Plant Germplasm Conservation in Australia

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

Edited by Catherine A. Offord and Patricia F. Meagher

The Australian Network for Plant Conservation (ANPC)
in partnership with
Australian Seed Conservation and Research (AuSCaR)

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Foreword

On reading this important book, I was struck by how much has changed for the better since I was first employed as the Western Australian government's one and only Flora Conservation Research Officer in 1977. In those days, a substantial divide existed among those who were describing and classifying plants, those who studied the biology of native plants, those who were involved in conserving plants in the wild and those who grew plants for horticulture or commerce. At the same time, wild biodiversity was being destroyed at an alarming rate for agriculture, or for industrial and urban development. Something had to change if there was to be any hope of caring for the jewels of Australian plant diversity, let alone the full range of variation in its species and wild communities of plants.

Then, as is so evident in these pages, the concept of integrated conservation emerged as a unifying approach, bringing disparate players together to work towards the common goal of conserving the rich diversity of native plants. The old walls of different disciplines began to tumble, especially as steadily increasing numbers of younger scientists and practitioners became interested and involved in plant conservation.

Today, we are witness to a remarkable transformation of concerted action, backed by cutting-edge science, global in reach, and motivated by the realisation that we must act together to conserve the plant diversity on which our very lives increasingly depend. Plants offer great potential to help with solutions to the inescapable environmental challenges we all face.

Plants absorb carbon, and therefore help cool the world. They provide oxygen, so you and I can breathe. They provide food at a time where the word 'crisis' is now being used for the supply of staples that feed the world. Plants provide filters in the landscape for clean water. They enrich and modify hostile soils. They are a source of medicines for human health; quinine from cinchona bark and now *Artemesia annua* remain the best defences against the world's greatest killing disease – malaria. In Australia there are plants known to have anti-cancer, anti-bacterial and anti-viral activity. Many solutions to sustainable living undoubtedly lie quiescent and as yet unrevealed in remnant patches of Australian native vegetation.

Foreword

If we are to retain and restore the Earth's vegetation carbon sinks to help minimise global warming, all people must look to their own backyards and manage biodiversity as though they were here to stay on this planet and in their lands, living lives enriched by biodiversity. Australians bear a special responsibility as custodians of one of the world's great repositories of biological richness.

Will we save it? I sincerely hope we do. We cannot afford to let such riches slip through our fingers, for self-preservation as much as for the intrinsic interest and wonderment for which Australian plant biodiversity has become internationally renowned.

The Australian Network for Plant Conservation (ANPC) plays an important role in facilitating the flow of scientific information to plant conservation practitioners. Australian Seed Conservation and Research (AuSCaR) is a new and vital nexus of collaboration of the Millennium Seed Bank's Australian partners. Together, the ANPC and AuSCaR have produced a succinct, well-researched and timely technical manual that will help Australia achieve its conservation goals.

This book provides a mine of information on recent advances in plant germplasm conservation in Australia and beyond. You will find accounts of the fine achievements of projects such as the Millennium Seed Bank, conservation of the remarkable 'pinosaur', the Wollemi pine, and discovery of smoke as a stimulus for germination of thousands of Australian native plants. Practical information, helpful summaries, case studies, contacts, and current scientific references are all here for the interested reader.

While not everything is known, and I suspect we are still some way off from developing a thoroughly Australian scientific perspective on some issues covered in the book, I cannot think of a better text to recommend on this subject. Congratulations to all involved in its production. May it help take us to the next level in caring for an irreplaceable natural heritage of global significance.

Professor Stephen D. Hopper
Director
Royal Botanic Gardens, Kew

Acknowledgements

There has been a rapid accumulation of knowledge of germplasm conservation in Australia in the last ten years. The need for revision of the ANPC's 1997 Germplasm Conservation Guidelines had been recognised for some time, but it was a suggestion for action by Anne Cochrane in 2006 that triggered the present project, with a collaboration formed between the ANPC and the Millennium Seed Bank Partners – Australia (now AuSCaR).

While the growth in knowledge and experience over the last decade has made this an entirely new book, it nevertheless owes much in its conception to the ANPC's 1997 Germplasm Conservation Guidelines for Australia, and the original ANPC Germplasm Working Group (Dave Coates, Kingsley Dixon, Peter Lawrence, Jock Morse, Mark Richardson, Dale Tonkinson, Anne Cochrane and Darren Touchell), who developed that earlier publication.

Since 2006, a large number of people have become involved in this project reflecting the growth in activity and experience within the Australian ex situ conservation community.

A significant amount of time has been donated by the authors and case-study contributors and they are acknowledged in the chapters and contributor section. Photographic and figure contributors are acknowledged in the captions.

We are grateful for the contribution of those who attended workshops at Mount Annan Botanic Garden (February, 2007), Kings Park and Botanic Garden (September, 2007) and the ANPC Conference at Mulgoa (April, 2008) which helped to scope and drive the project. These people include: Sally Dillon, Anne Parisi, Maurizio Rossetto, Paul Janssens, David Carr, Sarah Fethers and James Wood. Further input was provided by: Richard Johnstone, Rowena Long, Daniel McDonald, Andrew Orme, Tim Pearce, Zoe Smith, Kate Vleck and Magali Wright.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABSA	access and benefit sharing agreement
ANPC	Australian Network for Plant Conservation
ANZECC	Australian and New Zealand Environment and Conservation Council
AuSCaR	Australian Seed Conservation and Research Network
BAP	6-benzylaminopurine
BGCI	Botanic Gardens Conservation International
CBD	Convention on Biological Diversity
CHABG	Council of Heads of Australian Botanic Gardens
CPC	Centre for Plant Conservation
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DECC	Department of Environment and Climate Change
DEST	Department of the Environment, Sport and Territories
DMSO	dimethylsulfoxide
eRH	equilibrium relative humidity
FAO	Food and Agriculture Organisation
FDA	fluorescein diacetate
GA	gibberellic acid
GPS	Global Positioning System
GSPC	Global Strategy for Plant Conservation
H_2SO_4	sulphuric acid
IBA	indole-3-acetic acid
IPGRI	International Plant Genetic Resources Institute
ISTA	International Seed Testing Association
LN	liquid nitrogen
MC	moisture content
MD	morphological dormancy
MPD	morphophysiological dormancy
MS medium	Murashige and Skoog medium
MSBP	Millennium Seed Bank Project
NAA	1-naphthaleneacetic acid
NRM MC	National Resource Management Ministerial Council
PD	physiological dormancy
PGR	plant growth regulator
PLB	personal locator beacon
PVS	plant vitrification solution
PVS2	plant vitrification solution two
PY	physical dormancy
RBGM	Royal Botanic Gardens Melbourne
RH	relative humidity
SE	somatic embryo(s)
SID	seed information database
SPA	seed production area
TDZ	thiodiazuron
TTZ	tetrazolium

Chapter 1

Introduction

Catherine A. Offord and R.O. Makinson

1.1 Introduction

There are well over 20,000 native flowering plant taxa known for Australia; these occur across many bioregions and many demonstrate a high degree of adaptation and specialisation. Internationally, Australia's flora is considered megadiverse and is characterised by a high degree of endemism, especially in areas such as the biodiversity hot-spots of south-west Western Australia and the rainforests of northern Queensland. This diverse native flora faces a multitude of anthropogenic threats, especially habitat fragmentation and degradation. It is estimated that at least 10% of Australia's plant species are under some degree of threat of extinction. Some threats, such as the devastating effects of the introduced disease caused by the introduced *Phytophthora cinnamomi* and some invasive weeds, are documented and reasonably well-understood in terms of their interactions in ecosystems. Others, such as the effects of climate change, are less well-understood but pose no less a problem if predicted climatic changes occur over the coming decades (NRM MC, 2004).

In the mid-1990s, in light of such threats, the Australian Network for Plant Conservation (ANPC) initiated a Germplasm Working Group to prepare authoritative national guidelines for the conservation of plant germplasm, building on the work of the then CSIRO Australian Tree Seed Centre and the 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. The present volume was originally conceived as a revised edition of those Guidelines, but the amount of new knowledge and the needs of users have resulted in an essentially new book.

The 12 years since publication of the original ANPC *Guidelines* have seen a very 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). In Australia and some other countries, this process has been greatly aided by the collaborative programs offered by the Millennium Seed Bank Project (MSBP), a program of the Royal Botanic Gardens, Kew. MSBP's support of Australian State and Territory programs for germplasm (mainly seed) collection, banking, and research, has helped take those programs to new levels of achievement and publication, and to a new level of national collaboration among the MSBP Australian partners as Australian Seed Conservation and Research (AuSCaR).

1: Introduction

Figure 1.1
Australia has one of the most biodiverse floras on the planet; from arid rangelands (top), wet tropics (middle) and sclerophyll forests (bottom), and many other biomes, there are more than 20,000 known plant species. Images: J. Plaza, Botanic Gardens Trust, Sydney.

The present volume seeks to draw lessons from the vastly increased body of knowledge and experience now available, and to place these in the context of current conservation plans and strategies. As many existing international plant germplasm conservation procedures are still based on Northern Hemisphere crop species, they may not be appropriate for Australian plants (see for example Section 4.1). These guidelines on ex situ conservation methods therefore aim to be applicable to Australia's diverse and specialised native flora. Though written specifically for use with Australian vascular plants, many of the principles and procedures described here will be applicable to other floras.

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

1.2 Definition and scope of plant germplasm conservation

Germplasm is the genetic material of an individual, which may be transmitted by sexual or somatic tissue from one generation to another. In a general sense, germplasm is material that represents a species or population (see Chapter 3). In situ (on-site) conservation of plant germplasm incorporates the protection, maintenance, management, sustainable use, restoration and enhancement of the natural environment (DEST, 1996). Ex situ (off-site) conservation involves the identification, collection, and storage of germplasm away from the natural environment, in repositories such as seed banks or botanic gardens, and enables its subsequent use. Seeds, spores, vegetative parts, whole plants, tissue cultures, cell cultures and pollen are all types of plant germplasm that can be conserved ex situ. Plant germplasm conservation may also require the culture and storage of other organisms especially essential symbionts such as the mycorrhizal fungi required for germination and/or growth of some plant taxa.

Figure 1.2 *Collecting (left) and storing germplasm of threatened species, such as* Thelymitra *orchids (right), can aid in species conservation. Images: A. Orme and J. Plaza, Botanic Gardens Trust, Sydney.*

1: Introduction

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 number 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; rather, they complement the wider range of plant conservation-related activities (Smith, 2006). Germplasm conserved ex situ, for example, may serve as insurance against the loss of populations or species and may be used to re-establish extinct populations in the wild or to supplement populations on the verge of extinction.

Actively conserving plant diversity

'….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).

1.3 Objectives of germplasm conservation

In 1992, the Convention on Biological Diversity, Article 9, (CBD, 1992) formally recognised ex situ conservation as an activity that may be necessary to complement in situ measures. In particular, it recommended that ex situ actions should support the recovery and rehabilitation of threatened species, and enable their reintroduction into natural habitats. Similar recognition of the potential importance of ex situ actions for meeting biodiversity conservation goals appeared subsequently in domestic Australian Government policy documents (DEST, 1996; ANZECC, 2001).

Ex situ conservation contributes to the survival and continued natural evolution of species by:

- Providing material for propagation to remove or reduce pressure from wild collecting;
- Enabling the rescue of threatened germplasm;
- Providing material for translocation of rare, threatened or key species (e.g. for reintroduction or enhancement) or small-scale habitat restoration and management;
- Providing material for conservation biology research;
- Generating skills and knowledge to support wider conservation aims;
- Contributing to education and raising public awareness about plant conservation.

(After Smith, 2006; BGCI, 2007)

Thus, ex situ plant germplasm conservation contributes to in situ conservation through the provision of appropriate plant material for actions such as translocation, as well as generating essential biological information on the species and its interactions with the environment and other biota. Such information aids conservation efforts and may help ensure the success of translocations for example. Ex situ material can also provide a reference against which subsequent morphological or genetic changes in a population may be measured. In principle similar to herbarium collections, germplasm collections provide a rich resource for plant scientists.

The ultimate objective of ex situ germplasm capture and storage is to support the survival and continued evolution of species in self-sustaining populations in the wild. The Australian Network for Plant Conservation, from its founding in 1991, has strongly advocated the integration of in situ and ex situ techniques' i.e. 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.

1.4 Plant conservation in the international and Australian contexts

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), which addressed biological diversity at a global level and set the principles for broad scale conservation objectives. In response to the recognition that plants are an essential resource for the planet, the *Global Strategy for Plant Conservation* (GSPC), an instrument of the CBD, was developed (CBD, 2002). Target 8 of the GSPC relates specifically to germplasm conservation in stating that, by 2010, '60% of threatened plant species [will be] in accessible ex situ collections, preferably in the country of origin, and 10% of them [will be] included in recovery and restoration programmes……with additional resources, technology development and transfer, especially for species with recalcitrant seeds' (CBD, 2002).

The *National Strategy for the Conservation of Australia's Biological Diversity* (DEST, 1996) was prepared in response to the CBD and reflected, at Government level, the widening community support for biodiversity conservation. The Strategy aimed 'to bridge the gap between [then] current activities and those measures necessary to ensure the effective identification, conservation and ecologically sustainable use of Australia's biological diversity'. 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. A number of States and Territories have incorporated ex situ conservation into their conservation strategies. In South Australia for example, Target 12 is dedicated to the ex situ conservation of its State's threatened species (see <http://www.environment.sa.gov.au/biodiversity/pdfs/nsl_strategy.pdf>).

A subsequent *Review of the National Strategy* (ANZECC, 2001) noted that '*Australia has established and is maintaining a wide range of measures and facilities for ex situ conservation through Commonwealth, State and Territory agencies, tertiary institutions and scientific organisations. Ex situ conservation facilities include zoos, aquaria, botanic gardens, seed banks and collections of tissue culture and micro-organisms'.* It also states that *'On-going effort is required to maintain Australia's ex situ collections, both in terms of maintaining living specimens and also the information held about the items in the collections*'.

One recent Australian initiative has been the '*National Strategy and Action Plan for the role of Australia's Botanic Gardens in Adapting to Climate Change*' (CHABG, 2008), which outlines the role of botanic gardens and similar organisations in achieving plant conservation by:

1. Providing a safety net of germplasm collections, including seed banks and living collections;
2. Providing knowledge of horticulture, species distribution and taxonomy; and,
3. Increasing education and community awareness.

These Guidelines and the companion *Guidelines for the Translocation of Threatened Plants in Australia 2nd Edition* (Vallee et al., 2004) support the ongoing conservation activities in Australia.

1.5 Supporting information and technology

In the time since the original ANPC Guidelines (Touchell et al., 1997), a great deal of research and information has become available on the biology of the Australian flora, particularly in the area of seed biology. Much of this information still requires synthesis and evaluation for application. Globally, there have been advances in the technology associated with germplasm use and conservation. Seed banking and seed biology, in particular, have seen great leaps forward, including standardisation of techniques across institutions. Molecular genetic technologies have evolved to the point where it is now possible to consider the identification, isolation and utilisation of individual genes for plant improvement through breeding. The same sort of technology allows greater understanding of population structures and geneflow, assisting decision making in plant conservation efforts (Vallee et al., 2004). However, because of the high cost and level of technical input required, the scale of our biodiversity, and the difficulty of resolving complex genetic issues, little is known about the specific genetics of many Australian species, and decisions are generally made using inferred information from studied taxa. For highly specialised floras, such as those of the south-west of Western Australia, Tasmania and the rainforests of NSW and Queensland, practitioners should be cautious in applying generalisations about optimal storage conditions even among subspecies, let alone genera or families.

The citation of a large number of references throughout these guidelines provides a portal to the major sources of information available at the time of publication. See Appendix 2

for a list of useful and comprehensive publications and websites. It is advisable to check these sources regularly to keep abreast of the latest information and advances. Advice to ANPC from users of this volume about new or changed information is always appreciated (contact through <http://www.anbg.gov.au/anpc>).

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 in some way to the conservation, recovery and management of threatened plants and communities. They also have application to plants with non-threatened status, as these may become threatened at some point in the future, or their conservation may in some way contribute to the wider conservation picture e.g. habitat restoration (see Box 1.1). Some species may also be of economic, ethnobotanical, cultural, amenity or aesthetic value. These guidelines should therefore be seen as complementary to guidelines for non-threatened plant restoration (e.g. the Florabank revegetation resources maintained by Greening Australia <www.florabank.org.au>), or agricultural crops (e.g. Bioversity International, formerly IPGRI <www.bioversityinternational.org>), which often require similar procedures and facilities.

Box 1.1

Guidelines for sampling for conservation vs broadscale restoration: horses for courses?

In a recent 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 key endangered community, and the broadscale restoration of communities on a landscape scale (from small, 1-10 ha, to large 102 - 106 ha). These activities may have similar or complementary aims, including the need to restore long-term biotic function to habitats, but the scale and the nature of the germplasm sampling are often very different.

It is therefore important to try to anticipate both the currently intended and the potential future end uses of germplasm collections *before they are made*, in order to apply collection (sampling) strategies that will maximise the options for end use while still being consistent with available resources. 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.

Collecting strategies for broad-scale restoration are not specifically dealt with in this current publication, however, some of the conservation techniques described may be applicable, such as seed technology.

1: Introduction

The main objectives of these guidelines are to:

- address the needs of germplasm collection and storage of Australian flora;
- encourage the effective networking of germplasm banks and other ex situ conservation in Australia;
- 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 storage is most appropriate in a given situation;
- 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, including cryopreservation;
- 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 protocols for many species;
- highlight the importance of good record keeping, collaboration and data sharing;
- identify probable end uses for germplasm collections and timeframes for storage; and,
- show that stored germplasm can be used for a number of purposes complementing in situ conservation by providing material for translocation, bulk propagation, research, education, horticultural display and sustainable plant development.

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** details the development of a collecting strategy to represent the genetic diversity of a species over part or all of its range and explains the practical steps involved in seed and vegetative material collecting. **Chapter 4** provides an outline of seed storage techniques along with pre-storage operations, seed testing and seed bank management. **Chapter 5** explains the critical process of seed germination, including how to deal with the complexities of the various types of seed dormancy, with references to some of the latest information gained from research on Australian species. The last three chapters deal with techniques that may complement seed storage or be the only mechanism for ex situ conservation. The chapters begin with the associated benefits and risks of the specific option. **Chapter 6** explains applications and types of tissue culture conservation options and **Chapter 7** discusses the application of cryopreservation to germplasm storage, including reference to recent research in Australian species. Finally, **Chapter 8** covers the method of conserving germplasm as living whole plants and the alternative types of collections that can be held ex situ.

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

- **case studies** to illustrate particular points;
- **information boxes** to provide additional information on particular concepts or topics; and,
- **checklists** and **flow charts** to summarise processes.

These Guidelines have been developed as a companion to the *Guidelines for the translocation of threatened plants in Australia 2nd Edition* (Vallee et al., 2004), also published by ANPC. In line with the *Translocation Guidelines*, we have avoided the use of technical terminology where possible; however, whenever technical terms are used, definitions are in the glossary at the end of the publication. Please note that these guidelines are not a substitute for consultation with experts and therefore a list of useful contacts and key references are provided at the end of the publication.

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Chapter 2

Options and major considerations for plant germplasm conservation

Catherine A. Offord and R.O. Makinson

2.1 Introduction

This chapter provides a guide to ex situ plant germplasm conservation, from determining whether ex situ conservation is necessary for a particular taxon, to determining which method/s should be used and how the collection will be maintained. Bearing in mind that ex situ conservation is an adjunct action, and not an alternative, to in situ conservation (Section 1.2), three questions that are important to consider early in the planning process are:

1. Do you have all available information on the species, including information from other people who may be working in the same area?
2. Will the collection of material endanger the species being collected or others that may rely on it?
3. Can you get the necessary permission 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 on, 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

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 some 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 (see for example Case Study 2.1). Ex situ collections act as insurance against loss of wild germplasm.

2: Options and major considerations

Ex situ collections often provide material to conduct research on the biology of threatened species, away from the site, 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, fire, grazing, competition or nutrients etc.

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

Plant material is also available for educational and display purposes which in turn can assist in protection of the in situ population by raising public awareness.

Case Study 2.1

The role of ex situ conservation in recovery of Allocasuarina portuensis

An example of a translocation made from an ex situ collection is the Nielsen Park Sheoak (*Allocasuarina portuensis*), which is described in 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. Plants representative of the original population of this species have been reintroduced to the original site, and weeds and other threats minimised through intense management. However, it will take years to fully re-establish a viable self-sustaining population. Until such time, it is necessary to maintain viable ex situ collections. These are held as seed in the NSW Seedbank and as potted plants at Mount Annan Botanic Garden. 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, Botanic Gardens Trust, Sydney.*

Risks and limitations

Ex situ storage of material can be perceived as a substitute for the continued existence of a species in the wild, which may in turn lead to unnecessary or inappropriate destruction of habitat. As mentioned previously, ex situ germplasm storage can only ever be considered a complementary conservation measure, and not an alternative, to in situ conservation measures.

Germplasm storage poses a number of other significant risks, of which one of the most serious is the loss of ability of the stored material to regenerate whole plants. 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. Collection of material also poses the risk of damage to the wild population, which can be minimised by following the guidelines in Chapter 3.

A longer term risk associated with germplasm storage is related to the process of gradual adaptation. Populations in the wild have the opportunity to evolve over time in response to environmental changes while stored material is adapted to the environment as it was at the time of collection. If the original environment changes significantly, for example if the soil becomes saline or the average temperature rises several degrees, then the stored material may be adversely affected in terms of either reproduction or establishment when returned to the wild. In such circumstances, careful selection of recipient sites is vital.

2.3 Priority setting

The ultimate aim of an ex situ conservation program should be to reinforce or re-establish viable populations in the wild, and to minimise threats or the effects of threats. It is important to consider that there are a large number of species in Australia that potentially require ex situ conservation but resources for ex situ conservation are limited, with few repositories and personnel to manage collections, so priority setting is a critical activity.

Use the Decision Flow Chart (Box 2.1) and the following considerations in this chapter to assist decision-making on ex situ conservation options. An example of an ex situ program, integrated with other conservation considerations, is given in Case Study 2.2. Further considerations of the relative ongoing effort and resources needed for ex situ and in situ conservation methods are found in Maunder et al. (2004).

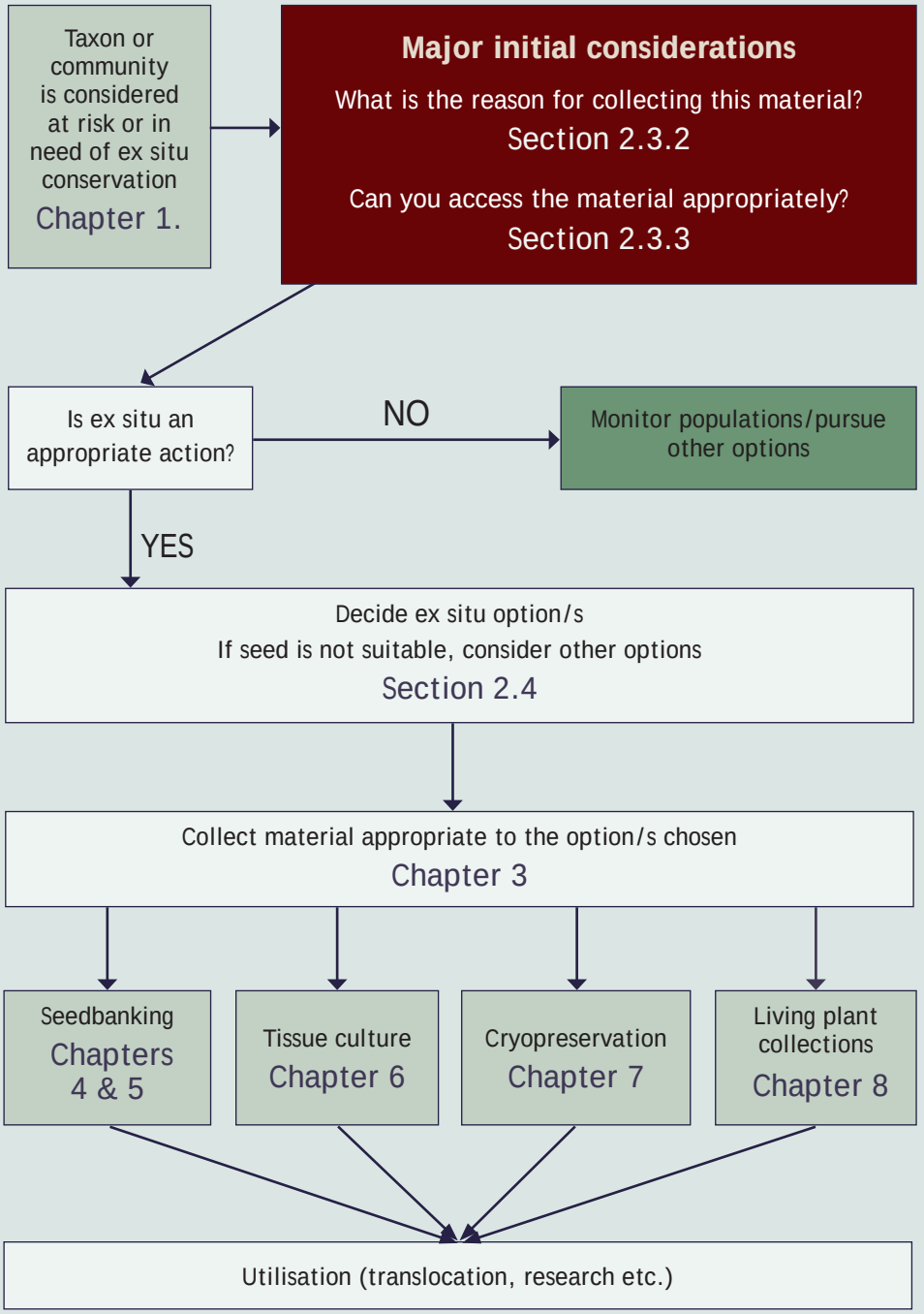
2.3.1 Which species to collect?

