of water, that resembles a solid brittle material but retains the disorder and physical properties of the liquid state. cf. liquid water; ice (a crystalline solid).

glyceronitrile a chemical germination cue found in plant-derived smoke, active through the release of hydrogen cyanide upon hydrolysis. See **smoke water**.

Gondwanan relating to the ancestry of flora and fauna species in the forests of the southern supercontinent Gondwana.

green globular bodies (GGBs) meristematic tissues of ferns that are produced in vitro and are like the protocorm-like bodies (PLBs) of orchids.

gymnosperms a group of plants – conifers, gingkos, cycads and Gnetophytes – that do not bear their ovules within closed megasporophylls (carpels); instead, they lie 'naked' on the megasporophylls (typically aggregated into a cone-like structure, although in some Gymnosperms, e.g., *Podocarpus*, reduced to one or two fleshy-coated seeds on a fleshy base) and are openly exposed to the environment (cf. **angiosperms**).

habitat the location and environment where an organism occurs naturally.

haustorium (plural: haustoria) highly modified stem or root of a parasitic plant that penetrates the tissue of a host plant and absorbs nutrients and water.

hemiparasite a parasitic plant that has chlorophyll and can fix carbon via photosynthesis but lacks a fully developed root system and forms connections with another plant to obtain some, or all, of its water and minerals and sometimes carbohydrates.

heterozygous having two different alleles at a given locus of a pair of chromosomes (cf. **homozygous**).

holoparasite a parasitic plant that lacks chlorophyll and relies on a host plant for carbon, water and minerals.

homozygous having the same allele at a given locus (place) of a pair of chromosomes (cf. **heterozygous**).

hybrid the progeny of a cross between different taxa.

hybridisation the exchange of genes (or alleles) between different taxa. In a cultivated or wild conservation setting, it is often undesirable to have hybridisation, as the more common parent will swamp the genepool of the rare species.

hygrometer an instrument used to measure the humidity in the air. Used for the non-destructive measurement of eRH in seeds.

hygroscopic tending to absorb moisture from the air.

hyperhydricity a condition in which tissue-cultured shoots develop a glassy or watery appearance.

hypha (plural: hyphae) a fungal filament that may form part of a loose network of mycelium or a dense mass, as in the fruiting body of a mushroom.

imbibition absorption of water by dry seeds from the surrounding medium. As seeds imbibe and hydrate, water causes turgor pressure (seeds swell). Imbibition is necessary to activate enzymes and transport nutrients to the developing seed embryo, as well as to break down starch into sugars.

inbreeding the mating of individuals with similar genetic makeup, usually causing a reduction in heterozygosity and genetic diversity.

inbreeding depression reduced fitness and ability to survive due to mating among closely related individuals; detected in F1 or subsequent generations. May be of concern in small populations.

indehiscent fruit a fruit that does not open at maturity to expose or release the seeds.

intermediate species in a germplasm content, species with storage behaviour neither truly recalcitrant not truly orthodox. Sub-categories include desiccation-intermediate (seeds can withstand desiccation to around 7–12% moisture content), and freezing-intermediate (survive drying to low moisture content but not freezing at -20 °C or begin to lose viability within 12 months of storage at -20 °C. .

inter situ conservation plants cultivated in near-natural (lightly managed) conditions for conservation reasons.

introgression the incorporation of genes of one species or subspecies into the gene pool of another, usually through the backcross of fertile hybrids to the more abundant species.

in situ the original place; pertaining to the maintenance of plants in the wild.

in vitro an artificial environment (such as a test tube). In vitro culture is used for vegetative propagation, as well as preparation for cryopreservation.

karrikinolide the butenolide, 3-methyl-2H-furo[2,3-c]pyran2-one, a seed germination stimulant present in plant-derived smoke; KAR_1. See **smoke water**.

laminar flow cabinet	an enclosed workstation used to create an environment free from contamination, through laminar airflow and filters that prevent particles entering the cabinet.
landrace	a crop cultivar evolved through years of selection and adapted to local conditions.
lipid peroxidation	a process in which oxidants such as free radicals attack carbon-containing double bonds.
longevity (seed, spore)	how long a seed or spore remains viable; the life span of a seed or batch of seeds or other germplasm.
lycophytes	also known as 'fern allies'; seedless vascular plants belonging to the phylum Lycophyta.
lyophilisation	the process of freeze-drying.
masting, mast-seeding	a pattern of highly variable levels of seed production from year to year (or from one seeding event to the next), and often synchronised within a population; a 'mast year' is at the high-productivity end of the variation curve. A pattern most often seen in some forest tree genera, but can occur in other taxa and ecological types.
maternal line	the offspring (seeds or plants) from a single mother plant; distinguished with a unique identifying number, stored in a separate package, and labelled if grown in a nursery. Knowing the number of maternal lines in a conservation collection is an estimate of the genetic diversity represented.
mating system	describes the mode of mating for a plant species. Can be: self-fertilisation (pollen donor and recipient are the same individual), outbreeding or outcrossing (with pollen from a different individual) or mixed mating combining outcrossing and selfing in varied proportions among species and among populations.
meristem	plant tissue consisting of actively dividing cells that differentiate into new tissues.
mesocarp	the fleshy middle layer of the fruit wall (**pericarp**).
metadata	data providing information about one or more aspects of data record, such as field collection data relating to a seed collection or herbarium specimen.
micropropagation	vegetative propagation of axillary buds or adventitious shoots through tissue culture.
moisture content (seed)	a measure of the amount of moisture in seeds; often expressed as the difference between the fresh weight and the dry weight divided by the fresh weight x 100 (%).
molecular data	genetic data.