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

- A high degree of threat;
- Local or regional rarity;
- A rapid decline in population numbers or size, as a result of anthropogenic causes;

Box 2.1

Decision flowchart for ex situ conservation options.



- Species that are poorly represented in collections;
- Taxonomic and evolutionary uniqueness;
- Potential for biological management and recovery;
- Genetic relationship to economically useful taxa;
- Probability of success of establishment in cultivation.

(After Center for Plant Conservation, 1991)

2.3.2 What is the reason for collection of this material?

The collection of propagation material from any plant growing in the wild must have a purpose. This is especially so in the case of rare and threatened species. Such plants have been listed because they are already threatened in some way. Needless collection only increases the threat. Even species that are not currently designated as threatened may be locally or regionally rare or may form part of an endangered ecological community. Over-collection from a small remnant site may also compromise the viability of the species persisting there.

The purpose of the collection, e.g. translocation, research, horticulture or breeding, 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 ;
- 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). The importance of provenance of conservation collections can not be over emphasised and we strongly advise seeking advice from conservation agencies and species specialists when designing a collection program to ensure the integrity of your collection.

2.3.3 Can you access the material appropriately?

By definition, most rare and threatened species are few in number; they may also be highly ephemeral, sparsely distributed, difficult to access because of terrain etc. Seeds are often in short supply and vegetative material sparse. So, before embarking on collection, it is important to identify the following:

- other people with an interest in the species or the site – it is vital to coordinate your proposed actions with others;
- whether a collection site has been subject to previous germplasm collection – cumulative impacts need to be considered;
- whether the species is conserved ex situ elsewhere;

Box 2.2

Provenance of germplasm – why is it important to conservation outcomes?

'Provenance' of seed or other germplasm refers to either:

- 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 zones or variants with maximum similarity and dissimilarity within the genome, or genotypes thought to be most suitable for purpose.

Assessing 'best provenance' for a conservation action cannot be divorced from intended end use, from the biological characteristics of the species (e.g. breeding system), from the characteristics and history of the parent population and environment, and from the characteristics of the recipient environment. For these reasons, detailed discussion of provenance is beyond the scope of these Guidelines. The ANPC *Translocation Guidelines* (Vallee et al., 2004) 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 guidelines at <www.florabank.org.au>. 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 (<l00-200 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;
- Seed sourced from remnant populations suffering poor genetic health will probably yield poor revegetation results;
- 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. Sampling of threatened taxa should always occur in close liaison with conservation agencies and species specialists.

DNA techniques are now allowing the genetic delineation of seed collection zones within species, and the identification of genotypes that may relate to end use (see for example the 'conservation genetics' pages at <www.bgpa.wa.gov.au>).

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 fairly local provenance for conservation plantings.

The experience-based evidence should not be downplayed, but also should not be universalised – survivorship 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.

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

- Source germplasm from large reproductive populations (>100-200 individuals);
- 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, publish and consider as much species-specific and site-specific knowledge as possible, including breeding system and ploidy levels if known.

Within those parameters, general rules include:

- Ensure taxonomy of the species is understood and identification of source material is correct;
- Source from geographically related and ecologically similar sites;
- Consult widely in the early planning phase – involve specialists;
- If planning to use seed production areas, control inadvertent narrowing of genetic base (see McKay et al., 2005).

- as much information as possible on the species, or, if not well understood, a related species;
- the required permits and permissions.

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 particular population. Examples includes government agencies, landcare groups and researchers. It is essential that these stakeholders are sought out 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 ex situ collections of the target species already exist. There may already be well sampled and viable collections that are available for other conservation actions. Even collections with low genetic representation may contribute to, or indeed 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 (Appendix 1).

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. It is important to consider the existing frameworks that apply to conservation of target species. For example, 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. In some States (NSW for example), only certain species and communities have full Recovery Plans, with management and recovery actions for all other threatened species being recorded in a database (in NSW, the Priorities Action Statement) which stores information on actions and the agencies, groups or individuals involved. Also, consider contributing information to appropriate sources such as databases.

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

Table 2.1 ***The major ex situ conservation options available in Australia and their relative advantages and disadvantages (see also individual chapters).***

Major conservation options available in Australia	Advantages	Disadvantages
Seed banking	Long-term storage of orthodox species; relatively low cost.	Desiccation sensitive species are not suitable. Germination protocols for many Australian plants are still to be developed.
Tissue culture	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 dependant on tissue/environment response; expensive to develop protocols for new taxa; high technical skills and facilities required; genetic changes possible over time.
Cryopreservation	Long-term storage of plant parts including seeds.	Success highly dependant on tissue/environment response; expensive to develop protocols for new taxa; high technical skills and facilities required.
Living plants	Living plants are available for a variety of conservation-related purposes e.g. species research and horticultural display and education; 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.

them. 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.

2.4 What are the possible ex situ conservation storage options?

For most species, seed banking is the major ex situ storage option because of the relative ease of storing genetically representative collections (Table 2.1). Other options to consider are: tissue culture, cryopreservation and living plant collections (i.e. nursery and garden

Box 2.3

Obtaining permission to collect, hold and utilise wild Australian plants

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 (Appendix 1). 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 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 in blind-alley trials.

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**, including the ACT (Canberra and Jervis Bay), and some Commonwealth-managed lands elsewhere, one or more permits must be obtained from the Commonwealth Department of Environment, Water, Heritage and the Arts or its successor (at 2009, www.environment.gov.au). 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 (but see same webpage for links), unless the export is from a scientific institution with a standing permit.

For the various **States and Territories**, the starting point for permits is always the main conservation agency (Appendix 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.

For **Aboriginal lands**, 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 identify benefits back to the local community wherever possible, and familiarise yourself with protocols for scientific or other work on Aboriginal 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 uses of sites may not be predictable in advance.

For **private freehold**, permission must always be sought from the land owner 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 land owners/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 or land owners 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.

collections, field genebanks). 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 Australian plants is the ability to effectively propagate target species for a variety of outcomes. Whatever ex situ option is used, specialist expertise and management is 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. (2004) 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 which ever option you choose.

2.4.1 Seed banking

For most dryland species, seed collection and storage is the most appropriate action. 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. The appropriateness of this option is determined by:

- Whether appropriate seed processing, storing and maintenance resources are available;
- Whether the plant produces seeds in sufficient quantity;
- The quality of seed produced;
- The seed storage behaviour of the species (2.4.1.1).

Often the last three of these information needs may not be easily met, but may 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.

2.4.1.1 Can seeds be stored?

Most species are orthodox in terms of seed storage, that is, they tolerate the drying required for long-term conservation at freezing temperatures. However, a minority of species have seeds that lose viability when dried to low moisture contents and cannot be stored in a conventional seed bank. Alternatives such as cryopreservation, tissue culture and living collections need to be considered for these species (see Chapters 6-8). Species are often classified according to the storage ability of their seeds:

- *Orthodox species* have desiccation tolerant seeds. These seeds can be dried down to very low moisture contents (4 - 7% moisture content [MC], around 15% relative humidity [RH], see Sections 4.3 and 4.4) and can potentially be stored for very long periods of time under dry, cold conditions. These types of seeds generally come from species that experience dry, harsh conditions for part of their lifecycle, and most can stay viable in the soil or canopy seed bank for an extended period of time. Orthodox seeds are relatively easy to identify due to their inherent ability to be dried and stored at low temperature and this ability is considered a qualitative trait ('all or nothing' feature) (Leprince, 2003). These species are generally stored at sub-zero temperatures (commonly -18 to -20°C) for long-term storage (expected to be viable for more than 10 years).
- *Desiccation sensitive species*. There are a number of species that have seeds that are sensitive to desiccation levels tolerated by 'orthodox' species, and are either killed or have poor storage ability when dried. There is a wide range of sensitivities and longevities under different storage temperatures, and the expression of this trait is thought to be quantitative (Leprince, 2003). Because of this wide spectrum of behaviour in non-orthodox seed, there is currently no general consensus on the categorisation of non-orthodox seeds. The literature, however, often allocates seed behaviour into the following groups:
    - *Recalcitrant species* have desiccation intolerant seeds. These are more common in certain plant groups, often those that occur in wetter environments such as mangroves, sea grasses and rainforests. Examples from agriculture are mangoes, avocados and coconuts. These seeds tend to have much higher seed moisture content at maturity (c. 40%) and generally do not survive drying to moisture contents below 15-25% (80-90% RH)(Pritchard and Dickie, 2003), although this varies between species. Indicators of potentially desiccation sensitive seeds are:
        - Large seed size;
        - Thin seed coat;
        - Shed in wet areas such as tropical rainforest or in wet seasons (Tweddle et al., 2003; Daws et al., 2006).
    - *Intermediate species* have seeds that display properties between orthodox and recalcitrant and can tolerate desiccation to generally around 10-12%, sometimes as low as 8%, moisture content (Berjak and Pammenter, 2003; Pritchard and Dickie, 2003; Black et al., 2006). Sometimes classified as (sub) orthodox, these species may not store well at sub-zero temperatures and may have better longevity at higher temperatures.

Further information on seed storage types can be obtained in Pritchard (2004). More information is required on the effect of drying and storage temperature, on Australian species, particularly those species from wetter environments.

Case study 2.2

Conservation strategy for the Wollemi Pine

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 canyon in Wollemi National Park, NSW. The species is extremely rare and is the only extant representative of the *Wollemia* genus. The few stands that have been discovered contain a small number of plants and face a large number of threats such as Phytophthora infestation and wildfire.

Because of their taxonomic rarity 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 such as that shown in Box 2.1, starting with major initial considerations and development of a conservation plan. Research since its discovery has resulted in the development of a complementary conservation strategy (DEC, 2006).

Firstly, 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. 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 NSW Seedbank and in the Millennium Seed Bank (UK) and germination experiments were conducted (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 not revealed any variation between any trees or their seedling-grown progeny. 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 is likely to be captured and maintained. There are fewer than 100 trees left 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 (some inaccessible trees are not yet included).

The clonal plants of this species are held as potted plants, in the nursery at Mount Annan Botanic Garden, and documented representatives have been planted out in this and other botanic gardens. The nursery collection comprises three replicates of 52 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 (no fewer than 20 scientific papers have been written on this species, mostly using this material, see <http://www.rbgsyd.nsw.gov.au/science/wollemi_pine/further_reading>) and as the basis for a major world-wide horticultural release (Offord and Meagher, 2006). 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, seed biology of this species is being conducted to improve long-term storage potential.

Figure 2.2
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 (bottom left); novel seed collection techniques were developed including by helicopter and suspended seed nets (bottom right). Images: J. Plaza, Botanic Gardens Trust, Sydney.*

There is some information available on the orthodoxy of Australian species; see for example the Seed Information Database (SID) (Liu et al., 2008). Additionally, information on the seed storage behaviour of more than 10,600 species from around the world can be found on SID.

Large seeded species from high rainfall areas should be suspected of having recalcitrant seed (Dickie and Pritchard, 2002; Ashmore et al., 2007). 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.

Prior to significant collection for seed storage, it is wise to check any unknown species for its desiccation sensitivity on a small test batch. Methods for identifying species with desiccation sensitive seeds are included in Gold and Daws (2008). An example of desiccation tolerance screening for a rainforest species is found in Case Study 2.3.

If germplasm of species with recalcitrant seeds is to be conserved ex situ, seeds must be collected fresh and germinated, or vegetative material used to establish a living collection in the ground, in pots, in tissue culture or in cryostorage (see Chapters 6, 7 and 8). For some species, extracted embryos may be cryopreserved, but this is a high investment conservation strategy and needs careful consideration.

Case Study 2.3

Screening for seed desiccation tolerance in the threatened species Myrsine richmondensis

Myrsine richmondensis Jackes (Ripple-leaf Muttonwood) is a rainforest shrub or small tree thought to be extinct until rediscovery in 1997. The species is now listed as Endangered under both the NSW *TSC Act 1995* and the *Commonwealth EPBC Act 1999*. The recovery plan for *M. richmondensis* (DEC, 2004) calls for establishment of an ex situ seed collection as an insurance strategy against loss due to catastrophic events.

However, since the first stage in the ex situ storage of seeds is drying, it was important to determine whether seeds of *M. richmondensis* would tolerate drying. The storage behaviour of this species was investigated by comparing the germination of fresh, dried and moist-stored seed (Martyn et al., 2008).

- Fresh seeds were germinated six days after collection, when the seeds had an equilibrium relative humidity (eRH) of 99.1%.
- Seeds in moist conditions (approx. 100% RH) were held over moistened vermiculite and sealed inside plastic zip-lock bags at room temperature.
- Seeds in dry conditions were placed in the drying room of the NSW Seedbank (approx. 15 °C and 15 % RH).

Figure 2.3 *Ripple-leaf Muttonwood (*Myrsine richmondensis*) leaf detail. Image: Simone Cottrell, Botanic Gardens Trust, Sydney.*

2.4.2 Other ex situ conservation options

If it is known or suspected that a species does not produce seeds, or seeds are difficult to store, various options may be available (Table 2.1).

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). These techniques are often applied where unique or elite genotypes need conservation, to ensure that clonal replicates are maintained. Taking whole plants (transplants) is rarely appropriate or successful, but could be considered in certain circumstances. Because living collections are often highly labour intensive, and therefore costly and often unsuccessful in terms of the ability to adequately conserve genetic variability, the options for living plant conservation should be carefully considered in terms of benefits and risks.

After 35 days, a random sample of seeds from moist and dry conditions was removed for germination tests. *M. richmondensis* was found to tolerate drying to an eRH of 23 %, with an average germination of 76 % after 132 days for dried seeds. By contrast, a desiccation sensitive species would show a significant decrease in germination of dried seed when compared to moist-stored and fresh seed.

These results demonstrate that the seeds of *M. richmondensis* can be successfully dried and have the potential to be stored ex situ. Viability of seeds will need to be monitored during storage to establish whether they can be stored for the short, medium or long term. Screening for desiccation tolerance, even with threatened species for which only low seed numbers are available, is worthwhile and practical.

— Amelia Martyn

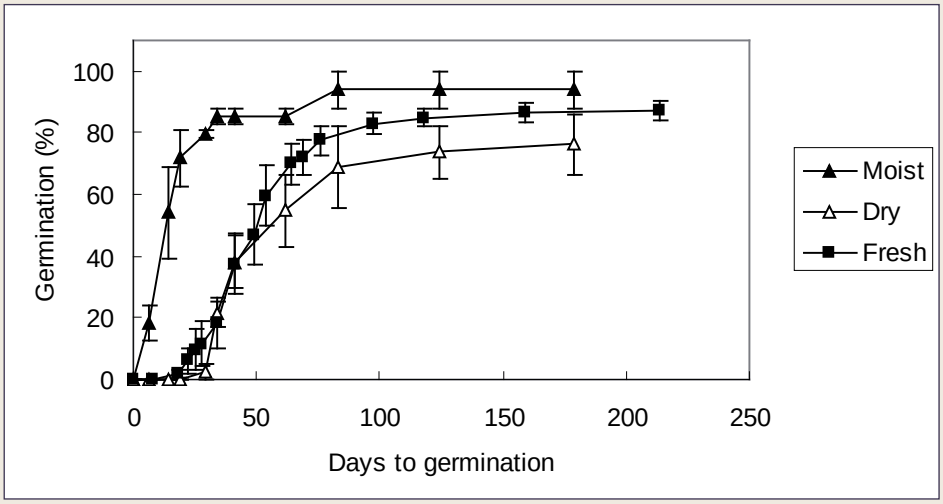


Figure 2.4 *Effect of moist (100% eRH) and dry storage (23.1% eRH) on seed germination of* Myrsine richmondensis*, compared to fresh seed germination. Data shown are averages ± standard error.*

2: Options and major considerations

The options available include:

- **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 6 and 7).

- **Living collections**

  1. *Botanic Gardens and other specialist gardens* generally contain dedicated documented collections for display, reference, research, education and other uses. Other cultivation options include agricultural collections (primarily for breeding), community or household gardens, commercial holdings and incidental collections (see Chapter 8). Many models for managing or indexing dispersed collections exist, especially in Europe and North America, albeit often with an emphasis on ornamental plants or crop varieties; for examples, see the [USA] National Collection of Endangered Plants <www.centerforplantconservation.org/NC_Choice.html>, and the PlantSearch database of Botanic Gardens Conservation International <www.bgci.org/plant_search.php>.
  2. *Field genebanks* include seed production areas and populations grown inter situ, which may be established from seeds or vegetative material and used as sources of material for conservation purposes in Australia. Field genebanks have the potential to be an intermediate cost option between seed banking and intensive cultivation (see Chapter 8) although they are not commonly used for wild species conservation.

There are other ex situ conservation options not specifically dealt with in these guidelines:

- **Sporebanking** - of Pteridophytes (ferns and fern-allies) and Bryophytes (mosses and liverworts) is not commonly practiced in Australia but may have applications for conservation in future; see Pence (2004) for more information.
- **Pollen storage** - is not addressed as its use in conservation is limited; see Towill (2004) for more information.
- **DNA storage** - at this time, whole plants cannot be regenerated from stored DNA (Hawkes et al., 2000); its current value in conservation is in genetic studies of population structure, gene flow etc.
- **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 bush-harvesting.

2.5 Are adequate storage resources for ex situ conservation available?

Adequate resources are required for storage of all forms of plant material. Different conditions are required for storage depending on the purpose of the collection, with the viability of the material being the governing factor. For example, seed being held for

long-term conservation is likely to require far more stable and permanent conditions than seed being accessed more frequently for research, display, short-term use or education purposes. For guidelines about the conditions required for different material types and storage purposes, see Chapters 4 to 8.

One of the most important actions in establishing ex situ conservation collections is ensuring their maintenance over time, as collections often outlast the personnel who made them. Good planning, documentation and recording systems should help to overcome this problem. 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.

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.

Partnerships can give considerable benefits to conservation programs, as can be seen in Case Study 2.4. Through such networks, information, expertise and germplasm can be shared, minimising risks to collections.

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.

2.6.1 Base and active collections

Give consideration to the development of a 'base' collection that holds essential material for long-term conservation, and an 'active' collection that can be used for a variety of purposes.

Essentially, a base collection is a limited number of accessions 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 used to conduct the necessary storage, propagation and establishment research. Base collections should therefore only be used where other suitable material does not exist, in active collections or from the wild, and there is a high chance of successful regeneration.

Once a collection is expended, particularly the active portion, consider recollecting the material.

Case Study 2.4

Australian Seed Conservation and Research (AuSCaR) – a network for conservation.

Since 2001, a number of Australian State and Territory institutions have been involved in an international seed banking effort that aims to store 25% of the world's plant species by 2020. The Millennium Seed Bank Project (MSBP) is coordinated by the Royal Botanic Gardens (RBG), Kew (UK) who negotiated bilateral Access and Benefit Sharing Agreements (ABSAs) with the participating organisations in Australia and around the world. The ABSAs are a means for parties under contract to gain access to resources within the spirit of the Convention on Biological Diversity (1992) Article 9. As the title suggests, an ABSA is a instrument designed to benefit both parties and improve the conservation of natural resources (see 2.6.2).

Each organisation involved is supported to help conserve their State or Territory's flora by collecting, storing and researching seed. Duplicate collections are held at the MSBP.

The Australian MSBP partners formed the Australian Seed Conservation and Research network, or AuSCaR, as a means to provide a national context for their work and to engage with stakeholders and potential funding bodies. With the development of a common strategy that covers activities, objectives and goals, it will be easier to achieve results that will benefit plant conservation nationally. The partnership creates a peak body providing statistical data to enable the Commonwealth government to report on internationally agreed targets such as the Global Strategy for Plant Conservation, Target 8 (CBD, 2002).

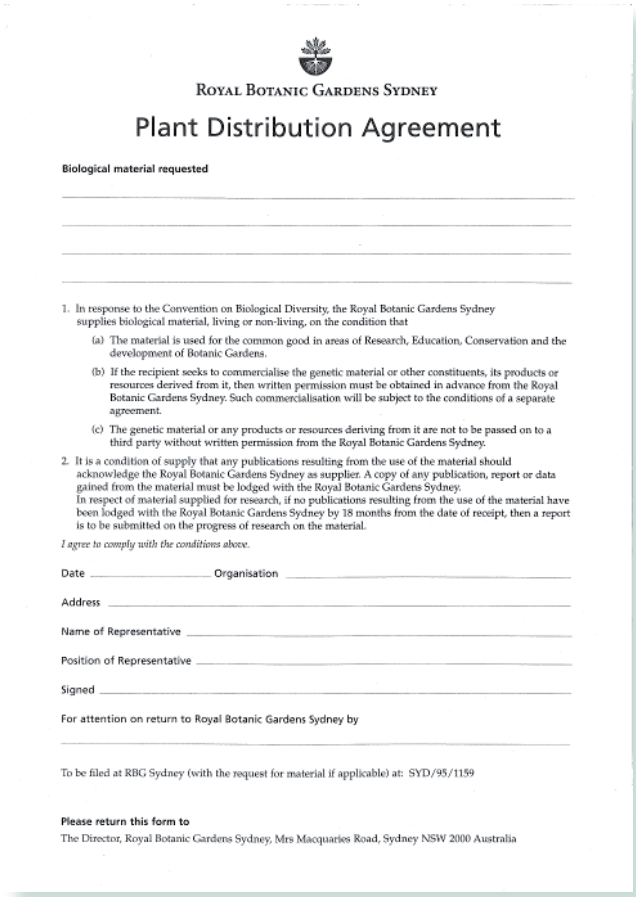
The group have adopted the vision of: 'An Australian network for the collection, storage, research and sustainable use of seeds for native plant conservation'.

— Tom North

2.6.2 Access and benefit sharing

The 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 (see example Figure 2.5). 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. Indigenous traditional knowledge and custodianship must also be respected (see <www.aiatsis.gov.au/-data/assets/pdf_file/3100/EthicsGuideA4.pdf>).

An example of a Plant Distribution Agreement, often known as a Materials Transfer Agreement, is below (Figure 2.5). Such agreements should be lodged with a registrar in the organisation and regular contact with the recipient be made to ensure correct use of the material. More information on this subject is found in Cheyne (2003).



ROYAL BOTANIC GARDENS SYDNEY

Plant Distribution Agreement

Biological material requested

1. In response to the Convention on Biological Diversity, the Royal Botanic Gardens Sydney supplies biological material, living or non-living, on the condition that
   (a) The material is used for the common good in areas of Research, Education, Conservation and the development of Botanic Gardens.
   (b) If the recipient seeks to commercialise the genetic material or other constituents, its products or resources derived from it, then written permission must be obtained in advance from the Royal Botanic Gardens Sydney. Such commercialisation will be subject to the conditions of a separate agreement.
   (c) The genetic material or any products or resources deriving from it are not to be passed on to a third party without written permission from the Royal Botanic Gardens Sydney.
2. It is a condition of supply that any publications resulting from the use of the material should acknowledge the Royal Botanic Gardens Sydney as supplier. A copy of any publication, report or data gained from the material must be lodged with the Royal Botanic Gardens Sydney.
   In respect of material supplied for research, if no publications resulting from the use of the material have been lodged with the Royal Botanic Gardens Sydney by 18 months from the date of receipt, then a report is to be submitted on the progress of research on the material.

I agree to comply with the conditions above.

Date _____ Organisation _____

Address _____

Name of Representative _____

Position of Representative _____

Signed _____

For attention on return to Royal Botanic Gardens Sydney by

_____

To be filed at RBG Sydney (with the request for material if applicable) at: SYD/95/1159

Please return this form to

The Director, Royal Botanic Gardens Sydney, Mrs Macquaries Road, Sydney NSW 2000 Australia

Figure 2.5 *Example of a Materials Transfer Agreement.*

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 other susceptible biota. Phytosanitation guidelines should also extend to moving material between organisations, as it is reasonable to expect that material is disease 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, a Phytosanitary certificate is required when moving material between countries, states and even adjacent areas. Information on the necessary steps and documentation is available from the State departments of Agriculture or equivalent, or from Biosecurity Australia.

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Chapter 3

Seed and vegetative material collection

Anne Cochrane, Andrew D. Crawford and Catherine A. Offord

3.1 Introduction

This chapter provides an overview of internationally adopted protocols for best practice collection of plant material for ex situ conservation of Australian plants. 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 3.1).

3.2 Planning for collection

This section will guide the planning and development of a collection strategy. It should be used in conjunction with Section 3.3 as the information provided here supplements that practical collecting information. Consideration of authorisation is important early in the planning process (see Box 2.3). As the demand for seed 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. Problems caused by seed collectors can alienate land managers and land owners, making future access to important collecting areas more difficult.