morphology	the form of the external structure cf. anatomy, which refers to the internal structure.
mutation	a change within the genome (at either a gene or chromosome scale) which produces in the mutant (or variant) a slight or profound effect.
mycoheterotrophic	a plant that lives in association with a fungus that supplies most of its nutrition (sometimes at a particular life stage).
mycorrhiza	a fungal species that maintains a non-pathogenic mutualistic association with a vascular plant (via the roots) or bryophyte (via the rhizoids).
non-orthodox	of seeds that cannot be stored under standard seed banking conditions including seeds that do not tolerate drying, seeds that tolerate some drying but not to low moisture content and seeds that tolerate drying but not freezing.
obligate-seeding	of plants that do not resprout and so rely on seed to regenerate after fire.
orographic	relating to mountains; for clouds or rainfall, resulting from the effect of mountains in forcing moist air to rise.
orthodox species	species that have seeds that remain viable when desiccated to 5 % moisture content or less (desiccation tolerant seed) and survive storage at sub-zero temperatures (cf. **recalcitrant** or **intermediate seeds**). The longevity of orthodox seeds is extended by storage at sub-zero temperatures compared to storage at temperatures above zero.
outbreeding	production of progeny by the fusion of distantly related gametes (cf. **inbreeding**).
outcrossing	reproduction relying on pollen from a different individual. Some species are obligate outcrossers.
ovary	the organ containing the female gametes; the enlarged, usually lower portion of the pistil containing the ovules.
ovule	consists of an inner embryo sac containing the egg cell surrounded by the nucellus and enclosed by one or two integuments; after fertilisation of the embryo sac, the ovule develops into a seed.
parasite	a plant that obtains or may obtain part or all of its food by parasitism, e.g. mistletoe, which also photosynthesises. See also **hemiparasite**.
pericarp	the part of a fruit formed from the wall of the ripened ovary, consisting of the **endocarp**, **mesocarp** and **exocarp**.
personal protective equipment (PPE)	refers to safety equipment, clothing or other items that can be used to minimise the risk of harm in the workplace. Common examples when working in a laboratory or nursery include safety eyewear, gloves for safe handling of chemicals, and laboratory coats for protection against spills.

phenology	the study of the influence of seasonal and other environmental conditions on the recurrence of flowering, seed production and other life cycle events; also used as a summary noun for these characteristics of a particular species.
phenotype	the observable characteristics of an organism, determined by the interaction of relatively unchanging genotype with varying environmental conditions.
photoautotrophic	a type of nutrition in which organisms synthesise the organic materials they require via photosynthesis.
phylogenetic	describes a system of classification of organisms that aims to show their evolutionary history.
pollination	the transfer of pollen from the anther to the stigma of the same flower or another flower; a pollination vector may be biotic (e.g., bird or insect, known as a pollinator) or abiotic (e.g., wind, water).
polyploidy	a condition in which cells of an organism have more than two paired sets of chromosomes.
population	a group of interbreeding individuals that belong to the same species from a particular location.
predation	the eating of seeds (or other plant parts) by animals which, in the case of seeds, can occur pre or post maturation; a major cause of seed loss.
preservation	maintenance of a taxon in the state of its existence at a given point in time cf. **conservation**.
probit analysis	statistical analysis examining the relationship between a binary response variable (e.g., germination or no germination) and a continuous variable.
progenitor	a parent or direct ancestor of an organism.
protocorm	a tuber-shaped body produced by orchid seedlings.
protocorm-like bodies (PLBs)	somatic embryo-like structures formed in vitro during orchid germination, from which shoots and roots arise.
post-harvest	processes that occur after harvest such as seed cleaning and drying.
post-zygotic	after the fertilisation event that forms a zygote.
pre-zygotic	before the fertilisation event that forms a zygote.
propagule	a unit of reproduction; includes seed, spores, or vegetative matter capable of independent growth (e.g., gemmae).
provenance (genetic)	the geographic location of seed or other germplasm collected from individuals within a population.

provenance (collection) the point of origin of a collected plant, primarily in a geographic (spatial) sense (see **provenance (genetic)**) but also – depending on state of knowledge – with some reference to habitat, environmental variables, and/or genetic structuring within the species. Often thought to be correlated with genetic adaptation to local environmental conditions.

pteridophyte a member of a taxonomic division of non-seed-bearing vascular plants which produce spores; ferns and fern-allies.

ramet a plant formed by asexual reproduction; a physically but not genetically distinct individual (cf. **genet**).

recalcitrant seeds seeds that are intolerant of desiccation and rapidly lose viability when dried; such seeds cannot therefore be stored at sub-zero temperatures (cf. **orthodox seeds**); typically found in wetter environments; often have large and/or oily seeds, thin seed coats and fleshy-fruits.

recombination the process by which pieces of DNA are broken and recombined to produce new combinations of alleles.

rehabilitation management actions that aim to reinstate a level of ecosystem functioning on degraded sites, where the goal is renewed and ongoing provision of ecosystem services rather than the biodiversity and integrity of a designated native reference ecosystem.

reintroduction an attempt to establish a population in a site or habitat type where it no longer occurs (locally extinct). This may be part of the process of restoration or reconstruction of a habitat where the species was previously known to occur. Also known as re-establishment.

relative humidity the amount of water vapour in the air expressed as a percentage of the amount needed for saturation at the same temperature. Note that this value varies with temperature, so it should always be recorded when relative humidity is recorded.

relict a group of organisms that survives as a remnant of a much larger group, in terms of taxonomic diversity or geographical distribution.

restoration any action, intervention, or treatment intended to promote the recovery of an ecosystem or component of an ecosystem, such as soil and substrate amendments, control of invasive species, habitat conditioning, species **reintroductions** and population reinforcements. See **ecological restoration**.

revegetation an action by which a barren or degraded site is provided with plants to provide functional habitat; it may not necessarily include original provenance or species composition.

rewilding the planned reintroduction of a plant or animal species, especially a keystone species, into a habitat from which it has disappeared, in an effort to increase biodiversity and restore the health of an ecosystem.

rhizobia	nitrogen-fixing soil bacteria from the genus *Rhizobium*.
rhizome	an underground stem from which new shoots or leaves may develop.
Sahul	a geographical and biogeographical term for landmasses that sit on the Australian tectonic plate, including the present-day Australia, Tasmania, New Guinea, Seram and many other islands. Greater proportions of Sahul have been exposed by past periods of low sea level, resulting in an enlarged continent extending well beyond Australia's current extent. The Sahulian flora comprises plant species and genera that arose in or are typical of that region; many have a more remote ancestry deriving from the flora of the Gondwanan supercontinent.
seed	a small embryonic plant in a resting state usually surrounded with some stored food (endosperm), enclosed in a protective covering called the seed coat; it is the product of the ripened ovule following fertilisation and some growth within the mother plant.
seed axis	the embryonic axis of a germinating seed comprising the plumule (shoot) and radicle (root). Plural: seed axes.
seed fill	the proportion of a batch of seed that contains an embryo (and endosperm, if applicable).
self-fertilisation (selfing/selfed)	reproduction with pollen and ovules from the same individual
self-compatibility	the absence of genetic mechanisms which prevent self-fertilisation; plants that can reproduce successfully via self-fertilisation and cross-pollination.
self-sustaining (population)	a population of plants that maintains itself and can reproduce without external assistance.
sequencing	of genetics, a laboratory technique to determine the exact sequence of DNA bases (A, C, G, T, U) in a DNA or RNA molecule.
serotinous	species that retain their mature seeds in a cone or woody fruit for a number of years, but release them after exposure to fire or other disturbance (syn. **bradysporous**, less preferred); serotinous species are characterised by the possession of an aerial seed bank (cf. **geosporous**).
smoke water	a liquid produced by bubbling smoke derived from the combustion of plant material through water; contains a variety of chemicals including **karrikinolide** and **glyceronitrile**; often used to alleviate dormancy in seeds of fire-responsive species.
soil seedbank	the 'store' of seeds within or on the soil; may be present for short (transient) or long (persistent) periods.