3.2.1 Information on target taxa

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

- Information sources (Appendix 1):
  - Herbaria (e.g. WA Herbarium *Florabase*, or NSW PlantNET)
  - Conservation databases and local land-use databases, e.g. forestry
  - Land managers
  - Conservation agencies
  - Species profiles, listing statements/advice and recovery plans
  - Field guides
  - Local expertise
  - Bureau of Meteorology
- Types of information:
  - Plant description and images (including any toxins and allergens)

— Best material for collection e.g. seeds, cuttings etc.
— Phenology (timing of seed maturity, best material for cuttings)
— Plant number and health
— Locality description
— 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)
— Climatic data and immediate weather forecast for collecting
— Permits required (see Section 2.3.3.4)

3.2.2 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 on a map or with coloured tape (in areas where it is unlikely to attract unwanted attention). The following are used to identify target species:

- Floras, guidebooks and botanical descriptions;
- Taxonomic keys;
- Images of target species;

Box 3.1

Collection checklist

Prior to collection

- Obtain necessary permits and permissions.
- Inform relevant seed bank or nursery of potential incoming material.
- Correctly identify target taxon/taxa and suitable population/s for sampling including the presence of hybrids or similar species that may be confused during collection.
- 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.
- Examine plants for signs of disease or stress, especially when collecting cuttings, and also for seed, the quality (insect damage, empty seed and maturity) and quantity available.

3: Seed and vegetative material collection

During collection

- Collect during dry weather conditions to reduce fungal contamination and spread of soil-borne diseases.
- Maintain hygiene precautions such as cleaning of secateurs, boots and other equipment.
- Collect only ripe (mature) fruit or seed, unless seed maturation off the plant is assured.
- Sample seed randomly and evenly from at least 50 widely spaced individuals to capture genetically unrelated plants and those exhibiting ecotypic variation.
- Where practical, keep seed samples from individual plants of threatened species or populations with 20 or fewer individuals separate, so maternal lines can be monitored in future translocations.
- The number of individuals to sample for cuttings should be determined by how many genotypes are required and can be maintained ex situ for the required duration.
- Keep cuttings from individual plants separate; 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 seed 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 fruit or seed, where possible. Discard twigs, stems and leaves if possible.
- Use appropriate collecting bags – for dry ripe fruit and seed use well secured breathable cloth or paper bags; for fleshy fruit use plastic bags that can be aerated to prevent decomposition or mould infestation; for cuttings, use moistened newspaper inside plastic bags; keep fleshy material and cuttings in car fridge or coolbox if possible at moderately low temperatures - do not freeze!
- Label bags and containers with relevant information for later identification.
- Record relevant collection information including 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).
- Collect herbarium specimen(s) for lodging with State or other Herbaria so taxonomic identity can be verified.
- To avoid overheating or mould in transit, dispatch material for processing in a timely fashion and keep cool and dry in the case of seeds, and cool and moist in the case of cuttings.

Post collection

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

- Expert local knowledge;
- Herbarium specimen/s to provide or confirm identification;
- Botanical expertise.

3.2.3 What material to collect

Plant material should be collected from known wild sources, unless the species is extinct in the wild or collection will further threaten wild populations. 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 due to the hybridisation of the plants 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 Chapter 8), in general, collecting from cultivated plants is not advisable so should only be used as a last resort. Material from such collections may be suitable for research purposes, such as seed biology studies when wild-collected seeds are unavailable.

When deciding which populations to collect, the end-use of the collection needs to be considered. In threatened species translocation and habitat restoration, the provenance of the material used is of paramount importance (see Box 2.2).

Once the target species and populations have been identified, the type of material to be collected must be decided (seeds, cuttings or divisions). Preferably this decision is made before collection, so as to allow the appropriate storage or propagation facilities and expertise to be secured (see Chapter 2). Often, information on the types of material that may be available is included in the sources mentioned in Section 3.2.2. However, for relatively unknown species, the decision may be based on the material available in the field or the circumstances of the collection expedition.

3.2.3.1 Seed and fruit

For most species, seed collection and storage is 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 are more likely to 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 angiosperms species (flowering plants) are often contained within fruits which nourish, protect and disseminate seeds. Some fruits are obvious, such as pumpkins, while other fruit types are barely distinguishable from the seeds. 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 and are openly exposed to the environment. For more information on fruits and seeds, see Kesseler and Stuppy (2006), Sweedman (2006), and Wilson and Wilson (2006).

3.2.3.2 Vegetative material

When viable seed is not available, or a clonal collection is being established for any reason, plants can be vegetatively propagated (see Chapters 2 and 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. Some species can be propagated by rhizomes, tubers or other structures that can be divided. 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.

3.2.3.3 Whole plants

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. To determine the chances of success, it is best to discuss this technique with a horticulturist/propagator prior to attempting transplantation.

3.2.4 When to collect material

3.2.4.1 Seed

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 3.2). 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. 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 3.4). At this point, seeds of orthodox species will have attained desiccation tolerance enabling them to be dried to low moisture contents for long-term storage (see Section 2.4.1).

3.2.4.2 Vegetative material

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. The choice of cutting type is dependent on the species and material available (see Mathews, 1999; Hartmann et al., 2002; Stewart, 1999).

Box 3.2

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 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 3.7).
- Serotinous plants (e.g. *Banksia*, *Hakea* and *Allocasuarina* and many small-seeded myrtaceous species like many *Eucalyptus*, *Callistemon*, *Calothamnus* and *Melaleuca*) retain seed on the plant within woody fruits for several years and the timing of collection after maturity is less important than for geospores (see Seed et al., 2006).
- Seed of orthodox species that is collected before it is fully mature will be of lower quality and, even if it does have germination potential, will not survive as long in storage as fully mature seed. This is because seed acquires the ability to be desiccated during the maturation period, at the end of which it reaches its greatest storage longevity potential (Hay and Smith, 2003).

Indications that seed has reached maturity include:

- Changes in fruit and seed coat colour;
- Splitting of fruit;
- Rattling of seed in fruit;
- Hard and dry seed;
- Some seed has already dispersed;
- Reduction in seed moisture content.

See 3.3.2 for methods to check seed maturity.

3.2.5 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);
- Communications (e.g. radio system or phone);
- Navigation (e.g. Global Positioning System (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);
- Vegetative material collection (e.g. secateurs, pole pruners, trowel, spade, plastic bags, moist newspaper for wrapping, spray bottle for wetting cuttings and newspaper, cooler/cool bricks, labels, data logger)
- Data collection (e.g. field data book, plant press);
- Photography;
- Camping;
- Hygiene control (e.g. cleaning vehicles, footwear, equipment, for disease control) (see Box 3.3).

Box 3.3

Protecting plants in the field

Regardless of the material being collected, it is vital that diseases, 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 even tent pegs can easily transfer potentially devastating organisms such as *Phytophthora cinnamomi* root rot, and such items should be cleaned between collections with disinfectant or alcohol such as methylated spirits.

More information on weed and Phytophthora control can be found on various States and Territories or the Federal Government websites (Appendix 1).

Figure 3.1 *(a) Advancing front of Phytophthora infestation through susceptible vegetation; (b) footwear and other items that come into contact with soil or plants should be cleaned between collection sites to stop spreading of diseases and weeds. Images: B. Summerell, Botanic Gardens Trust, Sydney.*

Box 3.4

Fruits and seeds of selected Australian taxa at maturity

*Images: Andrew Orme, Mishy Mckensy (*Syzigium*), Botanic Gardens Trust, Sydney.*

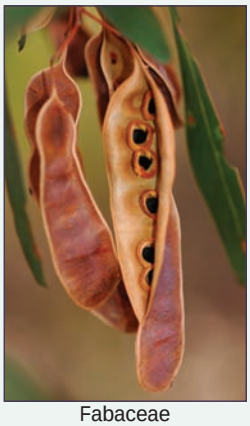
Asteraceae
Senecio diaschides

Casuarinaceae
Allocasuarina glareicola

Cyperaceae
Schoenus imberbis

Ericaceae
Epacris purpurascens

Ericaceae
Lissanthe sapida

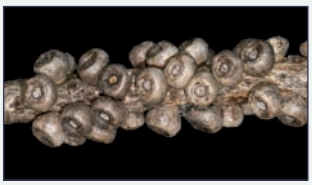


Fabaceae
Acacia nova-anglica

Goodeniaceae
Goodenia glauca

Lamiaceae
Westringia rigida

Malvaceae
Hibiscus splendens



Myrtaceae
Callistemon acuminatus

Myrtaceae
Corymbia eximia

Myrtaceae
Syzygium australe

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

Sapindaceae
Cupaniopsis anacardioides

Rutaceae
Zieria smithii

Solanaceae
Solanum petrophilum

Zamiaceae
Macrozamia plurinervia

Thymelaeaceae
Pimelea ligustrina

Xanthorrhoeaceae
Xanthorrhoea glauca subsp. *angustifolia*

3.2.6 Authorisation – permissions and permits

Prior to collecting, it is worth remembering that access to private land, Aboriginal Land, State Forests, National Parks or lands under other Federal, State or Local government control requires the consent of the land holder or manager. Collectors must also be aware of conditions that apply to rare and threatened species. For guidance on when a permit is likely to be required, see Box 2.3.

3.3 Seed collection

A collection strategy should aim to capture a significant proportion of the genetic variation found within a species. Emphasis should always be placed on sourcing material that captures high quality and genetically diverse seed 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 from 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). 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 3.3.6), or other species that rely on it for food, while maximising genetic diversity in the collection.

Read 'Planning for Collection' (Section 3.2) in conjunction with this section, as the information is not repeated here. The collection checklist is in Box 3.1.

3.3.1 Population assessment

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

3.3.2 Checking for seed maturity and quality

Seed should always be collected at the optimum stage of development i.e. maturity (Boxes 3.2 and 3.4). This will be assessed by observing phenology and seed development. The first signs of dispersal in a population can also be interpreted as showing that some, but not necessarily all, seed are close to maturity and therefore ready for collection. 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 seed. The collection should always contain a high proportion of fully developed (filled) and undamaged seed (see also Section 4.5.1). The maturity and quality of most seeds can be tested prior to collecting by conducting a cut-test (see Section 4.5.2) of a representative

sample and assessing it under a hand lens. Mature seed should generally contain firm, white endosperm and an embryo. Some taxa, such as orchids, lack endosperm and are dust-like and 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 seed. 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 seed 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 seed that is explosively dehisced), or for species with sequential maturation within an inflorescence (e.g. *Senecio*) bagging or seed traps are recommended (see Box 3.7e-f). These methods for seed collection should also be considered for high conservation value collections to ensure that seed is 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. If a collection contains a mixture of mature and immature fruits/seeds then these should be sorted and treated separately (Schmidt and Thomsen, 2003).

Labelling – seed is useless without it!

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 collection process, e.g. use a pencil or indelible marker resistant to water and rubbing during transport and storage. 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 4).

3.3.3 Collecting seed within a population

3.3.3.1 How many individuals?

The general aim of a conservation collection strategy is to capture as much diversity as possible in a collection, ideally 90-95% of the existing genetic variability found within a population (Falk and Holsinger, 1991). When the breeding system of a species is known, it is theoretically possible to capture population diversity by sampling seed as described in Box 3.5. In the absence of guidance from genetic analysis, this level of diversity can only be assumed to have been captured in a collection. In most cases, it is unlikely that this level of diversity will be captured due to a variety of reasons such as lack of access

to some plants (e.g. plants growing on cliffs), or because some plants may not set seed as a result of adverse weather conditions (e.g. drought) or a lack of pollinators. It may be necessary to assess collection of taxa on a case by case basis, and, if deemed important for conservation reasons, make further efforts to collect and capture the required diversity through recollection over subsequent seed production seasons. Repeat collecting (Section 3.3.7) is one strategy that may help achieve the required outcome, and should certainly be considered for small populations of threatened species where seed numbers are often low in any one season.

Box 3.5

Guidelines for collecting seeds within a population for ex situ conservation

(Brown and Marshall, 1995*; Guerrant et al., 2004†).

Breeding system	Number of randomly chosen individuals
outbreeding species	30 *
inbreeding species	59 *
not known < 50 individuals	from all individuals †
not known > 50 individuals	at least 50 †

In the absence of genetic information on the breeding system, the collection guidelines of Guerrant et al. (2004) recommend collecting material from at least 50 individuals if a population consists of more than 50 individuals, and from all plants if a population consists of fewer than 50 individuals. Increasing sample sizes greatly above 100 individuals is unlikely to result in greater diversity capture due to the logarithmic relationship between diversity and sample size, regardless of the mating system (Brown and Briggs, 1991; Holsinger and Gottlieb, 1991). In many cases, collection from 50-100 plants can be achieved without overstepping the safe limits of collecting (Section 3.3.6), but there are also many circumstances where this target is not appropriate or achievable. In the words of Guerrant et al. (2004) '... sample sizes will almost always be much smaller than these benchmark guidelines, reflecting the context in which particular taxa are found, our ability to work with them, and our purposes for collecting samples'.

As loss of genetic material may occur at any time through the storage, propagation and re-establishment phases (see Case Study 3.1), we suggest that this be carefully considered prior to collection to ensure that representation is as complete as possible within these constraints.

3.3.3.2 Sampling pattern in a population

Seeds should be collected randomly and evenly throughout the extent of a population. There are three main sampling strategies that can be used (Box 3.6). It should be noted that, generally, plants growing closely together are related, as seed falls below mother plants; a plant further away is more likely to be unrelated or less related. Sampling should aim to capture as many unrelated individuals as possible in the collection. It is better to sample from 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).

Where practicable, particularly for high conservation-value collections or where populations consist of fewer than 20 individuals, keep seed from each individual plant separate so as to permit future investigations.

Box 3.6

Sampling strategies for individual plants within a population

1. Simple random sample – each plant is chosen at random. Each individual has an equal chance of being selected.
2. Stratified random sample – where a population habitat is divided into differing and distinct patches, a random sample is taken from each distinct patch.
3. Systematic sampling – individuals are selected using a transect or grid approach, evenly spaced across a population.

(after Brown and Marshall, 1995)

3.3.4 Collecting multiple populations

When making ex situ conservation collections, consideration should be given to collecting from more than one population in order to capture diversity found in different populations, which is not always reflected at the taxonomic level. For example, some populations may contain traits that are not found in other populations. Multiple population collections are particularly important for inbreeding 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, different and diverse sites should be sampled within a species' range. To make a representative ex situ collection of a species with more than 50 populations, ideally collect from at least 50 populations, or from all populations if fewer than 50 populations are known (Guerrant

et al., 2004). 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. breeding 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 best represent as many genotypes of the species as possible, with subsequent populations increasing the genetic representation of the species. Populations of a species that are identified as being at high risk of loss or 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);
- degree of 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.

3.3.5 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;
- Monitor collection viability over time (see Guerrant and Fiedler, 2004 for a discussion);
- Duplicate the collection with another seed bank for safe keeping;
- Reintroduce or enhance viable population/s in the wild; and,
- Facilitate other uses such as research, education or commercialisation.

A collection size of 10,000 to 20,000 seeds is the recommended target number to meet these goals, providing that it can be obtained without threatening the survival of natural populations (Way, 2003). It should be remembered that this target may be difficult to achieve in a single collection, particularly for restricted or threatened species, but it provides a desirable target amount that should ensure adequate material for utilisation in species recovery. For some purposes, such as habitat restoration, seed numbers far in excess of this target will be required.

Generally, it is far easier to collect and conserve large numbers of small seeded species because they produce greater numbers of seeds than larger seeded species at any point in time (Moles et al., 2004). So, as seed size increases, it is more difficult to make large collections (of the order mentioned). Collection size will often be limited by population size and available seed, often making these ideal targets unachievable in any one collection. In these instances repeat collections to achieve the required size could be considered (Section 3.3.7). Further consideration is required before collecting numbers of large seeded species, as considerably more space is required to store them.

3.3.6 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 deplete the natural population, or individuals sampled of potential contribution of seed for natural recruitment. Sufficient material should remain to allow for soil or canopy seed bank accumulation in obligate seeding species, and natural regeneration. Further, it should also allow for natural predation of reproductive material as seed is often the major food source of native animals. Seed collections, particularly large in number or from threatened species, should not be made if there is no or limited ability to store them ex situ for the required length of time.

To reduce any adverse effects of over-collection, no more than 20% of the annual seed production of a plant population should be taken. In some States or Territories this amount may be legislated and may be far less. While a collection size of 10,000 – 20,000 seeds is recommended as a good size to cover a multitude of uses (Way, 2003), 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 limited to the maximum number of seeds that will reasonably avoid any impact on the long-term survival of the wild population. 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 seed. 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.

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.

3.3.7 Repeat collecting

Multi-year or multi-season sampling is a good strategy for obtaining sufficient material when one harvest may result in low seed numbers. If more than one harvest is made, then less material should be taken at each time than if making a one-off collection, as less intense, frequent harvests may have a lower impact on a population than more intense, infrequent harvests of seed (Guerrant et al., 2004).

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 collections through seed production areas may also be warranted (Chapter 8). Collections from different years should be kept separate.

Case Study 3.1

Seed collection sizes required for translocation success - allow for losses!

Analysis of the Western Australian Department of Environment and Conservation's seed bank germination data, botanic gardens nursery seedling mortality and translocated plant survival in the field allowed the calculation of the 'ideal' collection size required to successfully establish translocated populations of 10 threatened Western Australian species (Cochrane et al., 2007). The demographic cost of translocating threatened species was found to be high when losses were calculated (Figure 3.2). Survival of seed from collection, through germination, to reproductive maturity during the translocation process ranged from only 7% of the original seed used for *Daviesia bursariodies*, to 76% for *Grevillea humifusa*, with an average of 31% survival for the 10 threatened species assessed.

Modelling the survival of species from seed to reproductive maturity in this way informed the WA Threatened Flora Seed Centre as to whether sufficient seeds were available in storage to attempt to remove species from the threatened species list through a series of translocations. As an example, it was estimated that the critically endangered *Banksia* (formerly *Dryandra*) *ionthocarpa* subsp. *ionthocarpa* would require 12,500 seeds to create 1000 sexually mature plants. When these calculations were made, just over 4000 seeds were held in secure long term storage, sufficient to reliably produce fewer than 350 new reproductive plants in the wild. It was therefore considered necessary to make further conservation collections of germplasm of this species. In the case of the critically endangered *Acacia aprica*, only 28% of seeds used in the translocation process became reproductively mature

Repeat collecting is recommended when:

- Species display low reproductive output;
- Populations are small;
- Seed ripens 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;
- Stored seed has reduced viability (>20% of original).

3.3.8 Seed collection methods

The method of collection will be determined by the type of fruit and seed to be collected. Every plant type may require some modification to a general technique of collection, so assess each situation individually, be flexible and use some initiative. The basic considerations are:

plants in the field, but more than 55,000 seeds were held in conservation collections, sufficient numbers to enable full recovery of the species if necessary. No further collections were deemed necessary.

These types of data provide seed bank managers with vital information on the quantities of seed required to be held in storage to ensure future conservation success.

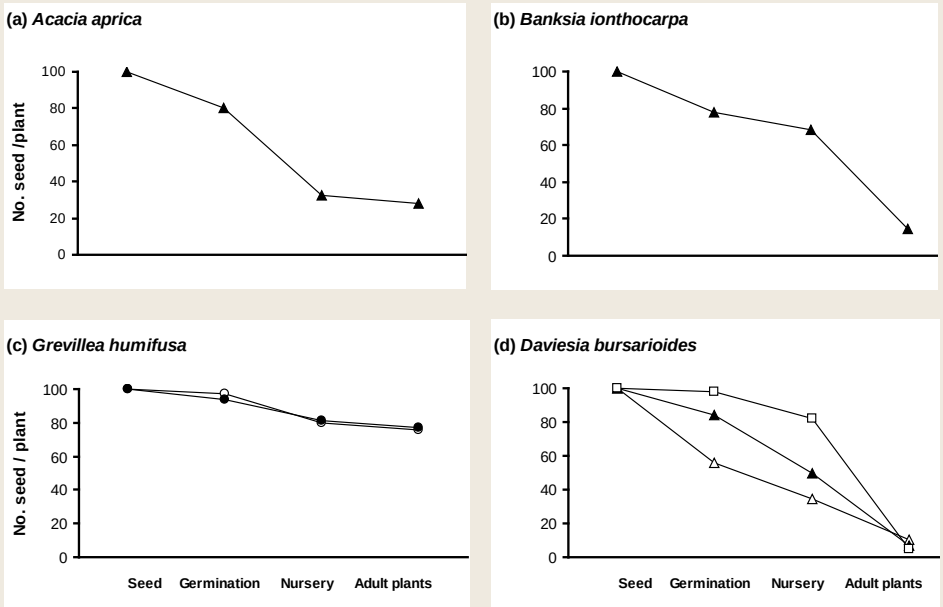


Figure 3.2 *Translocation of four critically endangered WA species: the proportion of seeds that survived from the seedling stage to become reproductively mature plants in the field. Data is calculated on a base of 100 seeds, germinated and grown to maturity, by year: 1998 (Δ), 1999 (▲), 2002 (□), 2003 (o) and 2004 (●) (from Cochrane et al., 2007).*

- When fruit is ripe, collect either individual fruits or heavily laden small branches.
- Collect fruit off the plant rather than from the ground as those on the ground may be empty or may have been damaged by rodents, insects or fungi.
- Collecting from the ground is impractical for fine seeds and there is the risk of contamination from morphologically similar seeds of nearby related species.
- Collect fruit into buckets, bags or straight on to a collecting sheet (see Box 3.7).
- Always make sure there are no holes in the container for small seeds to fall through.
- Use breathable bag materials, such as paper or calico, rather than plastic for transport of seed to minimize fungal attack through moisture build up. Be aware that there are various grades of calico. Chose one that is strong and breathable, with over-locked inside seams, so fine seeds don't get caught up in seams. Beware, not all paper bags are breathable.
- For fleshy, possibly recalcitrant seed, collect fruit in plastic bags as it may lose viability quickly if allowed to desiccate. Prevent the rotting of fruit and seed 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 seed if left intact. Place in a calico bag and squash the pulp before drying and placing in a clean bag.

Allergy note: *Be aware that certain plants may contain toxins or allergens e.g. some species of* Grevillea *(Proteaceae),* Ptilotus *(Amaranthaceae) and* Pomaderris *(Rhamnaceae). Long sleeves, gloves and a mask may need to be worn during collection and cleaning of material.*

3.3.9 Postharvest seed handling and cleaning in the field

Postharvest handling begins immediately following a collection and good practice is essential to maximise the quality and longevity of the collection. 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 seed is 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 it 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 on newspaper 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). If seeds requiring further maturation are still attached to plant stems, the cut stems can be placed in water for a few days.

Fleshy fruit 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 seed in the field by removing dry, bulky fruit parts or pulp from known orthodox fleshy seed (e.g. *Solanum*). Any extraneous material, such as leaves and twigs, can also be removed to minimize insect predation and disease. 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 4.3 deals with this issue in detail but it cannot be stressed too highly that fruit and seed should be handled with great care at all stages.

3.4 Vegetative material collection

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

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 seed, options involving vegetative regeneration of plants (e.g. cuttings, division, tissue culture and cryostorage) need to be very carefully considered in terms of costs, the diversity able to be represented and the required or perceived outcomes.

3.4.1 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 dependant on the diversity within the taxon. If the collection is made for conservation reasons (see Vallee et al., 2004 for information on sampling considerations for translocations), 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. If the collection is for display or educational purposes, it is generally sufficient to collect from only a few representative individuals.

Given that some genotypes perform better than others during propagation and cultivation,

Box 3.7

Some techniques and equipment used to collect ripe fruit

Image: A. Cochrane

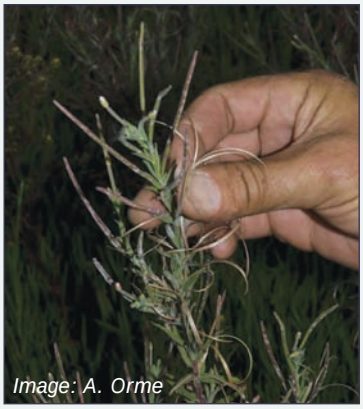


Image: A. Orme

(**a) Hand picking** allows an assessment of each individual fruit to be made on collection; heavily predated or damaged fruits can be 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.

Image: A. Cochrane

(b) Pruning of ripe fruit and seed bearing stems from plants that produce fruit 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. Fruit 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.


Image: A. Orme

Collecting fruit from tall trees requires long-handled pruners or saws. In some cases, ropes, rifles, or bows and arrows are used to bring down branches from very tall trees. Collecting from tall trees is hazardous and should only be done by experienced people who have the appropriate safety equipment and licenses. If possible, collect immediately after timber-felling operations.


Image: T. Tyson-Donely.

(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.


Image: A. Orme


Image: A. Cochrane.

(d) Shaking branches or plants

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

Box 3.7 continued

(e) Seed traps


Image: A. Cochrane.

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

(f) Bagging


Image: A. Cochrane.

Bags can be placed over stems containing immature fruit to catch seed when it is 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, pale in colour 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. 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.

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 3.2) in conjunction with this section, as the information is not repeated here. The collection checklist is in Box 3.1.

3.4.1.1 How many populations?

As with seed collections, this depends on the end-use and resources available to maintain the collections. Key populations should be represented if possible. See Section 3.3.4.

3.4.1.2 How many plants?

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 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 (see Case Study 2.2). 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 and 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.

3.4.2 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. 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 and great caution is required to avoid introducing diseases to the plant, either directly through unclean pruners, or indirectly by transferring contaminated soil on shoes for example (Box 3.3).

Always keep material from different plants separate and label as a replicate from the original wild plant. 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.

In order to successfully propagate cuttings, only healthy material should be taken. As a rule of thumb, particularly for threatened species, the conversion rate of cuttings taken, to plants established, may be low, typically 30-75%. 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 of material 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.

3.5. Botanical voucher specimens and field information

3.5.1 Botanical voucher specimens

Botanical specimens are taken to vouch for the identity of the collections and enable future taxonomic developments to be aligned with collections. One herbarium specimen should always be lodged with the relevant State or Territory herbarium. Often more than one specimen will need to be collected 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 Figure 3.3). 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. For further information on collecting herbarium specimens go to: <https://www.dec.wa.gov.au/images/stories/nature/science/herbarium/how_to_collect_herbarium_specimens.pdf>.

3.5.2 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 note book example Figure 3.4). It is also particularly helpful in monitoring threatened species populations. 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:

- Family, genus, species, subspecies etc.
- Date of collection.
- Collector's name and collection number.
- Locality (map details).
- Latitude / longitude or eastings / northings (use a GPS if possible) and record datum.
- Altitude.

- Number of plants sampled.
- Population status (number of plants and area occupied, or estimated frequency e.g. locally abundant).
- Plant description (tree, shrub, herb etc.).
- Site description (associated species, threats, disturbance, landform, aspect, slope, soils etc.).
- Phenology of population.
- 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.

Figure 3.3: *Example of a voucher specimen suitable for botanical identification and herbarium lodgement (*Banksia verticillata*, WA Herbarium). Image: A. Cochrane.*

3: Seed and vegetative material collection

Page No. _____

TAXON: _____ **ACCESSION No:** _____

POPULATION No: _____ **CONS.CODE:**_____ **DATE:** ____ / ____ / 200__

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 Kew ☐

PHOTO No's: _____

COMMENTS:

_____

_____

_____

_____

_____

_____

Figure 3.4: *Example of the basic field information to be collected.*

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Chapter 4

Seed banking

Amelia J. Martyn, David J. Merritt and Shane R. Turner

4.1 Introduction

Seed banking is the storage of seeds for ex situ plant genetic resource conservation (Smith et al., 2003). The genetic diversity captured in the seed collection can be used at a later date. The time between collection and use depends on user requirements, but is also governed by seed life spans. The longevity of a seed collection depends on the biology of the species and the nature (quality) of the material collected, as well as the storage conditions - particularly temperature and relative humidity.

Protocols for the handling, storage and testing of conservation collections of Australian seeds are based largely on those developed for other species, particularly agricultural crops and species required for forestry. However, the seed collections at Australian seed banks represent a much wider range of species and are generally collected in smaller quantities than crop seed collections. Handling and sampling protocols such as those recommended by Bioversity International (formerly IPGRI) (Rao et al., 2006) and the International Seed Testing Association (ISTA, 2007) have, therefore, been adapted to handle the diversity of seeds encountered in Australian species, and to make the best use of smaller seed collections whilst still following the principles of high-quality seed storage (see Box 4.1).

Conservation seed banks differ from restoration seed banks in that seed samples are generally cleaned to a higher standard, kept in smaller quantities and stored under more stringent conditions for longer storage than is required for broadscale restoration. Conservation collections are intended to be kept for decades, hundreds or potentially thousands of years and may be duplicated at a second location for risk management. Restoration seed banks have a more rapid turnover of seed collections (from months to years). 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 4.1, Table 4.2).

The information presented in these guidelines is considered best-practice at the time of publication. However, research into seed postharvest handling, desiccation tolerance, longevity, germination and dormancy is ongoing in Australian conservation seed banks. New information will inevitably lead to refinements and improvements in seed storage and utilisation techniques in the future.

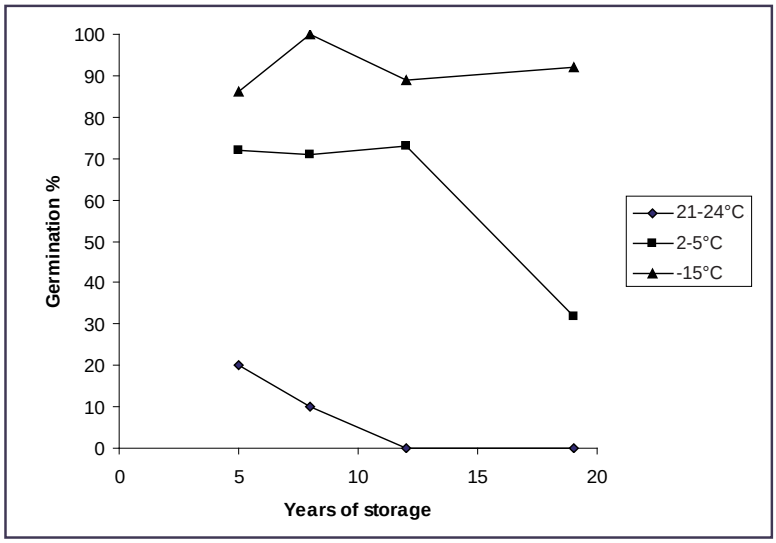

Figure 4.1 *The germination (indicating viability) of* Eucalyptus microtheca *seeds stored at three storage temperatures for 19 years (data from Gunn, 2001).*

4.2 Benefits and risks of seed banking

The benefits of seed banking as a method of ex situ conservation were briefly addressed in Section 3.2.3.1 and have been summarised by Maxted et al. (1997). Seed banking is an efficient and reproducible method of storing orthodox species over an extended period of time (Maxted et al., 1997). Collections require little maintenance once material is conserved and can be easily accessed for characterisation, monitoring and utilisation. Facilities can be scaled from desktop (seeds in an airtight container over silica gel) to the large walk-in vaults of gene banks (Maunder et al., 2004).

Perhaps the greatest risk is total catastrophic loss of the collection. Hence the need to duplicate (Section 4.4.4). Other disadvantages of seed banking include the inability to store desiccation sensitive species and the lack of evolutionary development during storage, as the collection is literally 'frozen in time'. Seed storage may lead to loss of genetic diversity during each regeneration cycle, although collections may last for decades before regeneration is necessary (Maxted et al., 1997). Risks of seed banking include mislabelling (compromising collection identification) and mishandling (compromising seed viability) during numerous operations, through collecting, cleaning, drying, packaging and storage. Once collections are stored, there is the risk of inadequate maintenance and management of the collections and of the temperature and humidity-controlled seed bank environment, particularly where on-going funding is not assured. Seed banks need to make provision for back-up generators in the event of power failure, and have plans in place to minimise the impact of natural disasters such as fire and flood.

4.3 Pre-storage operations

Careful observation, handling and cleaning of seeds prior to sorting is necessary to ensure that the effort put into making high-quality seed collections is not wasted. This section

Box 4.1

Seed banking procedures

Plan and collect seed and vouchers (Chapter 3)

↓

Unpack seed collections

↓

Unpack vouchers and confirm identification (Section 3.5)

↓

Record data for each accession (Section 4.3.1)

↓

Assess likely seed storage characteristics (Section 2.4 and 4.3.2)

↓

Seed cleaning
(possibly preceded by drying to release seeds from fruits)
(Section 4.3.3)

↓

Cut-test
(Section 4.5.1 and 4.5.2, also done during collecting Chapter 3)

↓

Seed quantity determination (Section 4.3.4)

↓

Drying (Section 4.4.1)

↓

Check seed moisture status (Box 4.2)

↓

Initial germination test (Section 4.5.2.1 and Chapter 5)

↓

Packaging and security duplication (Section 4.4.2 & 4.4.4)

↓

Storage (Section 4.4.3)

↓

Distribution of seed samples to users

Germination re-tests; regeneration/multiplication of collections, as required (Section 4.6)

From Smith et al., (2003) and Rao et al., (2006).

highlights important steps in postharvest handling of seeds, the importance of record keeping during curation, and briefly explains how the quantity of seeds in a collection can be determined.

4.3.1 Record keeping and labelling

During curation, it is essential to keep track of each seed collection and ensure that the data relating to each collection are stored appropriately for future access. Information collected during curation complements provenance and seed collection data and all of this information should be kept together.

Retaining seed (species and collection) identity during processing is essential if conservation collections are to maintain their value. It is imperative that individual seed collections are not mixed or confused with other batches of the same species, or other collections from the same field trip, and that provenance data collected in the field relates to each collection. One way to retain identity during processing is to give seed lots two labels – one inside the container with the seeds and the other on the outside of the container or on the processing equipment (e.g. jar, tray or cleaning apparatus). Make legible labels that are written in pencil or water-repellent ink and resistant to moisture. Jeweller's tags containing the collector's name or identification number and the species name written in pencil are ideal. After any partial or preliminary cleaning, seeds should be returned to the same container. Empty collecting bags, containers and processing equipment should be thoroughly cleaned before re-use.

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 collection information. Notes on the method 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. As well as adding to the value of the individual collection, these data can assist with decision making for germination testing, and help identify future collection needs such as the recollection of a particular species over several years to maximise seed numbers.

4.3.2 Postharvest handling and assessment

After collection, pay particular attention to the moisture content of the seeds, as seeds will age rapidly at high moisture levels. For orthodox species, seed moisture levels need to be reduced in order to be stored at low (sub-zero) temperatures. It is always wise to check whether a species is known to or is able to tolerate seed drying before the seeds are placed under conditions suitable for drying (see Section 2.4).

Seed moisture content is simply the amount of water present in a seed at a given point in time. Seed moisture content is influenced by the storage conditions (i.e. relative humidity and temperature) and the seed maturation state at collection. Seed moisture content influences all of the key processes associated with aging, dormancy and germination (see

Chapter 5). Relatively small changes in seed moisture content (1 – 2%) can be sufficient to induce significant changes in a seed's physiological state.

Seeds gain or lose moisture depending on the relative humidity of the surrounding air, eventually reaching equilibrium (Gold, 2008). The relative humidity of the air at this equilibrium point can be measured and is termed the equilibratium relative humidity (eRH). A 'safe' moisture level to prevent rapid aging is around <50% eRH. Most orthodox seeds tolerate desiccation to around 15% eRH (4-7% seed moisture content, depending on seed oil content) (Figure 4.2) and should be dried to this level for long-term storage (Gold, 2008). Seed eRH can be measured quickly and non-destructively using a hygrometer (Box 4.2). Seed moisture content is measured destructively, by oven drying seeds at 103ºC for 17 hours, and expressed gravimetrically on a dry weight basis (see Box 4.2).

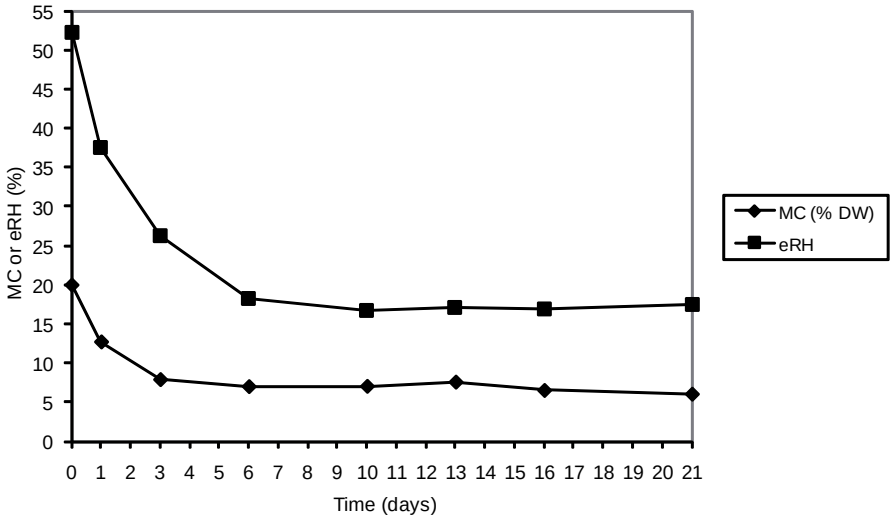

Figure 4.2. *Seed drying over time. This graph demonstrates the change in moisture status of seeds measured via two methods: seed moisture content (MC) and equilibrium relative humidity (eRH). The seeds are from* Bursaria spinosa*, freshly collected and dried at 15% RH and 15ºC (C. Offord, unpubl.).*

Seeds collected at the point of natural dispersal need to be dried as quickly as possible (see Box 4.3). Drying 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 4.4.1). Collectors and curators need to make sure that 'dry' 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 conditions that promote postharvest 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. Postharvest maturation can also be encouraged by storing seeds at high humidity (30 days at c. 80% for

Box 4.2

Seed moisture determination

Measuring equilibrium relative humidity (eRH) using a hygrometer (Gold and Manger, 2008a)

1. Place seeds into a sample dish supplied with the hygrometer, ideally filling the dish.
2. Place dish into the hygrometer chamber, close chamber and allow at least 30 minutes for seeds to reach equilibrium.
3. Record the eRH and temperature at equilibrium (eRH values are temperature dependant)

Measuring moisture content by oven drying (Rao et al., 2006 pages 28-35)

1. Weigh at least three samples (replicates) of 0.1g or a minimum of 10 seeds, depending on seed size and availability, on a balance measuring to three or four decimal places (0.001 – 0.0001g). This is the fresh weight of seeds.
2. Place containers with lids removed in an oven maintained at 103 ± 2°C for 17 ± 1 hours (as the oil content of many Australian species is unknown, this is the low constant temperature method as recommended for oily seeds and is also suitable for non-oily seeds).
3. Replace the lid of the container and transfer to a desiccator and allow to cool for about 45 mins.
4. Record the dry weight of each sample of seeds.
5. Calculate seed moisture content on a dry weight basis for each sample.

Seed moisture content (% dry weight) = ((wet weight – dry weight)/dry weight) x 100

Note: These guidelines recommend calculating seed moisture content on a dry weight basis, as this is more accurate when working with seeds over a large range of water contents. Moisture content can be calculated on a fresh weight basis, however. In any case, the method of calculation should be clearly stated.

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 a plastic bag until they can be cleaned but the bags must be opened daily to aerate and remove condensation. The storage potential of fleshy fruits should be assessed as described in Section 2.4.1.

4.3.3 Seed cleaning

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

Box 4.3

Postharvest handling (from Gold 2008)

<table>
<tr><th rowspan="2">Seed maturity stage</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 ambient conditions for 1-2 weeks *</td></tr>
<tr><td rowspan="2">Natural dispersal</td><td>'Dry'
<50% eRH</td><td>Hold in loosely packed bags in a well-ventilated shady 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>'Wet'
>50% eRH</td><td>Dry in a thin layer, in a well ventilated 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>

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 seedlot 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 and for a list of techniques and useful equipment for seed cleaning, see Terry and Bertenshaw (2008). 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 4.1), hand sorting and use of a gloved hand or rubber bung to roll seeds over a rubber mat (Terry and Bertenshaw, 2008).

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. The fruit pulp is usually removed via washing in a sieve and the seeds (or indehiscent, woody fruits) then dried and processed. It should be noted that seeds of some fleshy-fruited species are recalcitrant and desiccation tolerance may need to be assessed (Section 2.4.1). Fleshy fruits may alternatively be soaked for a few hours in a pectinase enzyme solution, similar to that used in the winemaking process, to speed up the process of pulp removal (Tieu et al., 2009, Box 4.4).

Box 4.4

Enzymatic depulping 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 *Persoonia*, *Leucopogon*, *Eremophila* and *Lomandra*, for example, and contain large amounts of pectins, sugars and water which can cause significant processing and storage problems, hence the need for their removal prior to storage. Indeed, if these surrounding layers are not eliminated, their continuing presence may remain a significant impediment to germination. Previous depulping techniques have relied on soaking in water for several days to promote natural fermentation, then manual removal of the remaining material, which is often a slow and laborious process. However, recent research has found that 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., 2009). Indeed, instead of taking days to process, fruits can now be depulped in only a few hours.

To begin the cleaning treatment, freshly collected fruits are quickly macerated and placed into the enzyme broth which is simply made by adding 1g of enzyme powder (two commercially available products are Ultrazym® EX-L and Lafase He Grand Cru) to 1 L of water. The mixture is then placed onto a magnetic stirrer and heated to around 40ºC. Constant agitation is then important, as it has been found to improve significantly the rate of depulping, completely liberating seeds and endocarps within 3-4 hours. These depulping methodologies have now been assessed on a range of pulpy native fruits and seeds including *Dianella revoluta*, *Leucopogon* spp., *Lomandra* spp., *Myoporum insulare*, *Nitraria billardierei*, *Rhagodia baccata* and *Solanum* spp.

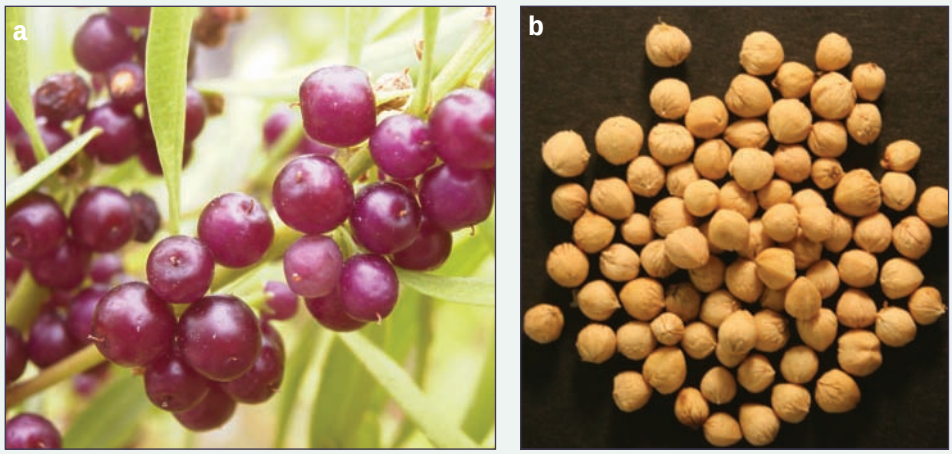

Figure 4.3 *(a)* Myoporum insulare *fruits prior to collection. Image: K. Dixon; (b)* M. insulare *endocarps after enzymatic depulping. Image: L. Commander.*

Case Study 4.1

Seed cleaning of Pomaderris lanigera *at the NSW Seedbank*

1. Fruits of Pomaderris lanigera *in situ*

4. Collection sieved to remove sticks and leaves

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

5. Collection cleaned in aspirator to remove more debris.

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

Case Study 4.1 **continued**

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, Botanic Gardens Trust, Sydney.

4.3.4 Seed quantity determination

After seeds are cleaned and dried, the collection can be assessed for its purity, total weight, the total number of viable seeds and the individual seed weight (Box 4.5).

4.4 Storage of orthodox seeds

The aim of conservation seed banks is to maintain the viability of high-quality seed collections for as long as possible. The emphasis on cool, dry storage for maximising seed longevity is expressed simply by Harrington's 'Rule of Thumb', Bioversity International guidelines (Rao et al., 2006) advocate drying seed at 10-15% RH and 10-25°C (a seed moisture content

Harrington's 'Rule of Thumb'

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

Box 4.5

Steps used to calculate seed purity and the total number of seeds in a given seed batch

Work out the total weight of the seed batch

⬇

Remove a small sub-sample (1 - 5 g) and weigh

⬇

Separate the seeds from the other debris in the sub-sample and count them all

⬇

Weigh all the seeds in the sub-sample

⬇

From the recorded data the following can now be determined:

1. Purity of the seed batch (%) = (total weight pure seeds in sub-sample/total weight of sub-sample) x 100
2. Total weight of seeds in seed batch = (% pure seeds in sub-sample/100) x total weight of seed batch
3. Individual seed weight = (total pure seed weight in sub-sample/total number of seeds in sub-sample)
4. Total number of seeds in the seed batch = (total weight of seeds in seed batch/ individual seed weight)

of 3-7%), and subsequently storing seed collections at -18°C or less. Recent studies have shown that these conditions are suitable for many Australian species (Offord et al., 2004; Crawford et al., 2007). However, other storage conditions may be more practical depending on the required longevity of seed bank collections. The conditions of an intended or current storage environment can be easily monitored using temperature and/or relative humidity dataloggers, for example, TinyTag™ or Thermocron™ loggers.

Note that seed testing for viability and germination (Section 4.5) may be conducted prior to or after storage, or ideally, both before and after seed storage.

4.4.1 Seed drying

Drying is the first step in limiting seed deterioration, as drying increases seed longevity and means that seeds can survive storage at sub-zero temperatures, avoiding the formation of lethal ice crystals. Seed drying also minimises damage due to insect or fungal attack.

Seed drying can begin as soon as possible after collecting, keeping in mind that immature seeds may benefit from slow drying (see Section 4.3.2) and some species may not survive drying (see Section 2.4.1). Seeds are usually placed in porous bags (e.g. calico bags), particularly if they have an explosive seed release mechanism, or spread out in trays until they are cleaned. After cleaning, seeds may undergo more controlled drying. Seed moisture levels (ideally 10-15% eRH or 3-7% moisture content) should be checked and recorded before packaging, either by measuring seed moisture content or equilibrium relative humidity (see Section 4.3.2).

Factors affecting seed drying include:

- Seed size and structure, particularly the permeability of the seed coat.
- Air velocity, as drying time is approximately halved when the velocity of air around each seed is doubled.
- Temperature, as water evaporates more rapidly as temperature rises. However, the use of high temperatures to accelerate seed drying is not recommended as it will speed up the seed ageing process and may unacceptably reduce viability. Temperatures of 10-25°C are recommended for seed drying.
- Relative humidity - aim to keep relative humidity of the air around 10-15% RH (see Section 4.3.2).
- Seed moisture content, with water being lost more rapidly at the beginning of drying than towards the end.

Source: Schmidt and Thomsen (2003)

Several options for seed drying are available including:

- Purpose-built low humidity drying rooms (operate at 10-15% RH and 10-25°C, as used in many state seed banks for Australian native seeds).
- Cabinet dehumidifiers e.g. Walsh and Jeanes (2008).
- Sealed containers with seeds dried over silica gel.
- Air-conditioned rooms.
- Sheds with drying racks.
- Polythene igloos.
- Ambient conditions (sun or shade).

More details can be found in Linington (2003) and Sweedman (2006).

4.4.2 Packaging dried seed

Packaging of seed collections is necessary to maintain the low moisture content achieved during drying. Ideally, seeds should be packaged quickly in a controlled humidity environment, to prevent them absorbing moisture from the surrounding air. Packaging also keeps seed batches separate and prevents insect and disease contamination in storage. Containers for packaging need to be air-tight, for example, heat-sealed tri-laminate foil bags (Figure 4.4) or glass jars with a rubber seal (see Gold and Manger, 2008b for more details). Advantages and disadvantages of different storage containers are presented in Table 4.1.

Storage containers for cryostored seed are discussed in section 7.5.4.1. The reabsorption of moisture during storage can be detected by including self-indicating silica gel sachets in the chosen container. Similarly, silica gel can be used in containers to ensure they are air-tight in high humidity and freezing environments (Gold and Manger, 2008b).

Figure 4.4 *Seeds stored in heat sealed foil bags in the walk-in freezer of the NSW Seedbank, Mount Annan Botanic Garden. Image: Simone Cottrell, Botanic Gardens Trust, Sydney.*

Table 4.1. *Options for packaging dried seed (from Gold and Manger 2008b).*

Container material	Advantages	Disadvantages
Glass containers	Transparent: seeds and moisture indicators can be seen	Heavy, potential for breakage Not space efficient
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 seeds e.g. grasses Do not allow seed or moisture indicators to be seen
Plastic containers	Lightweight May be transparent	Rarely seal effectively Potential effect of plasticizers on seeds Not space efficient
Metal containers (Not recommended)	Robust	Seal poorly Do not allow seed or moisture indicators to be seen May be prone to corrosion

4.4.3 Seed storage

Once seeds are dried and packaged, they should be placed directly into storage to further slow the seed ageing process. The temperature at which seeds are stored depends on the desired storage life of the seed collection and the facilities available (Table 4.2). Temperatures of -18°C or lower are preferred for conservation collections; for example, commercial deep freezers or purpose-built cold rooms at -18°C (recommended), (Figure 4.4); or cryostorage in liquid nitrogen (-196°C) (see Chapter 7). Higher temperatures e.g. household refrigerators (0-5°C) are suitable for shorter-term storage.

Table 4.2 *Potential seed life span for different storage conditions (after International Board for Plant Genetic Resources, 1991).*

Time frame	Conditions	Suitable for:
Short term (up to 5 years)	Air-conditioned room Ambient humidity	Restoration seed banks
Medium term (5-10 years)	Temperature 1-10°C Relative humidity <15% Seed moisture content 10-12%	Plant breeding
Long term (>10 years)	Temperature ≤ minus 18°C Relative humidity 10-12% Seed moisture content 4-6%	Conservation seed banks

Conservation collections may be divided into active and base collections (see Section 2.6.1) prior to storage. In some seed banks, for example, those used in plant breeding, the active collection may be held at a higher temperature (1-10°C) than the base collection (-18°C) (International Board for Plant Genetic Resources, 1991). The proportion of seed held in active and base collections varies from seed bank to seed bank and depends on the taxon and the intended usage.

The conditions suitable for seed storage may also be appropriate for storing banked collections of other genetic material for conservation purposes. Examples include fern spores and the fungal mycorrhizae required for germination of orchid seeds. Handling and storage of orchid seed and mycorrhizae is described in Section 6.4.3.

4.4.4 Duplication of banked collections

Duplication refers to a genetically similar sub-sample of an accession being stored at another location to provide insurance against the loss of material. Ideally, duplicate seedlots should be maintained under similar conditions to the main collection. 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 Section 2.6.2), a memorandum of understanding is worthwhile to ensure that seed use is discussed before collections are sent.

4.5 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 (4.5.1), viability (4.5.2) and germination (Chapter 5). Initial seed viability is also a significant factor in seed longevity (4.6.1). General suggestions for sampling and testing are presented in Box 4.6.

Box 4.6.

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 only estimates 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.