somaclonal variation	variation seen in plants derived from somatic tissue through tissue culture and other vegetative means; variation may be genotypic, or phenotypic i.e., of genetic or epigenetic origin.
somatic embryogenesis	the production of embryos from somatic cells of explants (direct embryogenesis) or by induction of callus (indirect embryogenesis).
somatic tissue	tissue derived from a body cell i.e., of non-sexual origin.
sporophyll	a modified leaf that bears sporangia, the structures on or within which spores (reproductive bodies) are formed.
sporophyte	the generation in the life cycle of a plant that produces spores; it is the dominant part of the life cycle in clubmosses, horsetails, ferns and seed plants.
stochasticity	1. demographic – random variation in the birth and death of individuals; changes to the structure and size of a population; more pronounced in small populations; 2. environmental – random and unpredictable environmental processes and events causing change in community or landscape structure, such as a catastrophic event like flood or fire.
stratification	chilling or heating treatments of moist seeds used to alleviate dormancy in some species.
Sunda	a biogeographical region that comprises the Malay Peninsula and the Indonesian Archipelago islands west of and including Borneo and Java. Sunda and Sahul are separated by the Wallace Line, an important division between ancestral floral and faunal zones.
suspension cell culture	a type of cell culture in which cells function and multiply in an agitated growth medium.
symbionts	1. (narrow sense) organisms living together to their mutual benefit (syn. mutualism); 2. (wide sense) other relationships such as commensalism – which benefit only one partner.
taxon (plural: taxa)	the named classification unit to which individuals are assigned e.g. genus, species, subspecies, variety etc.
temporal	relating to time.
testa	that part of the seed coat derived from the outer or single integument (layers covering the nucellus).
tetrazolium test	a staining test (known as the TZ or TTC test) using the chemical 2,3,5-Triphenyltetrazolium chloride to detect seed viability; a healthy embryo usually stains red, but staining patterns vary from species to species.
thallus (plural: thalli)	relatively undifferentiated vegetative body with no true roots, stems, leaves or vascular system.

threat	a factor potentially or already causing degradation, damage or destruction.
tissue culture	the cultivation of plant parts under **axenic** conditions in synthetic media in vitro; a method of plant propagation.
totipotency	the ability of a plant cell to dedifferentiate into an undifferentiated cell which is then capable of developing into any type of plant cell; ability of a plant cell (type) to form into an organ or regenerate a whole plant.
translocation	the deliberate transfer of plants or regenerative material from an ex situ collection or natural population to a location in the wild. Often used in the context of threatened species. Translocation actions can include reinforcement, reintroduction, introduction and assisted migration.
tuber	a swollen underground stem or root.
viable population	an interbreeding group of organisms of the same species that possesses the ecological, demographic and genetic attributes required to persist in both the short and long term.
viable seed	living seed that can potentially germinate if sown under suitable conditions. Viable seeds include dormant seeds, in which case the dormancy must be alleviated before germination. Thus, a non-viable seed is dead, and therefore will not germinate even under optimal conditions, including following treatments for breaking dormancy.
vitrification	rapid cooling of liquid medium in the absence of ice crystal formation.
voucher specimen	a specimen that can be stored for future reference (e.g., in an herbarium) to confirm identification etc.; it should include key plant parts used in formal identification including reproductive parts such as flowers and fruits/seeds.
zygotic embryo	embryo formed following fertilisation or union of haploid male (sperm) and female (ovum) sex cells, inheriting traits from both parents.

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‘This third edition and update of the Guidelines is timely, with significant advances in knowledge gained through research and experience at a time when threats to plant diversity are increasing. The emergence of new pests and diseases, such as Myrtle Rust, the increasing frequency and intensity of bushfires, and recurring extreme weather trends and events are not just confined to Australia. They are a worldwide phenomenon. This gives added urgency to our mission to conserve and manage plants for future generations. Fortunately, we continue to learn from what does and doesn’t work and – crucially – to share that learning through publications like this.’

Dr Paul Smith, Secretary General, Botanic Gardens Conservation International

Strategies to conserve Australia’s unique plant treasures depend on understanding how plant species function. The collection, storage and study of plant germplasm – whether as seeds, spores, tissue cultures, cryopreserved seeds or tissues, or whole plants – helps us gain this knowledge. Ex situ collections provide research material to unlock the secrets of seed dormancy in the lab and in the wild; allow us to establish new wild populations of threatened species with the best chance of success; and provide an ‘insurance policy’ of genetically representative samples, to guard against the risk of extinction. The scale and pace of biodiversity decline resulting from climate change and threatening processes, such as past and ongoing habitat loss, inappropriate fire regimes, invasive weeds, and disease mean that germplasm capture and its rigorous maintenance and development ex situ are of critical importance.

These Guidelines include the latest advances in ex situ plant germplasm conservation. Written by many of Australia’s foremost experts, with significant input from international collaborators, they place conservation of this country’s unique and varied plant life in a national and international context, outline the key conservation policies and strategies, and provide an evidence-based handbook for programs requiring germplasm collection, storage, research and utilisation.

The book is written as a guide for a range of users, including conservation agencies, scientists, students and anyone interested in applied plant biology.