4.5.1 Seed-fill

Seed-fill (see also Section 3.3.2) describes the proportion of seeds that are outwardly undamaged and have all the tissues essential for germination (that is, an intact endosperm and/or embryo). Seed-fill can be determined by a number of methods. They include:

1. Cutting seeds in half with a scalpel and examining under a dissecting microscope (called a 'cut-test', Figures 4.5 and 4.7).
2. Seed can also be X-rayed to determine seed-fill (Figure 4.6). The accuracy of this technique depends on the size and structure of the seed, and the ability of the

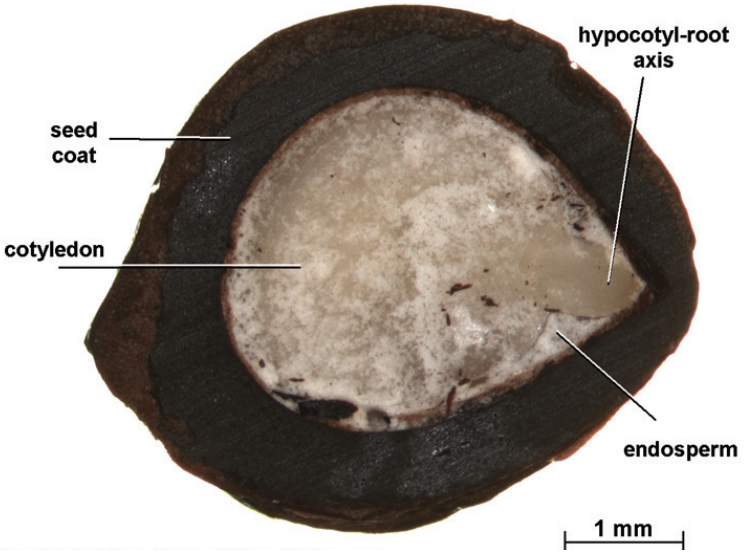

Figure 4.5 *Cut-test showing a firm white embryo (hypocotyl-root axis + cotyledon) and endosperm surrounded by a thick seed coat in* Geijera salicifolia *var.* latifolia *seed. Image: Mark Ooi.*

operator to interpret the results. The advantage of X-ray is that the technique is non-destructive (although seed used for x-ray should be kept separately to the rest of the collection) and it provides a permanent record of the collection.

3. In very small seeds such as *Callistemon* and some *Eucalyptus*, crushing seeds and counting oil dots left by crushed seeds can give an indication of filled seeds.
4. Seed-fill can be determined by flotation in some species; for example, *Geijera* spp. (A. Martyn, unpublished data). Filled seeds will generally sink while empty seeds will float. The correlation between flotation and seed-fill should be checked using a cut-test for each species before flotation is used as a reliable test. Following flotation, seeds should be dried appropriately before being stored.

Some families tend to have low seed-fill, due to either aborted embryos or predation by insect larvae (for example, seeds of families Rutaceae and Fabaceae). Seed-fill can also be lower than expected due to production of seed mimics (for example, *Callitris* and *Allocasuarina* species).

4.5.2 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 intact living and dead seeds often look exactly the same. Ideally, conservation collections should have a high viability when initially placed in the seed bank and this viability should be maintained during storage. Monitoring of seed viability during storage is addressed in the following section on seed longevity (4.6.1). Since seed viability varies with environmental conditions during maturation on the parent plant, collection season and seed maturity at collection, every effort should be made to maximise seed viability by following collecting guidelines and handling seed carefully after harvest. However, some Australian species may have inherently low viability.

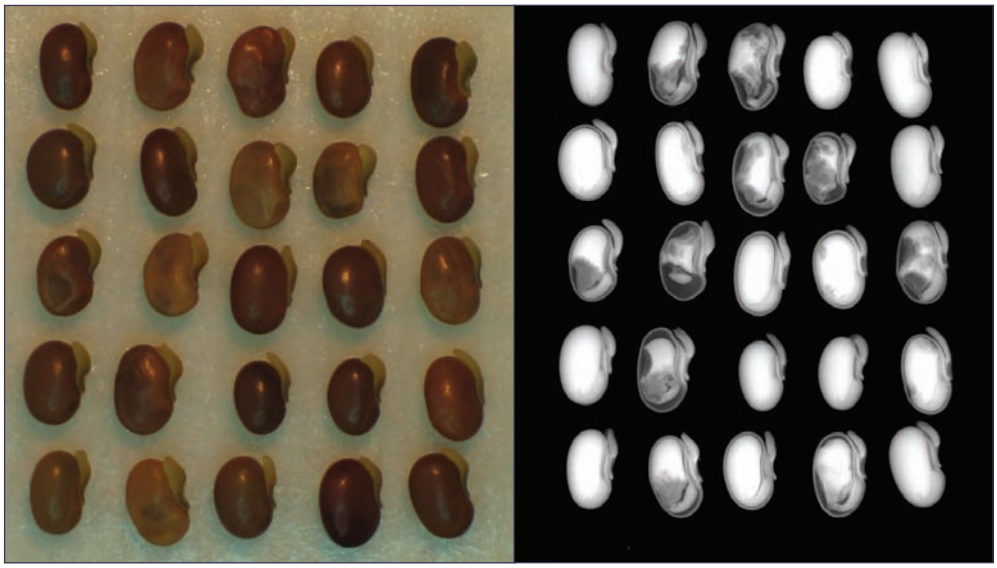

Figure 4.6 *X-ray of* Davesia cordata *seeds showing the before (left) and after (right) X-ray assessment of seed fill. Out of the 25 seeds assessed nine show signs of significant damage and a further four show a smaller amount of damage. Images: R. Tuckett.*

4.5.2.1 Germination

The most reliable 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 test conditions for that species are unknown, or seeds are dormant, there may be a proportion of viable seeds that do not germinate. This can lead to a significant underestimate of viability. For more information on germination and dormancy, see Chapter 5.

4.5.2.2 Cut-test

A cut-test (described in Section 4.5.1) can be used on imbibed seed to estimate seed viability, with the added step of interpreting whether the tissues inside the seed are firm, fresh and healthy. The endosperm and embryo tissue in viable seeds is usually firm and white. The cut-test is simple, quick and inexpensive but destructive (Figure 4.7).

4.5.2.3 Staining

Biochemical tests such as the tetrazolium test can also be used. Tetrazolium chloride stains viable tissues red, while dead tissues stain a pale pink colour, or remain unstained. The tetrazolium test is more time-consuming and requires experience and skill. For example, fungal infection of seeds can lead to false red staining, as the fungal mycelium is also alive (Miller, 2005). Other biochemical stains, such as fluorescein diacetate and Evan's Blue, have been used to determine the viability of orchid seeds. Biochemical viability tests have been developed for crop species but standard methods for testing

Figure 4.7 *Comparison of cut-test results from two different seedlots showing intact seed of* Beyeria lechenaultii *(a) and* Prostanthera eurybioides *(b) and cut-tests respectively. For* B. lechenaultii *(c) all 20 seeds (100%) are considered potentially viable. Comparatively for* P. eurybioides *(d) only 3 seeds (15%) are considered potentially viable. These results highlight the potential use of the cut-test in assessing seed quality. Images: P. Ainsley.*

Australian species have not been developed. In particular, seed preparation and staining time may differ markedly between wild species and crop or ornamental species, where the technique is more widely applied (Miller, 2005; ISTA, 2007). Examples of tetrazolium testing for Australian species are provided by Gravina and Bellairs (1999) and Thompson et al. (2001). If biochemical tests are to be used for testing particular species, steps should be taken to correlate final germination and viability indicated by staining (e.g. Ooi et al., 2004). This method is also destructive.

4.5.2.4 Embryo culture

Another test to determine seed viability is to extract zygotic embryos from seeds and culture in vitro under sterile conditions (Chapter 6). In many cases seed embryos will grow rapidly (even if the seed does not germinate) once removed from the seed and 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 you are working with are rare, unusual or from a threatened species. The medium used for culturing extracted embryos can be as simple as water agar (effective for cotyledonous embryos e.g. *Eucalyptus* or *Acacia* species) or as complex as MS (Murashige and Skoog, 1962) based media with added vitamins, sugars and plant growth regulators (Chapter 6). Prior to embryo-extraction seeds need to be surface-sterilised and embryos gently removed without damage in a laminar flow 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 6).

4.6 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 (4.6.1) and possibly seed vigour (4.6.2). Storage environments need to be monitored to ensure that containers maintain seeds at the correct moisture content (4.4.2) and fridges or freezers maintain the correct temperature (4.4.3). Seeds may also be distributed for various purposes (4.6.4), and particular caution should be taken with the small collections that are often present in conservation seed banks (4.6.3).

4.6.1 Seed longevity

The maximum longevity of seeds stored under conservation storage conditions is a topic of ongoing research, with little published information on long-term storage for Australian species. While few long-term studies have been completed, seeds of many Australian Fabaceae, Myrtaceae and Proteaceae can be stored at 5-10% moisture content and 5°C for up to ten 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).

Since the longevity of Australian seeds in storage is largely unknown and cannot be easily predicted, it is important to monitor the viability of seed collections during storage. This will ensure that seeds do not lose their capacity to produce viable plants when needed and will inform future seed practitioners. Seeds with high initial viability survive longer in storage. For highly viable seeds, the decline in seed viability during storage is initially slow, and then more rapid as seeds age. Monitoring seed viability at regular intervals allows accessions with declining viability to be identified and regenerated or re-collected before germplasm is lost. Rao et al., (2006) suggest that monitoring is carried out every 10 years for seeds with initial high viability (>90% germination), and every 5 years for oily seeds (oily seeds are seeds that contain a substantial quantity of storage lipids, such as canola) and those with lower initial viability (<85-90% germination). For poorly understood species, including most Australian species, a more cautious approach to testing is suggested, perhaps after one, two and five years of storage, particularly for high value seeds (Merritt, 2006). The frequency of testing will also depend on staff and resource availability, and the number of seeds available for testing.

When monitoring seed viability over time, it is important to use consistent testing methods (e.g. dormancy breaking treatments), and consistent test conditions (e.g. temperature and light). 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 need to be regenerated from the original batch and used to multiply seed numbers (Chapter 8), or plans made for timely re-collection.

4.6.2 Seed vigour

Seed vigour can be defined as 'the inherent ability of seeds within a seed lot to establish normal (or usable) seedlings under diverse growing environments' (Genève, 2005). Seed vigour is not monitored in all conservation seed banks, but seed vigour measurements can provide an additional indicator of successful plant establishment in the field. Seed vigour is generally reduced before decreased seed viability can be observed, providing an early indication of seed deterioration.

Vigour can be indicated by the time taken for a seed to germinate, with more rapid germination indicating more vigorous seeds. Time to germination increases with seed age. A better indication of how seeds are likely to perform in the field may be obtained by growing-on seeds following germination, to determine how many produce normal, healthy seedlings (e.g. Offord and Meagher, 2001). Other vigour tests measure germination percentage following the imposition of temperature or moisture stress, or following accelerated ageing (see Genève, 2005 for details of vigour testing in flower seeds).

4.6.3 Handling small collections

Storage of relatively small quantities of seeds is one feature that differentiates conservation seed banks, particularly for threatened species, from crop and restoration seed banks. Small collections (fewer than 1000 seeds) may be stored for a variety of reasons. For example, the species in question may set limited seed even in good conditions, or drought or predation may have reduced the amount of seed available for collection and storage. Collections over several years may allow accumulation of a larger collection of seeds 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 (see Chapter 5).

4.6.4 Seed distribution and end-use

Seed samples from banked collections (usually active collections only) may be distributed to users. People using conservation collections are generally involved in restoration of plant diversity, research or plant breeding. Distribution of seed samples may depend on how the seed is 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 Chapter 2 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 Box 2.2), 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 (Section 2.6.2). A copy of this agreement should be supplied with the seeds to be dispatched. Records of seed distribution should 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 e.g. phytosanitary certificate and export permit number. 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 5

Seed germination and dormancy

Shane R. Turner and David J. Merritt

5.1 Introduction

Seeds form the primary means of reproduction and species persistence for the majority of flowering species. Therefore, seed germination is a critical process in the lifecycle of plants. In order to maximise the chances of seedling survival after germination, seeds have evolved a range of dormancy mechanisms to sense the external environment and germinate only during periods 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 (Finch-Savage and Leubner-Metzger, 2006). A dormant seed is one that does not have the capacity to germinate (within a specific time period) 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 Australian species possess some type of seed dormancy 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. This means that germination triggers need to be determined, both 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 (Finch-Savage and Leubner-Metzger, 2006). Dormancy types are classed 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. In order to germinate a seed, it is necessary to first define the type of dormancy and then determine the conditions necessary to alleviate the dormancy. 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.

5.2 Gathering species-specific information relevant to germination and seed dormancy

Prior to commencing germination of a species seed collection 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. Good sources of literature are:

- The major public libraries around Australia.
- Libraries within universities and technical colleges.
- Botanic Gardens.
- Publicly accessible databases available on the internet which include:
  - The Seed Information Database (SID) of the Millennium Seed Bank Project, Royal Botanic Gardens, Kew, <http://data.kew.org/sid/>
  - Greening Australia's Florabank, <http://www.florabank.org.au/>

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 5.4.1) and require a hot-water or scarification treatment for germination to proceed. Many species of Asteraceae respond well to a period of dry after-ripening (Section 5.6.2).

It is important to 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.
- The average monthly maximum and minimum temperatures.
- The rainfall figures.

Seasonal response

Seeds naturally germinate during the wet season, when water becomes non-limiting. Average temperatures at this time 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, some knowledge as to the type of dispersal mechanism of the species in question can also be useful (Box 5.1). Determine whether seeds are:

- Persistent (termed serotinous); or,
- Shed into the soil seedbank (termed geosporous).

Box 5.1

Dispersal mechanisms – serotiny and geospory

Species that store their 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, most often after a disturbance such as fire. Common examples of serotinous plants 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. In addition, 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 common examples of geosporous taxa. Other characteristics of serotinous and geosporous species are shown in Table 5.1.

Table 5.1 *Characteristics of serotinous species, that store their seeds in the plant canopy, and geosporous species, that release mature seeds into the soil.*

Characteristic	Serotinous species	Geosporous species
Release true seed from fruit	all	many
Release seeds while still contained within fruits (indehiscent fruits – see Box 5.2)	none	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

5.3 Seed testing

5.3.1 Determining the natural germination unit

As the anatomical features of seeds and fruits can significantly affect germination characteristics and seed dormancy status it is important to identify these features and the dispersal characteristics of the parent plant. Seeds and fruits vary immensely in size, shape and structure and in some cases the germination unit of a plant is not always obvious (see Box 3.4). True seeds comprise an embryo and storage tissue (either endospermic

tissue or cotyledons) surrounded by a testa (e.g. *Boronia* spp. Figure 5.1). However, seeds can also be encased in an indehiscent fruit that acts as both the dispersal unit as well as the germination unit (e.g. *Persoonia* spp. – Box 5.2).

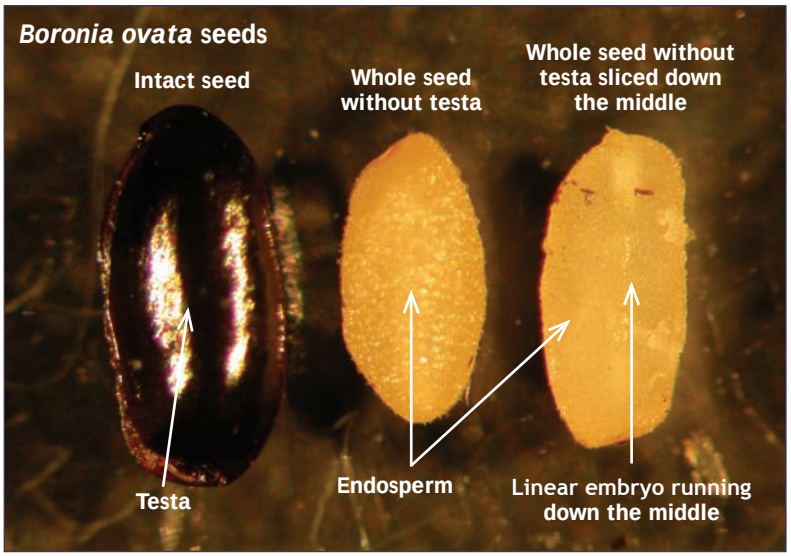

Figure 5.1 *Example of a true seed -* Boronia ovata. *Image: S. Turner.*

Box 5.2

Indehiscent fruits

Commonly encountered indehiscent fruits include those comprising a fleshy outer exocarp/mesocarp surrounding a woody endocarp (e.g. many genera of Ericaceae), and those that are dry, such as an achene (e.g. Asteraceae) or nut (e.g. some Cyperaceae). 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 push through the surrounding tissue.

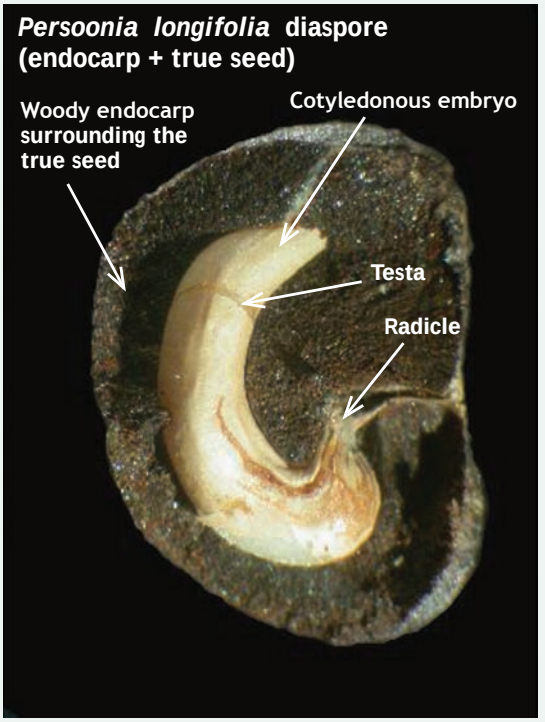

Figure 5.2 *Indehiscent drupe of* Persoonia longifolia *is an example of a diaspore consisting of a true seed + encasing fruit. Image: D. Merritt.*

5.3.2 Imbibition testing

Prior to any germination testing it needs to be determined whether the seeds are capable of imbibing water. If not, the seeds have a water-impermeable seed (or fruit) coat (Section 5.4). These seeds will not germinate under any set of conditions unless first rendered permeable to water. An imbibition test will establish whether seeds can imbibe water (Box 5.3).

Box 5.3

Imbibition test

Replicate batches of dry seeds are weighed and then placed onto moist germination papers. After several minutes (long enough for the seed coat to become wet) seeds are initially removed, patted dry, re-weighed, and placed back onto moist germination papers. Seeds are re-weighed after 1, 2, 4, 8....hrs etc. until there is no further increase in seed weight, or for at least 24 – 48 h. If seeds have water-impermeable seed coats there will be very little increase in seed weight (< 10%). However, if seeds do imbibe then seed weight increases > 30% would be expected (Fig. 5.3). To calculate increases in seed mass the following formula is used:

% increase in weight = [(Wi-Wd)/Wd] X 100,
where Wi and Wd = weight of imbibed and dry seeds, respectively.

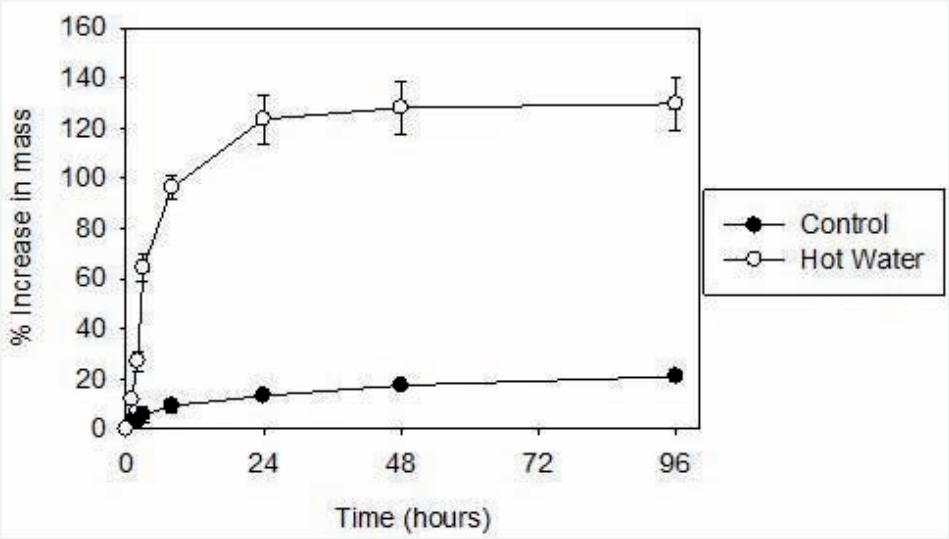

Figure 5.3 *Imbibition curve for* Diplopeltis huegelii *(Sapindaceae) seeds. No significant increase in seed mass is evident in untreated (control) seeds, indicating seeds do not imbibe water and thus possess physical dormancy. Seeds treated with hot water rapidly increase in mass, indicating seeds readily imbibe water and the treatment has removed physical dormancy. Figure from Turner et al. (2006a).*

5.3.3 Initial germination testing

Fundamentally, germination testing is necessary to establish the presence or absence of dormancy and, if present, classify the dormancy type. For dormancy studies it is important to use freshly matured seeds wherever possible as seed dormancy status can change over time in storage and result in difficulties in developing standardised germination protocols. 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 5.4.

Box 5.4

Basic principles of germination testing

To germinate, a non-dormant seed requires exposure to a suitable germination environment consisting of an appropriate temperature and light regime, and ample moisture and oxygen.

Laboratory testing

Where?

Ideally, a germination test should be undertaken in Petri dishes placed in an incubator to ensure a level of accuracy and repeatability between tests.

In/on what?

A number of germination substrates are commonly used in the Petri dishes, including

- filter paper (either cellulose-based or the more inert glass-fibre based),
- solidified water-agar (between 0.7% – 1% w/v),
- washed and sterilised sand.

With what?

These substrates can be irrigated with (or comprise of)

- de-ionised water, or
- solutions containing a germination-promoting compound such as gibberellic acid or smoke products (Section 5.6.2.5).

Temperature regimes?

The incubation temperature should be

- based on indicative habitat temperatures during the wet season, and/or
- on published research on species from similar habitats.

Light regimes?

If complete darkness is required then Petri dishes can be

- wrapped in two layers of aluminium foil, or
- placed inside light-excluding boxes.

Often it is useful to test seeds under a light regime and complete darkness to identify a positive, negative or neutral photoblastic response (i.e. to see if germination is promoted, indifferent or suppressed by light). Even small 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 common regimes being

- 12/12 hours or
- 16/8 hours light/dark.

Seeds can be exposed to

- artificial lighting (cool white fluorescent tubes), or
- incubated in darkness.

Avoiding fungal contamination

For many species fungal contamination can become a problem 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.

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

- operating under sterile conditions in a laboratory (e.g. by using laminar flow cabinets and sterilised water, and flaming forceps and other instruments), or
- by using accredited nursery facilities.

If fungal and bacterial contamination continue to be a problem, consider the use of

- fungicides (such as Previcur) and/or
- Plant Preservation Mixture (http://www.ppm4plant-tc.com) may be considered.

Nursery testing

As an alternative to laboratory germination testing seeds can, of course, also be germinated in soil in a nursery. Important considerations in the nursery include

- seed burial depth (generally only 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

Box 5.4 continued

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 time of year at which they naturally germinate to ensure temperatures are not too hot or cold. Do not discard pots if seeds fail to germinate. The seeds may possess physiological dormancy and if the pots are left to experience natural seasonal changes in temperature and drought they may germinate during the next wet season.

Recording results

Record germination number and express as a percentage of the total sampled e.g. 18/25 seeds = 72%. This could also be expressed as the number of seeds germinated per number of viable seeds in the sample (determined after the germination test) e.g. 18/20 viable seeds = 90%.

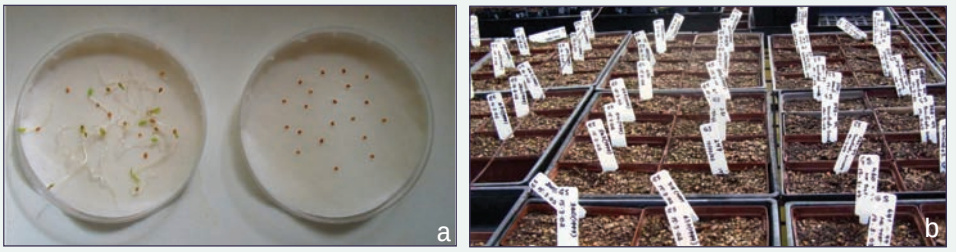

Figure 5.4 *(a) Laboratory testing: Germination of* Spyridium globulosum *on seed germination papers in Petri dishes. (b) Nursery testing: Soil germination under nursery conditions for a range of native species. Images: S. Turner.*

5.4 Seed dormancy types

If seeds do not germinate readily within 4 – 6 weeks when incubated under suitable conditions, they are either non-viable, require a germination stimulant, or are dormant. At present our understanding of the seed dormancy mechanisms and germination requirements of many Australian plants is limited. Even many dominant plant families produce seeds with poorly-known germination requirements (e.g. Cyperaceae, Dasypogonaceae, Dilleniaceae, Ericaceae, Restionaceae and Rutaceae). However, it is well established that dormancy blocks or interrupts the germination process by various physical and/or physiological means along the sequence of events from seed imbibition of water to seedling emergence (Baskin and Baskin, 1998). Seed dormancy is either imposed by the embryo or by the seed coat or outer coverings, with two basic states of dormancy recognised – primary and secondary dormancy.

Primary dormancy is imposed whilst seeds are maturing on the parent plant. Secondary dormancy refers to seeds which are released from primary dormancy after being shed from the parent plant, but then re-enter dormancy due to exposure to unfavourable environmental conditions (Baskin and Baskin, 1998; Black et al., 2006). Five classes of seed dormancy are currently recognised (Baskin and Baskin, 2004a).

5.4.1 Physical dormancy (PY)

Physical dormancy is imposed by seed coverings (the seed coat or fruit) that are impervious to water. Physical dormancy can be broken by:

- Scarification of the impervious coat by precision nicking to create a small hole in the seed coat (e.g. using a scalpel) (see Section 5.6.2.4).
- Hot-water pre-treatment (see Box 5.6).
- Concentrated acid treatment.

In nature, seed coats are thought to become water permeable by repeated heating and cooling over many months, and sometimes years, in the soil seedbank. Physical dormancy is known within 16 plant families world-wide, including many which are common in Australia, such as Convolvulaceae, Malvaceae, Fabaceae, Rhamnaceae and Sapindaceae.

5.4.2 Physiological dormancy (PD)

Physiological dormancy is the most common dormancy form world-wide and is most easily distinguished from physical dormancy by the water-permeable nature of the seeds. It is caused by low growth potential of the embryo, and a mechanical restriction to embryo growth imposed by the surrounding tissues (e.g. endosperm, seed or fruit coat). The embryo must be cued to increase its growth potential and allow penetration of the surrounding tissues.

Physiological dormancy may be overcome by:

- Moist stratification in warm (≥ 15˚C) or cool (0 - 10˚C) temperatures for weeks or months prior to incubation at cooler or warmer temperatures (see Section 5.6.2.2).
- A period of dry after-ripening prior to incubation (see Section 5.6.2.1).
- Treatment with gibberellic acid.

5.4.3 Morphological dormancy (MD)

Morphological dormancy refers to seeds in which the embryo is not fully developed at the time of seed dispersal. Species of the Apiaceae family, for example, are known to possess morphological dormancy. For these seeds to germinate the embryo requires time to mature and grow following imbibition. This generally occurs within a month of incubation.

5.4.4 Morphophysiological dormancy (MPD)

Seeds with morphophysiological dormancy possess both morphological and physiological dormancy. In these seeds the embryos in mature seeds are underdeveloped and also possess a physiological constraint to their growth. Species of Dilleniaceae, Haemodoraceae, Stylidiaceae and Ranunculaceae have morphophysiological dormancy. The embryos require:

- Exposure to appropriate conditions to alleviate embryo dormancy.
- Suitable conditions to allow embryo maturation and growth inside the seed prior to germination.

5.4.5 Combinational dormancy (PY + PD)

Seeds demonstrating combinational dormancy possess a seed or fruit coat that is impervious to water, plus a physiologically dormant embryo. Some species within the 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.

Box 5.5

Key for dormancy classes (Baskin and Baskin, 2004a)

1. Seed/fruit coat not permeable to water, embryo fully developed..... 2

   2. Germination occurs within about 2 weeks (usually within a few days) when seed/fruit coat is scarified ..... PHYSICAL DORMANCY

   2. Germination does not occur within about 2 weeks (usually not even within a somewhat longer period of time) after seeds/fruit coat is scarified, although seed becomes fully imbibed within a few hours following scarification..... .....COMBINATION OF PHYSICAL AND PHYSIOLOGICAL DORMANCY

1. Seed/fruit coat permeable to water; embryo either fully developed or underdeveloped.....3

   3. Embryo not differentiated, or if differentiated it is underdeveloped (small) ...4

      4. Embryo not differentiated .....SPECIALIZED TYPE OF MORPHOLOGICAL DORMANCY

      4. Embryo differentiated but underdeveloped (small).....5

         5. Embryos in freshly-matured seeds begin to grow (elongate) within a period of a few days to 1-2 weeks, and seeds germinate within about 30 days..... MORPHOLOGICAL DORMANCY

         5. Embryos in freshly-matured seeds do not begin to grow within a period of even a few weeks, and seeds do not germinate within 30 days ....MORPHOPHYSIOLOGICAL DORMANCY

   3. Embryo differentiated and fully developed (elongated)..... 6

      6. Seeds do not germinate within about 30 days.....PHYSIOLOGICAL DORMANCY

      6. Seeds germinate within about 30 days.....NON-DORMANT

5.5 Seed dormancy classification

Classification of dormancy types for Australian species has received little attention. However, a recent review of seed dormancy in Australian species suggests the most common forms of dormancy are likely to be physical dormancy and physiological dormancy (Baskin and Baskin, 2003). Understanding the type of dormancy a species has is useful for identifying 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 5.5.

5.6 Approaches to overcoming dormancy in Australian species

Dormancy classification is a useful first step to eliciting germination of a 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. Species differ in the complexity of treatments required for dormancy loss and the rate at which this occurs. Germination experiments may vary from a few weeks or months duration, to one or two years or more. In the case of taxa that have deeply dormant seeds, it is often useful to develop a greater understanding of ecological aspects of dormancy induction/loss in situ to pinpoint key factors influencing dormancy status. Laboratory experiments can then be designed to develop reliable ex situ germination. Careful experimental design, documentation and patience is necessary. Dormancy can sometimes be alleviated by more than one factor, and/or seeds may require exposure to multiple cues to become germinable.

5.6.1 Alleviating physical dormancy

To determine whether a species has physical dormancy, an imbibition test (Section 5.3.2, Box 5.3) is conducted. If imbibition testing confirms the presence of physical dormancy there are several options to removing the barrier to water uptake. For small numbers of seed, scarification is possible. Hot water treatment (85 – 100°C for 15 seconds to 5 minutes) is suitable for large numbers (Box 5.6), as is exposure to concentrated acid (98% v/v H_2SO_4) for several minutes to many hours (Turner and Dixon, in press).

5.6.2 Alleviating physiological and morphophysiological dormancy

There are several methods for promoting germination of seeds with physiological or morphophysiological dormancy. Dormancy may be alleviated in these species via dry after-ripening, stratification of imbibed seeds at warm or cool temperatures, or combinations of these treatments. Precision nicking (Figure 5.7, Case Study 5.1) and/or the use of germination promoting compounds in combination with these treatments is often necessary. For these species an understanding of the time of year and environment into which the seeds are shed is vital to selecting treatments. For example, seeds from mediterranean and arid climatic zones that are shed into dry soil during the hot temperatures of late spring and summer often require weeks or months of after-ripening. Seeds may also

Case Study 5.1

Overcoming dormancy in Australian Mint Bushes

The genus *Prostanthera* belongs to the Family Lamiaceae (Labiatae), and consists of approximately 100 known species endemic to Australia. Commonly known as Mint Bushes, many species are well known in the horticultural industry for their fast growth and spectacular appearance when flowering (Figure 5.5a). However, germination has proved difficult for many species, and the ability to propagate plant material from seed for restoration, conservation or horticultural purposes has been limited.

Fruit of *Prostanthera* consist of four mericarps enclosed by a persistent calyx, with individual seeds characterised by the presence of a 'mericarp plug' (Figure 5.5b). Research undertaken at the Botanic Gardens of Adelaide has focused on understanding the role that the plug structure plays in seed dormancy in the genus. Contrary to previous reports, seed imbibition tests (refer to Section 5.3.2) showed that the seed coat was not impeding water uptake, and that physical dormancy (refer to Section 5.4.1) was not present in the genus. Subsequently, a technique was developed whereby seeds are soaked overnight in water, after which the mericarp plug is excised using a scalpel (Figure 5.5b). Due to the relatively small size of the seeds, the mericarp plug is excised under a microscope. Care is needed to ensure that the embryo is not damaged during the plug removal process. Using this approach, germination levels (when compared to untreated control seeds) improved up to 4-fold (Figure 5.6). A pulse dry heat treatment (80°C for 10 minutes) also improved germination, although this was to a lesser extent, with the effectiveness of this treatment varying between species, and being dependent on the thickness of both the seed coat and mericarp plug.

It is thought that the mericarp plug acts as a mechanical barrier preventing germination (physiological dormancy; refer to Section 5.4.2). Under natural field conditions, this dormancy mechanism would be overcome by either fire (similar response to pulse dry heat) or a wetter than average autumn/winter, whereby the attachment mechanism of the mericarp plug is weakened allowing germination to occur. — ***Phil Ainsley***

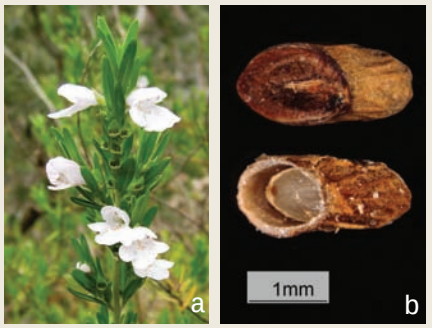

Figure 5.5 *(a) Downy Mint Bush (*Prostanthera behriana*) in flower. (b) Seed of the Monarto Mint Bush (*Prostanthera eurybioides*) with mericarp plug intact (top) and excised (bottom).*

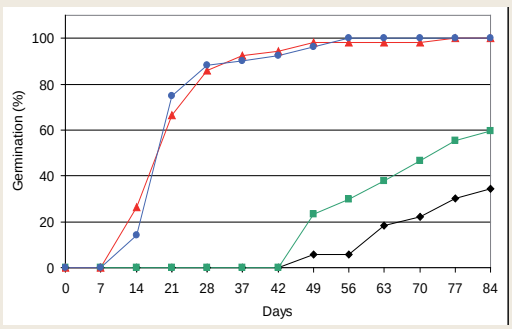

Figure 5.6 *Germination frequency of seed from the West Coast Mint Bush (*Prostanthera calycina*) following various pretreatments (♦ = control; ■ = pulse heat; ▲= plug dissection; ●= pulse heat + plug dissection). Seeds were incubated at 10/22°C with a 12 hour photoperiod.*

Box 5.6

Hot water treatment to break physical dormancy

Perhaps the easiest treatment to use, hot-water ruptures a small region in the seed coat known as the water gap, allowing water entry. Hot-water is only applicable to species with physical (or combinational) dormancy. No other dormancy type appears to be overcome with hot-water treatment. 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 5.6.2.1) or stratification (Section 5.6.2.2) will suffice.

Method: Generally a water temperature greater than 90°C is used, but cooler temperatures can also be effective. Boiling water should be avoided, 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 3 minutes, depending on the species, the seed size and the hardness of the testa.

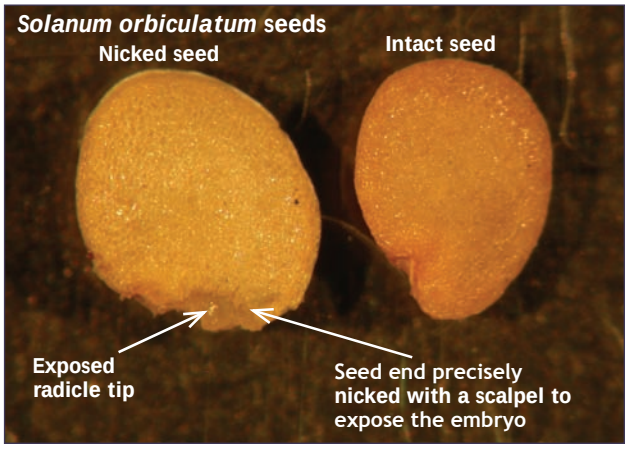

Figure 5.7 *Example of precision nicking to overcome physiological dormancy as a means of reducing the mechanical restriction of the surrounding endospermic and testa tissues on the embryo. Image: L. Commander.*

respond to warm stratification; conditions analogous to those experienced during the first rains of autumn. In contrast, many species originating in cooler temperate climates (common in the northern hemisphere and temperate and alpine Australia) respond to cold stratification, as the seeds over-winter in the snow and germinate in spring.

5.6.2.1 After-ripening

A dry after-ripening requirement for dormancy loss appears to be common in the Australian flora, with many species of Apiaceae, Asteraceae, Brassicaceae, Haemodoraceae, Poaceae, Solanaceae and Stylidiaceae responding to this treatment. Seeds that after-ripen do so more rapidly at warmer temperatures, and are usually placed at 20 - 30°C to facilitate dormancy

loss. In some cases even higher temperatures (e.g. ≥ 40°C) can be used to accelerate the after-ripening process, although more care must be taken to moderate seed water content to prevent rapid seed ageing. Short periods of very high temperatures (80 – 120°C for up to 3 hours) can be useful for some species with an after-ripening requirement. This treatment is known to be effective in species of Anthericaceae, Haemodoraceae, Poaceae, Restionaceae and Stylidiaceae (Tieu et al., 2001b; Merritt et al., 2007; Turner et al., 2009).

The after-ripening treatment can be as simple as storing seeds on a laboratory bench at room temperature (c. 23°C) for a number of weeks or months. At regular intervals seeds can then be tested for germination at different incubation temperatures, with and without germination promoting compounds.

During the after-ripening treatment, some control over seed moisture content may also be desirable, as there is often a narrow range of seed moisture contents conducive to after-ripening (Black et al., 2006; Turner et al., 2009). Factorial experiments testing germination of seeds stored at different water contents (eRH of c. 15 – 50% - see Section 4.3.2) and temperatures are useful to identify conditions that will alleviate dormancy in the shortest time.

5.6.2.2 Stratification

Stratification refers to the moist incubation of seeds at warm (≥ 15°C) or cool (0 - 10°C) temperatures, prior to incubation at an optimal germination temperature, and is a technique known to alleviate physiological and morphophysiological dormancy (Baskin and Baskin, 1998). Whilst this technique has not yet been extensively applied to the Australian flora, recent studies have demonstrated the applicability of stratification to a range of Australian families, including several previously considered 'deeply dormant'. Several south-west Western Australian species, including those of Dasypogonaceae, Iridaceae and Pittosporaceae respond to a short period (6 wks) of warm (> 25°C) stratification (Merritt et al. 2007; Turner et al. 2006b). Cool stratification (at 5 – 10°C) has been shown to be effective for some species of *Eucalyptus*, *Allocasuarina* (Beardsell and Mullett, 1984; Moncur et al., 1997) and *Wollemia nobilis* (Offord and Meagher, 2001).

One approach to testing stratification is to use a 'move along' experimental design (Baskin and Baskin, 2004b) (Table 5.2). Seeds are placed at a range of constant or alternating temperature regimes indicative of different seasons. Example temperatures for temperate regions of Australian are 5/15°C (winter), 15/25°C (spring/autumn), and 20/35°C (summer). In a 'move-along' experiment seeds are moved every 4 – 8 weeks starting at cooler (e.g. 5/15°C → 15/25°C→20/35°C → 15/25°C → 5/15°C…) or warmer (e.g. 20/35°C → 15/25°C → 5/15°C…) temperatures. The experiments can be run concurrently to test if seeds respond to particular seasonal changes. If seeds respond to stratification then increased germination should be observed after seeds are moved in one direction or another (relative to the control seeds incubated at one temperature regime for the duration of the experiment). It may take two or more cycles to alleviate dormancy in some species. Additionally, seeds may be either incubated under light/dark conditions (12 hr/12 hr) or constant dark conditions during the

move-along experiment as both have been shown to interact with photoregimes and either promote germination or suppress germination. Unless prior knowledge is known about the response of the study seeds to different light conditions it is recommended to use a 12 hr/12 hr light/dark regime to begin with.

A more simple approach to stratification is to incubate imbibed seeds at a single stratification temperature (e.g. 5˚C or 25˚C) for 4 – 12 weeks, and then move the seeds to a second temperature appropriate to germination (e.g. 15°C).

Table 5.2. *'Move along temperatures'. Proposed experimental approach for determining whether seeds respond to seasonal changes (modified from Baskin and Baskin, 2004b). Temperatures are indicative only and represent conditions approximating winter (5/15°C), spring/autumn (15/25°C) and summer (20/35°C) in temperate and mediterranean regions of Australia.*

	Incubation temperatures	
Incubation time (week number)	Control treatments (°C)	'Move along' treatments (°C)
0	5/15 15/25 20/35	5/15 20/35
6	5/15 15/25 20/35	15/25 15/25
12	5/15 15/25 20/35	20/35 15/15
18	5/15 15/25 20/35	15/25 5/25
24	5/15 15/25 20/35	5/15 20/35
30	5/15 15/25 20/35	15/25 15/25
36	5/15 15/25 20/35	20/35 5/15
42	5/15 15/25 20/35	15/25 15/25
48	5/15 15/25 20/35	5/15 20/35

5.6.2.3 Wetting and drying cycles

Another approach that combines the concepts of after-ripening and stratification is the use of alternating wetting and drying cycles. In these experiments, dry seeds are incubated at warm temperatures punctuated with periods of hydration (e.g. imbibed for 24 hours and then re-dried) every week or two. This appears to mimic natural hydration/dehydration cycles 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).

5.6.2.4 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 (Figure 5.7, Case Study 5.1). For species with physiological or morphophysiological dormancy precision nicking can remove the

mechanical restriction imposed on embryo growth by the surrounding tissues. The location and depth of nicking is species specific, though as a general rule the testa needs to be breached and the nicking undertaken at or near the radicle end of the seed. Care must be taken not to damage the embryo. This technique is also very effective for species with physical (or combinational) dormancy as it overcomes water impermeability.

5.6.2.5 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 karrikins), the plant growth regulator gibberellic acid, and nitrate

Smoke - a germination stimulant

Germination of seeds with physiological, morphophysiological, or occasionally physical dormancy, is promoted by smoke. It is important to note that in many seeds the smoke response is complex and seed sensitivity to smoke varies depending on seed dormancy status. Thus, germination promotion by smoke may only be achieved after seeds have been exposed to appropriate dormancy-breaking treatments (e.g. after-ripening or stratification) (Merritt et al., 2007). Whilst the active component in smoke (karrikinolide) has now been artificially synthesised (Flematti et al., 2005), it is yet to be made commercially available. See Box 5.7 for details on production and use of smoke.

Gibberellic acid (GA) – another germination promoting compound

Another commonly used compound to promote germination is the plant growth regulator gibberellic acid (GA). Gibberellins promote germination of a wide range of Australian species (Bell, 1999), in many cases promoting germination of dormant seeds with no other treatment. Seeds with physiological or morphophysiological dormancy can respond to gibberellic acid.

Whilst there are many types of gibberellins, the most commonly used and readily available form is GA_3. This gibberellin can be purchased in commercial volumes from nursery supply companies and can be used at concentrations ranging from around 5 mg/L - 10 g/L (w/v). Whilst effective concentrations are species specific and high concentrations can suppresses germination of some species, a concentration of between 5 mg/L - 1 g/L should indicate whether a species is GA responsive. Seeds can either be soaked for 24 - 48 hours in a solution of GA prior to sowing, or incubated in Petri dishes irrigated with the GA solution. Gibberellic acid often replaces other dormancy-breaking treatments such as after-ripening and stratification, and may substitute for smoke application or exposure to light.

Box 5.7

Production and use of smoke

- Crude smoke products are produced from the combustion of plant material. Straw is the easiest material with which to produce smoke.
- Smoke can be applied in an aerosol form using a smoke tent (Figure 5.8). Smoke generated from a small fire is pumped into the tent using an electric pump and piping.
  - ➢ Smoke in this form is usually applied to dry seeds (placed out on trays), soil-sown seeds (punnets) or filter papers onto which seeds can be later sown. An exposure time of one hour should give good results.
- Smoke can also be applied as an aqueous solution.
  - ➢ To create a smoke water solution, smoke is pumped into water for one hour to make a smoke concentrate (Dixon et al., 1995).
  - ➢ This concentrate is then ready for use at a standard dilution of 1:10 (v/v), although dilutions of 1:100 and 1:1000 can also be effective.
  - ➢ The best way to apply smoke water is to soak the seeds for 24 – 48 hours prior to sowing in soil or in Petri dishes irrigated with water.
- Incubating seeds on Petri dishes irrigated with smoke water is not advisable as crude smoke water contains many toxic compounds. Using too strong a concentration or too long an exposure time can be detrimental to germination.
- Other ways to apply smoke include the use of commercially available perlite or vermiculite-impregnated smoke products. Seeds can be sown into these carriers and placed in soil punnets.

Figure 5.8
In situ use of a smoke tent for mine site restoration in Banksia *woodland, Western Australia. Image: Deanna Rokich.*

compounds. Smoke products are of particular interest to Australian species. Smoke has been found to have a stimulatory effect on the germination of at least 95 genera of Australian plants (Merritt and Dixon, 2003).

5.6.3 Seed burial experiments

For many native species dormancy is a considerable impediment to ex situ germination and one that is not easily overcome. It is apparent that many species require several seasons or years in the soil seedbank before dormancy is lost (Tieu et al., 2001a; Baker et al., 2005; Ooi et al., 2007). It should also be recognised that within any population of seeds, individual seeds may lose dormancy at different rates – a strategy to increase the chances of seedling recruitment, but one that can create difficulties in the laboratory or nursery. For many Australian genera in families such as Cyperaceae, Dilleniaceae, Ericaceae and Restionaceae, germination in the laboratory is difficult. Species in these families are thought to require a complex sequence of cues for dormancy break and germination stimulation.

In such cases soil burial experiments and studies of germination ecology in the field can assist in identifying the cues seeds require to break dormancy. See Box 5.8 for details of seed burial experiments and Case Study 5.2.

Case Study 5.2

In situ seed burial aids understanding the germination requirements of a nationally threatened tree species

The Slender Bell Fruit (*Codonocarpus pyramidalis*) is a nationally threatened tree species (Figure 5.9a) restricted to the arid zone of eastern South Australia. Getting its name from the shape of its fruit (Figure 5.9b), this species belongs to the fire responsive family Gyrostemonaceae. It is thought that alteration of fire regimes

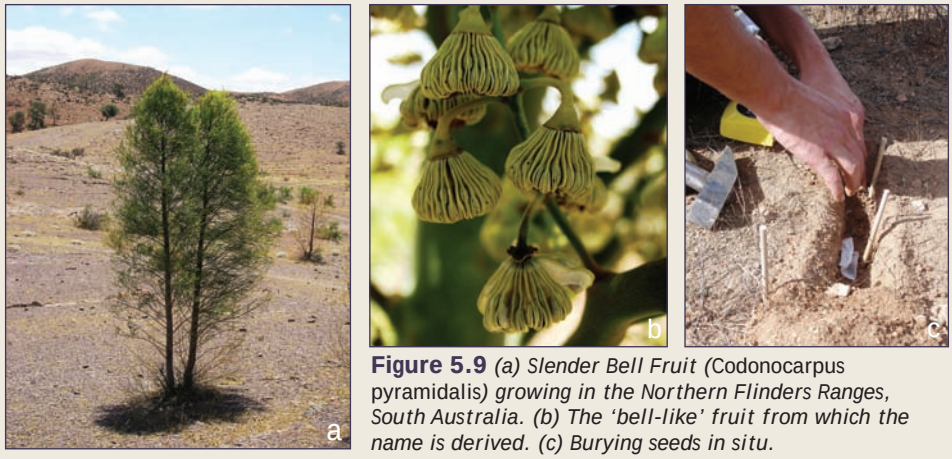

Figure 5.9 *(a) Slender Bell Fruit (*Codonocarpus pyramidalis*) growing in the Northern Flinders Ranges, South Australia. (b) The 'bell-like' fruit from which the name is derived. (c) Burying seeds in situ.*

and vegetation structure in its natural environment since European settlement has resulted in its decline.

Preliminary research conducted under laboratory conditions using freshly collected seed, tested a series of fire related treatments including aerosol smoke and dry heat. Whilst it was possible to generate a limited germination response with freshly collected seed, reproducibility of the result proved difficult. In an effort to understand why this was happening, an in situ seed burial experiment (Figure 5.9c) was set up in the northern Flinders Ranges in South Australia. Seeds were buried in nylon bags approximately 25 mm below the soil surface, with environmental conditions (soil temperature and rainfall) recorded for the duration of the experiment. Every 3 months a fraction of the buried seeds were extracted and returned to the laboratory where they were exposed to a range of treatments including pulse dry heat (90°C for 10 minutes), cooled aerosol smoke (15 minutes in a smoke tent) and combinations of both treatments. Results showed that fresh seed required a 6 month after-ripening period before becoming responsive to the range of germination cues being tested (Figure 5.10). Whilst a steady increase in germination response to dry heat was observed over subsequent extractions (peaking at 80% in seeds buried for 12 months), the response to cooled aerosol smoke and the combined treatment of heat followed by cooled aerosol smoke was more variable (Figure 5.10). Results proved that the Slender Bell Fruit is a fire responsive species and that fire would play a key role in the natural recruitment process.

In situ seed burial provided a unique opportunity to expose seeds to variations and extremes in natural environmental conditions that are extremely difficult to simulate ex situ. It also highlighted that seed response to germination cues varies depending on the physiological state of the seed, and how under natural field conditions, seeds respond differently to different cues at different times of the year. This information has informed management plans for this species. — ***Phil Ainsley***

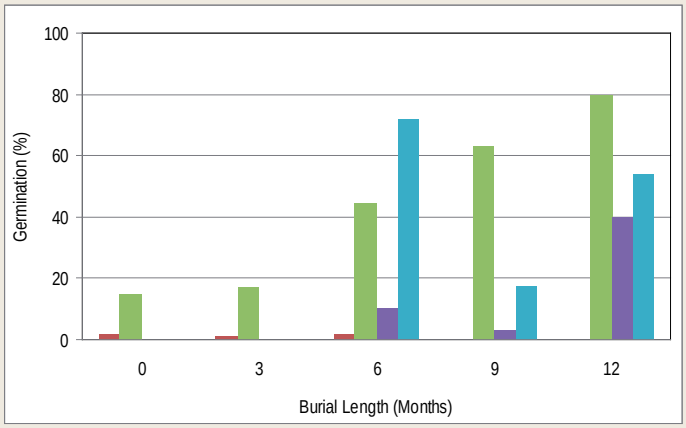

Figure 5.10
Germination response in seeds of Codonocarpus pyramidalis *buried over 12 months following various germination treatments (■ = control; ■ = 90°C for 10 minutes; ■ = 15 minutes aerosol smoke; ■ = 90°C for 10 minutes followed by 15 minutes aerosol smoke). Seeds were incubated at 10/22°C with a 12 hour photoperiod for 100 days.*

Box 5.8

Seed burial protocol

After assessing seed viability and germination under standard laboratory conditions:

1. A known number of seeds are sealed in porous bags (e.g. 0.75 µm nylon mesh) containing sand.
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 data logger can be buried at the same time to document soil temperatures and allow identification of critical temperatures 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).
5. Once germination has been attained, temperature and rainfall (or soil moisture) data can be used to assess the environmental cues the seeds received during burial, and prior to germination/dormancy break.
6. These factors can then be incorporated into future ex situ germination treatments.

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Chapter 6

Tissue culture

Catherine A. Offord, Eric Bunn, Shane R. Turner, Karen D. Sommerville, John Siemon and Sarah E. Ashmore

6.1 Introduction

For ex situ plant conservation, tissue culture is often seen as the first resort for multiplication of highly threatened taxa. It is rare that large numbers of genotypes can be conserved using this technique and it is most suited to conservation of very rare species that have poor seed set, low germination or problematic storage requirements. Therefore, it is often considered a technique complementary to broader conservation strategies for threatened plants (Bunn et al., 2007) and can be used in combination with other ex situ conservation techniques such as seed storage (Chapter 4), cryopreservation (Chapter 7) and living plant collections (Chapter 8). The specialised techniques described in this chapter have been used in the conservation of a number of highly threatened Australian species.

The growing of plants or plant parts in vitro is commonly referred to as 'tissue culture' and, in the case of propagation of whole plants, 'micropropagation'. In vitro literally means 'in glass', and refers to the culture of cells, organs, seeds or other plant parts in a sterile environment inside a test tube or flask (Figure 6.1). Often these plant parts, known as 'explants', do not have functional roots; the nutrients required for growth are supplied to the tissue via a support medium such as agar or in liquid culture. Plant growth regulators (PGR), chemicals that mimic natural plant growth hormones, may be added to the support medium, in precise concentrations and combinations, to elicit a particular type of growth. For example, cytokinins may be added to stimulate shoot multiplication, gibberellins to promote shoot elongation, and auxins to initiate root formation.

6.2 Benefits and risks of tissue culture

Tissue culture is a useful technique for replicating large numbers of plants for specific purposes, where genetic variability is not a major consideration, in some types of revegetation programs, and for propagation of desirable cultivars for horticulture. It is also useful for maintaining base collections of important vegetative material in a small space. Plant tissue culture relies on the concept of totipotency; that is, the ability of a living plant cell to produce an entire new plant. Theoretically, it is possible to grow entire

new plants from root cells, leaf cells, pollen cells, and even petal cells. Tissue culture is a very effective way of producing large numbers of plants rapidly; for example, up to 15 million Kangaroo Paws (*Anigozanthos* spp.) can be produced over 12 months from one starting plant (Stewart, 1999).

Tissue culture for plant production and storage purposes is a highly specialised area that requires specific skills and equipment. Techniques and procedures may need to be developed and optimised for a given species, which might take considerable time and effort. Trained personnel are required to perform most tissue culture procedures as the manipulation of material must be carried out in sterile conditions. Therefore, tissue culture is usually carried out in a laboratory or specialised nursery, though fern and epiphytic orchid cultures are commonly produced in less specialised environments. Australian health and safety standards must be adhered to when applying these procedures as the chemicals and equipment used may pose a health hazard.

It is often necessary to develop complex protocols for tissue culture that may involve varying nutrients, light levels, plant growth regulators, and other growth factors (see for example Bunn et al., 2005). It is also common for different genotypes of a species to exhibit a range of responses to the various phases of culture. Some species, or individual genotypes, cannot be established in culture (at least within a reasonable time frame) and are therefore unsuitable for this technique. Unfortunately, this recalcitrance may not be apparent for some time and after much trial and error.

In vitro culture systems can lead to genetic changes known as somaclonal variation, which can be the result of either heritable genetic mutations or epigenetic changes (not transmitted to the next generation) (Ashmore, 1997). Somaclonal variation is mainly associated with callus cultures (i.e. undifferentiated cells) or embryogenic suspension cultures, but is uncommon using direct micropropagation of shoot material (e.g. shoot apices, nodal cuttings) (Reed et al., 2004).

One of the most difficult stages of tissue culture is the transition out of in vitro culture and back into the ex vitro environment (Barlass and Hutchison, 1996). This is particularly the case for many woody species. 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 manipulation 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 the reestablishment of leaf cuticle integrity.

6.3 When should tissue culture be used?

For the reasons given in Section 6.2, and because of the costs involved, other propagation techniques such as seed or vegetative cuttings should be considered before tissue culture is attempted for conservation purposes (see Box 2.1 and Box 6.1). Quite often, it is necessary

to establish plants from seed or cuttings before attempting tissue culture in any case as wild-grown plant material may be unsuitable due to heavy microbial contaminant loads or poor plant condition. For many species, the process of plant tissue culture should only be seriously considered when other more accessible options have proved to be impractical because of poor seed set, deep seed dormancy, slow rate of division, low cutting success, the need for specific clonal or elite genotypes, or low remaining numbers of a critically endangered species (Fay, 1994).

Box 6.1

Checklist - Is tissue culture an appropriate option?

Before tissue culture is utilised for any particular plant species, the following issues need to be considered:

- Why does the species need to be propagated?
- What is known about the propagation and cultivation of this species?
- Can it be propagated by another method?
- What are the characteristics of the species to be propagated?
- How many plants are required?
- What type, condition and amount of plant germplasm are available?
- Are technicians with the appropriate skills available?
- Are appropriate resources and equipment available?

There is a vast body of literature relating to tissue culture, reflecting the extent and complexity of its uses, so only a cursory examination of the basic procedures will be given in this chapter. References such as George (1993, 1996) should be consulted for general tissue culture procedures, media formulations, and working concentrations of plant growth regulators. Taji and Williams (1996) and Burchett and Johnson (1996) provide more specific information on, and examples of, the tissue culture of Australian plants. 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.

In vitro techniques are used for the following purposes which may have direct or indirect conservation outcomes:

- Multiplication of genotypes of interest (usually for mass propagation of horticultural cultivars, but also for large scale revegetation of clonal species).
- Propagation and storage of clones of conservation value e.g. where the number of individuals (genotypes) is severely reduced in the wild; movement of endangered species under CITES legislation.

- Propagation and storage of species that are recalcitrant to standard seed storage conditions (e.g. many tropical rainforest species), which do not produce seed (e.g. sterile plants), or which have complex regeneration systems (e.g. ferns).
- Culture of seeds with specific nutritional or other requirements (e.g. aseptic culture of orchids).
- Co-culture of species requiring or preferring the presence of a symbiont (e.g. terrestrial orchids with mycorrhizal associations).
- Somatic embryogenesis for the production of synthetic seeds.
- Embryo rescue, i.e. the culture of embryos which would otherwise abort due to incompatibility of embryo and endosperm.
- Research, for example testing cell, organ, partial or whole plant interactions with introduced factors or genetic manipulations.
- As a substitute for pot or garden collections when space is limited.
- Shipment of material certified free of contaminants, between countries (thus avoiding soil borne diseases).
- Virus eradication.
- As a substitute for cryopreservation where resources or skills in this area are lacking.
- As a source of explants for long-term storage using cryopreservation.

6.4 Types of tissue culture

Virtually any type of plant tissue is theoretically suitable for initiating a tissue culture. However, the most common types of tissue used are buds or shoots taken from established plants, or zygotic embryos taken from seeds. The culture environment determines the type of growth response observed. For example, concentrations of growth regulators and other media components can be manipulated, to stimulate the production of callus (a mass of undifferentiated [parenchyma] cells), which can then be 'turned' into other tissue types such as somatic embryos, new shoots or plant organs e.g. leaves or roots.

The main types of tissue culture systems used are:

- Micropropagation and slow-growth storage (Section 6.4.1).
- Somatic embryogenesis (Section 6.4.2).
- Terrestrial orchid culture (Section 6.4.3).
- Callus cultures.
- Suspension cell cultures.

For further information on in vitro conservation approaches, see Bowes (1999).

6.4.1 Micropropagation

Micropropagation is the most basic plant tissue culture system used to propagate clonal plants. It involves the initiation and multiplication of shoot cultures for plant production which are in many respects treated like miniature cuttings and it is commonly used for many rare woody species (see Case Study 6.1).

6.4.1.1 Initiation of cultures

The success of this phase often relies on the source of explant material. Material straight from the wild is often not suitable due to the large number of associated microbes that can contaminate cultures even after stringent sterilisation. Healthy, pot-grown plants, seed and seedlings are used as sources of explant material (Figure 6.1a). Once the plants for initiation are identified, the target material is made as clean as possible, often by encouraging healthy growth so that the microbial load is low. Suitable branches or stems are then selected and cut to lengths of about 2-5 cm, with each section containing an axillary bud (nodal section). These are thoroughly washed under running water for several hours (the longer the better) to remove surface contaminants. Following washing, the shoots are placed into a sterilant solution (e.g. 1-2% sodium hypochlorite or 70% alcohol, with a drop of detergent) and agitated gently for 5-15 minutes. All subsequent procedures must be performed in a laminar flow cabinet using aseptic techniques to retain the sterility of the samples (Figure 6.1b) and everything that comes into contact with the material must be sterile. Explants are then 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-sterilized and washed again). Washing and sterilising are vitally important procedures as any contaminants (fungi and bacteria) not removed will rapidly overwhelm and destroy cultures placed in growing medium. When using seeds to establish cultures, they must also be sterilised in a similar fashion to shoot explants; once germinated, the seedlings are treated as shoots.

The choice of seeds or extracted embryos over shoots to start a culture may be guided by availability of material, ability of seeds to germinate, and whether the exact genetic material needs to be replicated. Seeds have an advantage over shoots in that they are inherently juvenile and often respond better to culture conditions; however, they may take longer to establish an adult plant ex vitro. Shoot cultures may also revert to a juvenile stage during culture but this is highly variable.

Once the material has been sterilised, nodal sections are placed onto a sterile tissue culture medium which has been autoclaved at 103.4 kPa at 121°C for 15 min. The tissue culture medium contains a variety of components that can be modified to suit individual species and specific outcomes. A typical tissue culture medium consists of:

- A nutrient formulation e.g. Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), which act as a complete inorganic nutrient source (like fertiliser);
- Vitamins, a few of which are essential for healthy growth;
- Sucrose (or other sugar), which provides energy to the explant;
- Plant growth regulators, which shape the plant's growth responses by mimicking natural hormones; and,
- Gelling agent (except in the case of liquid culture) e.g. agar, which binds the various components together and provides a suitable substrate to support growth of 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).

Case Study 6.1

*Micropropagation of Tree Waratah (*Alloxylon flammeum*)*

Alloxylon flammeum (Tree Waratah) is a rare and threatened Australian rainforest species that features spectacular red flowers and contrasting dark green foliage giving it potential as a cut flower crop. It also has potential as a rootstock for difficult to grow tree waratah species. *A. flammeum* can be propagated by vegetative cuttings and in vitro (Donovan et al., 1999). Pre-treatment of *A. flammeum* stock plants with gibberellin (A_4 and A_7) spray before initiation into culture increased initiation success. The use of tip material resulted in minimal explant contamination and 1% sodium hypochlorite for 15 min was adequate for surface disinfection of material. Well elongated shoots were multiplied using BAP (6-benzylaminopurine) or 2iP (6-dimethylallyaminopurine [isopentyladenine]), whilst TDZ (thidiazuron)-cultured shoots were numerous but short and unusable. Plantlets were established using IBA in the culture medium to promote root growth prior to exflasking into a high humidity glasshouse environment.

Figure 6.1 *(a) Harvesting material for culture initiation using axillary buds (b) Tissue culture transfers are made under sterile conditions in a laminar flow cabinet. Cultures are incubated under controlled temperature and light conditions. (c) Axillary shoot culture of* Alloxylon flammeum *(d) Acclimatised plantlets of* Alloxylon flammeum, *(e) root development on explants of* Alloxylon flammeum. *Images: (a-c) J. Plaza Botanic Gardens Trust, Sydney, (d-e) P. Meagher Botanic Gardens Trust, Sydney.*

6.4.1.2 Shoot multiplication

To encourage shoot multiplication, the explants are usually placed onto a medium containing a cytokinin such as kinetin (N-(s-furanylmethyl)-1H-purin-6-amine) or BAP (6-benzylaminopurine). Cytokinins tend to suppress apical dominance, and encourage rapid development of axillary shoots, if applied at suitable concentrations in the culture medium. Once the explants have been established, they can be subcultured (subdivided under sterile conditions) every 3-4 weeks. The resulting plantlets can be placed onto fresh multiplication medium where they will continue to grow and multiply rapidly. Over-multiplication should be avoided as somaclonal variation may occur. Hyperhydricity (glassy/watery shoots) can lead to poor re-establishment of plants. Often this can be controlled by manipulation of the growing environment within the culture vessel, through increasing the amount of agar or sucrose, decreasing the concentration of cytokinin or reducing humidity by growing in a vessel with a vented lid.

6.4.1.3 Root initiation

Once the plantlets are of a sufficient size, they can be moved onto a root induction medium. This involves moving plantlets onto fresh medium devoid of plant growth regulators (which can stimulate rooting in some species such as kangaroo paws because of the natural endogenous hormone levels) or, more commonly, onto a medium supplemented with an auxin such as IBA (indole-3-butyric acid) or NAA (1-naphthaleneacetic acid) that actively stimulates adventitious root production. Auxin application may be chronic i.e. applied at low concentration over a number of days or weeks, or acute i.e. applied for minutes or hours at high concentrations, usually as a liquid overlay on the medium or as a concentrated dip. Root initials should appear within a few weeks. Care should be taken to encourage root formation on the shoots and not on callus growth as, for most species, a vascular connection is essential to the success of the next phase.

6.4.1.4 Removal and hardening-off

Once the explants have initiated roots they can be gently removed from the agar (use warm water to remove all agar) or other support medium and potted into an open propagation mix (e.g. seed raising mix with added perlite). The plantlets should then be placed into a controlled environment with high humidity (>90% RH), such as a greenhouse with misting, shading and, preferably, temperature control.

A variation on this procedure is to 'direct root' the microcuttings which bypasses the final in vitro rooting stage. This involves applying an auxin to the microcuttings, placing them into propagation mix, and growing them in a high humidity environment.

The plantlets are initially sensitive to desiccation stress as they tend to possess incomplete or reduced waxy cuticles on their leaves, and have a limited ability to regulate water loss through stomatal opening and closing. As explants establish, air movement is essential to prevent fungal diseases like botrytis which easily take hold in still, humid conditions.

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 seed or cuttings.

Slow growth storage

Reduced growth (slow growth) storage is a method of maintaining plant cultures through alteration of the chemical composition of the culture medium, and/or a lowering of culture temperature to 4 - 15°C to reduce the growth rate and extend the subculture period from six weeks to up to two years. For information on slow growth methods, readers are referred to Englemann (1991), Ashmore (1997) and Touchell and Dixon (1999). Examples of large collections held in tissue culture are the world banana and cassava collections held respectively at Bioversity's International Transit Centre (ITC) in Belgium and CIAT (International Center for Tropical Agriculture) in Columbia. There is scope for greater use of tissue culture conservation of Australian species, particularly for woody subtropical and tropical taxa.

6.4.2 Somatic embryogenesis

Somatic embryogenesis describes the process of inducing zygote-like 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 zygotic embryo. 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 is capable of producing 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 soils.

6.4.2.1 Production of somatic embryos

The most common way to induce somatic embryogenesis is to apply the synthetic auxin analogue (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 1970's – 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-medium 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).

Case Study 6.2

Translocation of tissue-cultured Corrigin Grevillea (Grevillea scapigera)

The Corrigin Grevillea is a critically endangered species that was once presumed extinct. By 1986, the last known remaining plants were held at the Royal Botanic Gardens, Sydney (NSW). One of these plants was sent to Kings Park and Botanic Garden (Perth, WA) in 1989 for propagation. Material from this plant was successfully established in tissue culture (Figure 6.2a) and, by 1992, many micropropagated plantlets had been produced. Further plants had been found by this time in the West Australian (WA) wheat belt (near the locality of the last recorded plant) and a program to bring all the known plants into tissue culture was launched. By 1994 this was achieved and by 1996 the Corrigin Grevillea had become the first rare and threatened species in WA to be returned to field sites in its natural habitat via micropropagation (Figure 6.2b). Further introductions of micropropagated plants were made in 1998, 1999 and 2000 in other sites. Up to 90% of translocated plants survived the first 12 months following planting out. The majority of these flowered (92% in 1995) and seeded after one year, but usually with only a few fruit (I.R. Dixon and M. Rossetto, pers. comm.). The reintroduction program has been very successful and hundreds of plants have now been introduced back into the wild. The Corrigin Grevillea is now recruiting naturally (Figure 6.2c) and is more secure than thought possible back in 1986 (Bunn and Dixon, 1992).

Figure 6.2. *(a)* Grevillea scapigera *growing in vitro. Image: E. Bunn. (b) Translocated* Grevillea scapigera *derived from tissue cultures growing in situ. Image: B. Dixon. (c) Close up of* Grevillea scapigera *flowers from tissue cultured reintroduced plants. Image: B. Dixon.*

There are two main routes to somatic embryogenesis: direct and indirect. Direct somatic embryogenesis can be induced from highly responsive explant material such as seed embryos, or the etiolated hypocotyl segments of extracted embryos or seeds germinated in vitro. Clusters of somatic embryos develop directly from; the intact zygotic embryo explant, excised hypocotyl or coleoptile explants derived from embryos germinated in vitro (Figure 6.3) following application of an embryo-inducing compound (2,4-D or TDZ). Indirect somatic embryogenesis involves the generation of a distinct callus or wound tissue (disorganized cells) from the primary explant in response to auxin (or a combination of auxin and cytokinin) treatment. This tissue can be cultured separately and later induced to form somatic embryos following further treatment with an embryo-induction compound. The formation of a distinct callus phase is essential for plant cell culture 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 off-types or somaclonal variation (see Section 6.2) and is generally not recommended for conservation purposes, unless there is no alternative.

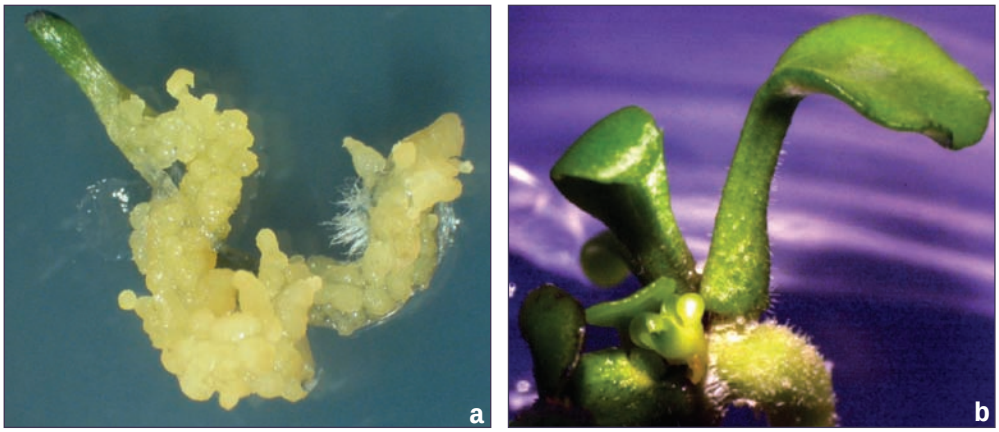

Figure 6.3 *(a) Direct somatic embryogenesis from coleoptile segment of* Anigozanthos manglesii. *Image: E. Bunn. (b) Germinating somatic embryo of* Citrus inodora*; original explant was seed tissue. Image: K. Hamilton.*

6.4.2.2 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, leading 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 in recent years with the development of somatic embryogenesis protocols for Australian sedge species (Panaia et al., 2004b – Case Study 6.3), native citrus (Hamilton, 2007) and species of Ericaceae (Anthony et al., 2004a,b) with potential for both conservation and commercial production of plants.

Case Study 6.3

Somatic embryogenesis in Baloskion tetraphyllum *(Restionaceae).*

The Koala Fern (*Baloskion tetraphyllum*) (Figure 6.4a) is a commercial species grown from seed or harvested from the wild in eastern Australia for the cut flower trade (with potential for use in habitat restoration). These methods of providing plants are relatively inefficient and in the case of wild harvesting also considered environmentally deleterious and unsustainable. Hence research was undertaken to develop an advanced in vitro mass propagation protocol i.e. somatic embryogenesis (Panaia et al., 2004a) that would provide a cost-effective alternative to conventional micropropagation which tends to be achievable albeit with slow multiplication with Restionaceae species (Meney and Dixon, 1995a,b). As a result of this research, high frequency somatic embryogenesis (Figure 6.4b) was achieved with *B. tetraphyllum* and potentially up to 22,000 somatic embryos (SE) produced per gram of plant tissue using ½ MS medium with 1 µM 2,4-D as the primary induction signal for SE production from coleoptile explants (Panaia et al., 2004a). The protocol developed would be commercially viable with this level of plant production and would also allow production of plants for large-scale rehabilitation/restoration of mine-sites or degraded wetlands.

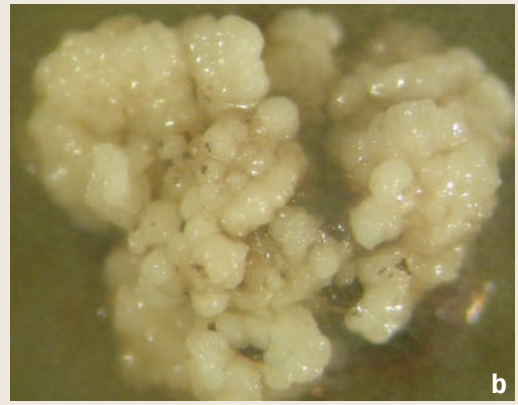

Figure 6.4 *(a)* Baloskion tetraphyllum *growing in situ (Fraser Island, Qld). Image: S. Turner. (b) Close up of* Baloskion tetraphyllum *callus and somatic embryos (small round bumps). Image: M. Panaia.*

The potential for somatic embryos to be desiccated and encapsulated to produce 'artificial seed' has been reported; however, this system has only been developed for a relatively restricted number of high value forestry, amenity or crop species (Jain et al., 1995; Zimny et al., 2003). Only a few instances of somatic embryogenesis have been reported for Australian plants (Panaia et al., 2008; Anthony et al., 2004a,b; Wang and Bhalla, 2004; Watt et al., 1999).

6.4.3 Terrestrial orchid culture

Orchids are generally grown in culture from seed, rather than other plant parts, using tissue culture techniques. Epiphytic and lithophytic orchids are relatively easy to grow in this manner but the propagation of terrestrial orchids is complicated by their reliance on mycorrhizal fungi. While all orchids are thought to have mycorrhizal associations that initiate germination (Clements, 1988), terrestrial orchids rely on these associations to a greater extent than epiphytic genera, especially during seed germination (Zettler, 1997).

6.4.3.1 Orchid seed production and storage

Orchid seed for propagation can be derived from open- or hand-pollinated plants, located in either in situ populations or in ex situ collections. Seed pods can take from a few weeks to several months to mature. However, in hot, dry periods, developing pods can change from green to brown in a matter of days, with the majority of dust-like seed being dispersed within minutes. One of the advantages of ex situ-derived seed is that pod maturation may be monitored more closely, minimising the chance of seeds dispersing prior to pod collection. In the field, such losses may be minimised by covering maturing pods with fine mesh bags (empty tea bags work well) when frequent visits to the site are not feasible. Alternatively, flower stems with developing pods may be cut and kept in a vase of water until the pods are mature.

Orchid seed pods may be harvested when they start to change colour from green to yellow. If the seed is to be introduced immediately to tissue culture, sterilise the surface of green pods and open them under aseptic conditions to remove the seed. If the seed is to be stored for later use, place the pods in a paper bag or envelope and store in a cool, dry environment until they dehisce and the seed is released naturally. Although other containers may be used for this purpose, the static forces present in some plastic, glass or metal containers can make it difficult to remove the seed from the container. This is a particular problem when the species is threatened and seed numbers are limited.

The viability of orchid seed stored at ambient conditions can decline rapidly. As the seed of a number of terrestrial genera have been found to be orthodox in their response to storage conditions (Seaton and Pritchard, 2003; Liu et al., 2008), long-term storage is likely to be most effective under cool, dry conditions. Thus, orchid seed to be stored long-term should first be dried to approximately 5-6% moisture content, then stored at low (preferably sub-zero) temperatures. If facilities are available storage at -80°C and -196°C (in liquid nitrogen) may give greater longevity than storage at -18°C. For a historical perspective on orchid seed storage refer to Pritchard and Seaton (1993).

An essential component of long-term seed storage is the periodic testing of seed viability, either through germination in the presence of the correct fungal symbiont, or on specially formulated asymbiotic media. Orchid seed viability may also be detected using biological stains such as fluorescein diacetate (FDA), tetrazolium (TTZ) or Evan's blue (see Chapter 4). In some instances, however, biological stains have been found to be poor predictors of viability (Batty et al., 2001).

6.4.3.2 Co-culturing orchid seeds and mycorrhizal fungi

The seed of most epiphytic species and many species of terrestrial orchids can be germinated on specific nutrient media without a mycorrhizal fungus (asymbiotically) (Knudson, 1922, 1930; Rasmussen, 1995). 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, 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, natural seedling recruitment cannot occur and the species will be unable to establish a self-sustaining population (Zettler et al., 2003). For this reason, it is now more common to germinate such species on a culture containing a suitable fungal symbiont. In the presence of the correct fungal strain, the seed will first produce a protocorm (a tuber-like structure), and will then progress to green leaf formation.

The fungal symbiont of an orchid is normally isolated through extraction of fungal pelotons (coils of fungal hyphae) (Figure 6.5a) from root, rhizome, stem or collar tissues; the location of these pelotons varies depending on the orchid species. The isolated pelotons are first grown on an agar-based medium then the tips of hyphae growing out from the pelotons are removed and cultured on a medium such as oatmeal agar. As each peloton may contain more than one fungal organism, this process is repeated until pure cultures are obtained. An alternative method for isolating an appropriate fungal symbiont is to germinate seed on filter paper placed over site soil, or soil from an ex situ potted collection, and then culture the resulting protocorms as above (Brundrett et al., 2003). Both methods require a laboratory environment and a good knowledge of aseptic techniques.

Fungal specificity is highly variable among terrestrial orchids, with some species compatible with a diverse range of fungal groups and others requiring a highly specific relationship (Bonnardeaux et al., 2007). Orchid seed should be challenged with any fungal strains isolated using the above methodology to confirm compatibility. It should also be noted that if a particular orchid associates with a fungal species that is widespread, it is possible for asymbiotically raised seedlings to establish self-sustaining populations when transplanted to the wild (Ramsay and Stewart, 1998; Gangaprasad et al., 1999).

Seedling recruitment may also be achieved by sowing orchid seed on the pot surface of ex situ collections of established plants of that species.

6.4.3.3 Storage of seeds and fungi

Collections of orchid seed and associated fungi are generally stored as separate entities (Seaton and Pritchard, 2003). The fungal symbionts associated with each species (which are usually stored for research and restoration purposes, rather than forming part of any seed bank collections) are commonly grown on an agar-based culture medium and then stored at either room temperature or 4°C. The fungal cultures are sometimes placed in storage as is, and are sometimes covered with either sterile water or mineral oil. Fungal cultures stored on agar at room temperature or at 4°C require subculturing on a regular basis to maintain viability (Smith and Onions, 1994; Zettler et al., 2003). This time-consuming process can lead to gradual changes in culture morphology including the loss of key functional characteristics such as the ability to increase plant growth (Sneh and Adams, 1996) and to initiate germination (Dixon, 1987). Fungi may also be treated with a cryo-protectant (Chapter 7) and stored under liquid nitrogen (Batty et al., 2001). Germination of orchid seed then requires retrieval of the fungus from storage and the re-establishment of active growth on fresh culture medium prior to sowing the seed. An alternative storage method currently under investigation involves the encapsulation of both fungal hyphae and seed in an alginate bead, thus allowing the simultaneous storage of orchid seeds and their symbionts (Wood et al., 2000; Sommerville et al., 2008).

6.4.3.4 Re-establishment from culture

Due to the natural cycle of dormancy in terrestrial orchids, the timing of germination in the wild is critical to ensuring an orchid seedling has stored sufficient resources during the growing season to re-emerge the following season. Laboratory-grown seedlings must mimic this natural cycle. Therefore, they should be removed from culture and transferred to potting media sufficiently early in the growing season to ensure they have time to

Case Study 6.4

Translocation of symbiotically cultured terrestrial orchids

Australia's diverse terrestrial orchid flora, found largely in the southeast and southwest, is becoming increasingly threatened with extinction. Orchids represent 33 of the 81 Critically Endangered plant species listed under the Federal Environment Protection and Biodiversity Conservation Act 1999 (DEWHA, 2008).

Reintroduction is increasingly used as a conservation method and relies on successful ex situ propagation, including isolation of suitable fungi from plant tissue and use of these fungi to germinate seed in vitro. Both seed and mycorrhizal fungi are sourced from the target species and population, as appropriate fungi is important for effective germination and subsequent plant growth (Wright et al., 2009). The success of these

introductions not only relies on the successful establishment of the plants but on the presence of pollinators for seed production. However, pollinators can be highly specific and their presence restricted (Bower, 2007; Swarts and Dixon, 2009). Seedling recruitment, and therefore seed production, is essential for development of a self-sustaining population.

The collaborative approach of a number of organisations has helped the conservation of Victorian threatened orchid species, bringing together research and management resources. Examples of reintroductions include:

a) Symbiotically germinated *Caladenia hastata* (Mellblom's Spider Orchid) from the Portland region was reintroduced during the growing season, monitored for six years, with natural pollination and seedling recruitment observed (Wright et al., in press).
b) Although 50% of plants established (Smith et al., 2007), limited natural pollination has been observed four years after the reintroduction of 700 *Diuris fragrantissima* plants in urban Melbourne.
c) Reintroduction sites for *Caladenia calcicola* (Limestone Spider Orchid) in Victoria's coastal southwest were selected by baiting for the pollinator species with freshly cut *C. calcicola* flowers prior to plant reintroductions in August 2007. The reintroduced symbiotically germinated plants achieved a natural pollination rate of 7.5% in the first year.

By meeting both mycorrhizal and pollinator requirements in orchid reintroductions the likelihood of creating self-sustaining populations is vastly increased.

— Rob Cross

Figure 6.5
(a) SEM of fungal pelotons in cells of the common species Caladenia tentaculata. *Image: Magali Wright. (b) Pollinator on* Caladenia calcicola *flower. Image: David Pitts. (c) Reintroduced ex situ propagated* Diuris fragrantissima. *Image: Zoe Smith.*

develop a tuber before the onset of the dormant dry season. As with micropropagated plantlets, the transition of terrestrial orchid seedlings growing in a laboratory to the glasshouse is often problematic (Clements et al., 1986; Anderson, 1991; Zettler et al., 1995; Zelmer and Currah, 1997). For techniques refer to Batty et al. (2006) and Sommerville et al. (2008). Epiphytic orchids, in most instances, are easier to deflask from tissue culture as they are less prone to desiccation, probably as a result of the waxy cuticle covering the leaves in many species.

6.5 Other considerations

6.5.1 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 including the history in, and prior to, culture. Each subculture flask should be coded to reflect the following separately recorded details:

- Species.
- Clone/Accession.
- Date, location and collector of wild collection or accession number.
- Date put into culture.
- Subculture number.
- Treatments.

6.5.2 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 (see Section 2.6.3).

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Chapter 7

Cryopreservation

Kim N. Hamilton, Shane R. Turner and Sarah E. Ashmore

7.1 Introduction

Cryopreservation, which is the storage of germplasm at -130 to -196°C in liquid nitrogen (LN), is a method of securing conservation collections on a long-term basis (i.e. >25 years) in a relatively low cost way, free from deleterious processes such as diseases, contamination, and general tissue senescence. The suspension of metabolic processes which occurs at ultra-low temperatures (below -130°C) extends the storage life of germplasm and minimises genetic drift (i.e. random mutation or loss of specific genotypes).

There is an increasing interest in the use of cryopreservation for conservation of Australian threatened species, non-orthodox seeded (see Section 2.4.1) or vegetatively propagated species, or economically valuable species (Ashmore et al., 2007a; Bunn et al., 2007). 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.

7.2 Benefits and risks of cryopreservation

Cryopreservation represents the only long-term option for ex situ storage of germplasm of non-orthodox seeded species and vegetatively propagated species (Engelmann and Engels, 2002) and is often coupled with in vitro culture (see Chapter 6). Cryopreservation also brings a number of practical (e.g. prolonged storage) and economic benefits by reducing the need to perform labour-intensive tasks such as regular monitoring of viability and subculturing of tissue cultures, so reducing quantity and space required.

However, there is no single approach that can work as a 'magic bullet' and facilitate the cryostorage of all species and tissues types. Depending on the species and tissue to be cryopreserved, different protocols may need to be developed and optimised through empirical experimentation altering factors such as desiccation conditions, cryoprotectant (antifreeze) mixes, and preculture and recovery environment. In addition, 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. This is to make sure that the techniques and approaches adopted are working across the whole group of interest and the results achieved are reproducible.

An on-going problem for many germplasm collections is the loss of critical accessions through equipment failure (of freezers and incubators), disease and microbial contamination (particularly in tissue culture collections) and even human error (through misslabelling or poor sterile technique). Through adopting cryogenic storage, many of these risks are greatly minimized and in some cases eliminated. For example, for vegetative selections (container or tissue culture collections) LN storage can eliminate pests and diseases, somaclonal variation, microbial contamination due to mites and reduce the chance of human error as the material, once frozen, is virtually maintenance free.

However, the single biggest risk to LN collections is securing LN supplies and having a fool-proof LN replenishment schedule (on average once per week). Therefore managers must be mindful of this and plan refills accordingly, taking into account public holidays, employee sickness and other chance events.

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 (e.g. normal morphology for recovered plants) of model species through different methodologies (e.g. genetic analysis, growth studies, biochemical analysis) is essential for the long-term development and utilisation of robust cryopreservation protocols.

In addition to the risks associated with the maintenance of cryogenic collections, there are also some associated occupational health issues and safety standards that are required. These include the provision of adequate ventilation and low-oxygen alarms in areas where LN dewars are situated, and the use of appropriate protection such as gloves and face masks to protect from LN burns and exploding vials when handling frozen materials.

7.3 When should cryopreservation be used?

As with other forms of germplasm storage used for conservation collections, cryopreservation must ensure the survival and recovery of a species (Centre for Plant Conservation, 1991).

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 is desired such as for critical base collections;
- Be used to establish corresponding base collections for species where adequate

germplasm can be collected and stored.

Cryobiology has become an important scientific discipline for the conservation of wild plant diversity (Touchell, 2000). Plant species for which cryopreservation is useful include:

- Rare or threatened;
- Socio-economically important (e.g. horticultural crops and wild relatives);
- Non-orthodox seeded (e.g. recalcitrant see Section 2.4.1.1);
- Orchids (both seeds and fungal symbionts);
- Lower plants;
- Symbiotic microbes.

7.4 What form of germplasm can be stored?

Virtually any plant germplasm tissue can be cryopreserved, including:

- Seeds;
- Zygotic embryos or axes;
- Vegetative tissue (shoot tips);
- Cultured tissue (callus, suspension cell cultures, embryogenic cultures or somatic embryos);
- Pollen.

It is critical to take into account the desired purpose of the collection when deciding which form of germplasm to use. Also consider the advantages, limitations and restrictions of each form of germplasm. Use the following points as a guide in deciding the type of germplasm to use.

- **Seed** is 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.
- **Zygotic embryos and embryonic axes** may be the best type of tissue to use for species that produce non-orthodox seeds.
- **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., 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. Therefore, where species are reduced to very small populations or material is derived from elite genotypes, investigations into the cryopreservation of shoot tips should persist 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 seeds or produce seeds that are deeply dormant and therefore difficult to germinate.
- **Cultured tissue** (callus, suspension cell cultures, embryogenic cultures or somatic embryos) is used for specialised biotechnology programs (e.g. alkaloid producing cultures) or when alternative approaches are not possible.

- **Pollen** can be used to store genetic diversity, particularly for specialist breeding programs (not dealt with in these guidelines) when species (to be hybridised) flower at different times of the year or pollen from elite or desired genotypes is saved if the parental plant is likely to die (i.e. annual species).

7.5 Overview of cryopreservation techniques

The choice of cryopreservation technique depends on the plant species, tissue availability, propagation system and intended use.

- For somatic tissues, vitrification procedures are the preferred option for several reasons and for Australian species are the most advanced. Nevertheless, many species are still recalcitrant to these approaches.
- The encapsulation/dehydration technique is another procedure which has several advantages (and disadvantages), but has also been shown to be widely applicable, and under some conditions may be more appropriate for cryoprotectant-sensitive species.
- The final technique which has been widely utilised is slow cooling, but due to significant disadvantages this procedure is currently not widely used.
- Another possibility is to use combinations of these procedures (i.e. encapsulation/dehydration/vitrification), which have had varying degrees of success for different species.

Box 7.1 gives an overview of cryopreservation procedures. Table 7.1 provides some examples of the most commonly used techniques, their advantages and disadvantages. Figure 7.1 shows some tissue types and cryopreservation techniques, and Figure 7.2 shows some of the typical equipment needed to carry out cryopreservation.

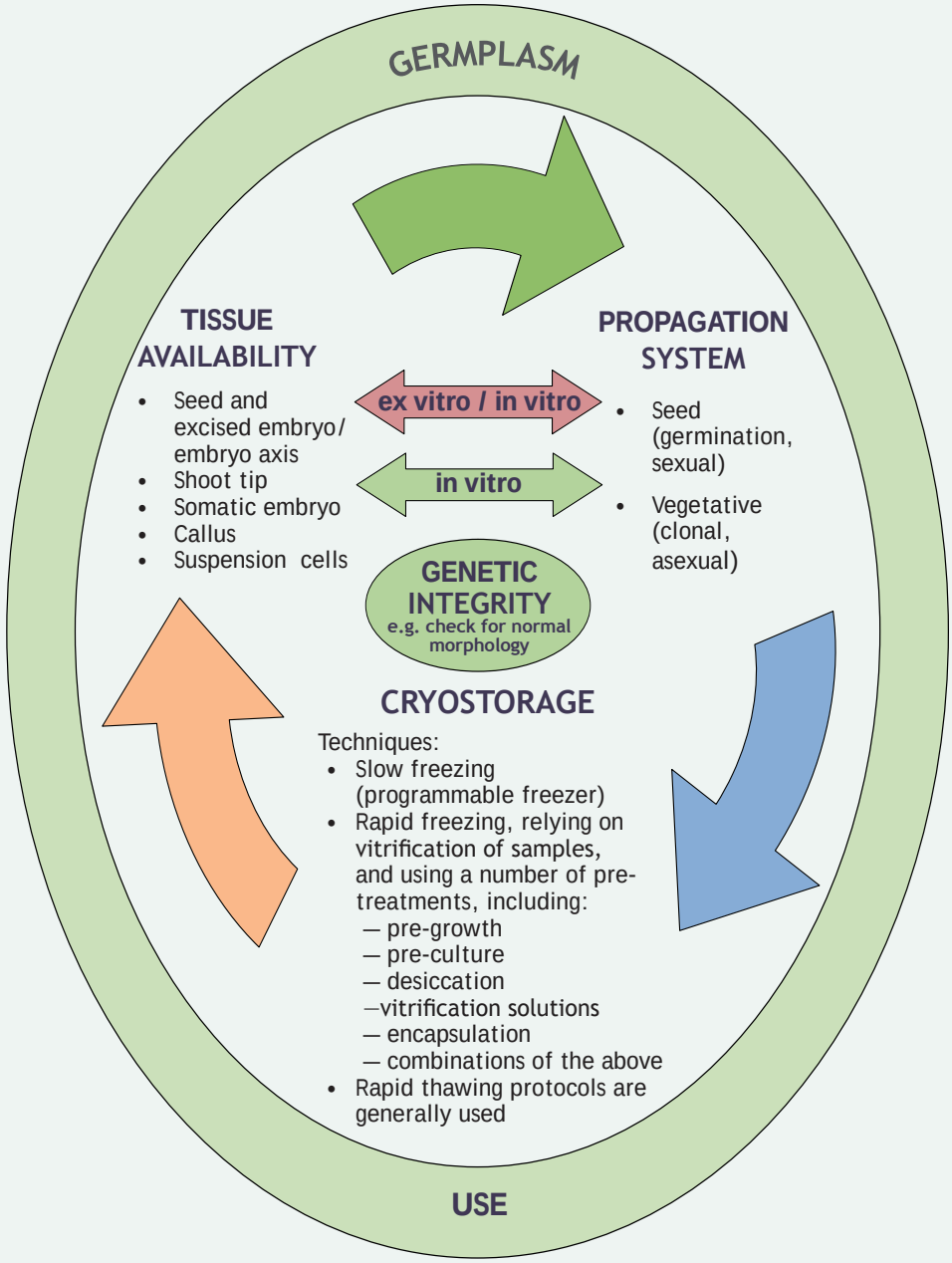
Plant health is crucial to survival of cryopreserved material, i.e. survival rates are generally much lower if material is in poor condition (Reed et al., 2004). Thus, physiological conditioning of plant material prior to processing and LN immersion can improve survival to the extreme stress (i.e. desiccation, freezing and thawing) involved in cryopreservation. Additionally, any successful cryopreservation protocol needs to achieve a relatively high level of recovery of genetically stable plants, though equally important is reproducibility of results when the same techniques are applied at different times. Generally, the following components are included:

- Preculture, particularly in vitro culture;
- Pretreatment;
- Cryoprotection;
  - Freezing
  - Storage
  - Thawing
- Recovery and plant regeneration.

Box 7.1

Overview of cryostorage procedures

Cryopreservation procedures vary considerably between species in terms of both tissue type and technique.



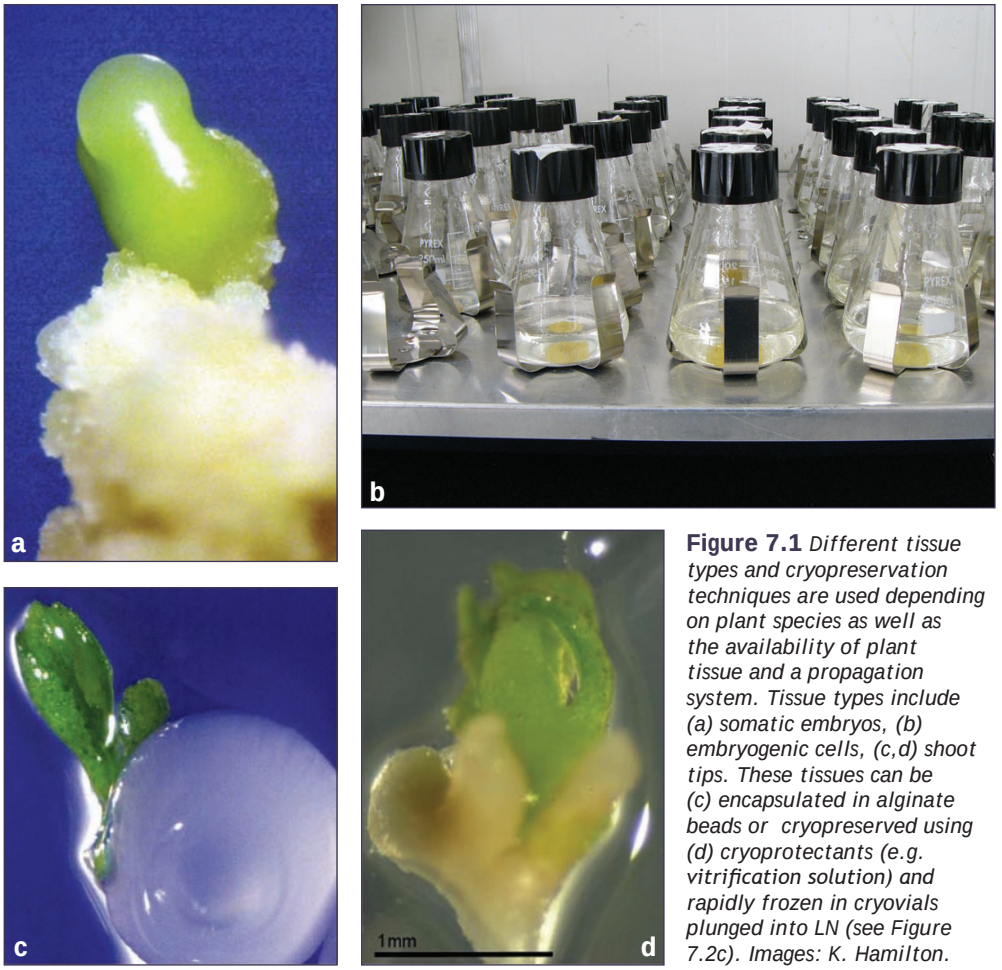


Figure 7.1 *Different tissue types and cryopreservation techniques are used depending on plant species as well as the availability of plant tissue and a propagation system. Tissue types include (a) somatic embryos, (b) embryogenic cells, (c,d) shoot tips. These tissues can be (c) encapsulated in alginate beads or cryopreserved using (d) cryoprotectants (e.g. vitrification solution) and rapidly frozen in cryovials plunged into LN (see Figure 7.2c). Images: K. Hamilton.*

7.5.1 Preculture

Maintain cultures under conditions highly conducive to healthy vigorous growth. Both the type and concentration of nutrients and the Plant Growth Regulator (PGR) regime should be manipulated to achieve this aim (see Chapter 6). When ready for harvesting, select only healthy well-formed tissue and cells (e.g. shoot tips) during optimal growth.

7.5.2 Pretreatments and cryoprotection

Pretreatments (e.g. incubation on media containing high concentrations of sucrose) reduce cell size as well as the cytoplasm to vacuole ratio which may:

- enhance the ability of cells or tissues to take up cryoprotectants; and/or,
- modify cell walls and membranes to resist deformation during freezing (Luo and Reed, 1997).

Cryoprotectants, such as amino acids, polyols, sugars and lyotropic salts, help protect membranes during freezing and protect proteins and nucleic acids from inactivation

Table 7.1 *Advantages and disadvantages of some commonly utilised cryopreservation techniques* (after Reed, 2001).

Technique	Advantages	Disadvantages
Vitrification	• no special equipment needed • fast procedure • low technical input • fast recovery time • widely applicable	• vitrification solutions can be highly toxic • requires careful timing of solution changes
Encapsulation/ dehydration	• no special equipment needed • non-toxic cryoprotectants used • simple thawing procedures	• requires high technical input (each bead is handled several times) • some plants do not tolerate high sucrose concentration • currently not widely applicable
Slow Cooling	• stability from cracking • relatively non-toxic cryoprotectants used • low technical input	• requires sophisticated equipment • slow recovery rates • low applicability

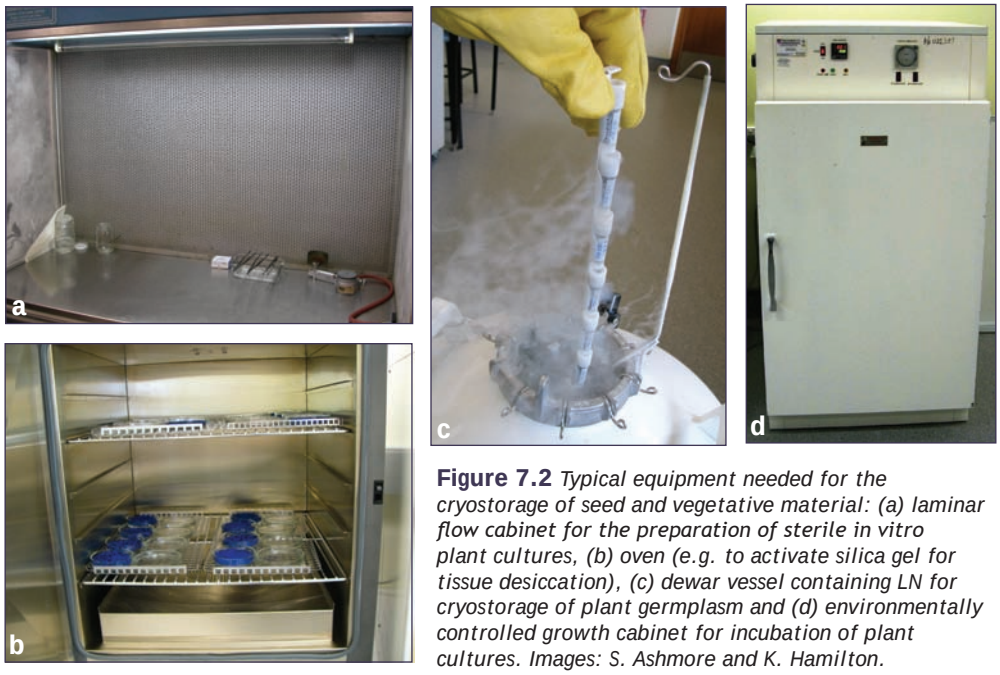


Figure 7.2 *Typical equipment needed for the cryostorage of seed and vegetative material: (a) laminar flow cabinet for the preparation of sterile in vitro plant cultures, (b) oven (e.g. to activate silica gel for tissue desiccation), (c) dewar vessel containing LN for cryostorage of plant germplasm and (d) environmentally controlled growth cabinet for incubation of plant cultures. Images: S. Ashmore and K. Hamilton.*

(Towill, 1990). Three techniques of pretreatment and/or cryoprotection commonly used are:

1. **Desiccation**

   This method involves simple dehydration 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. The reduction of ice formation is dependent on the extent of water removal. Moisture contents of between 10 to 20% (fresh weight basis) are often optimal for survival of freezing (Engelmann, 2004). Thus, this technique is only viable for somatic tissue (e.g. somatic embryos, Figure 7.1a), seeds and embryonic axes that will tolerate this level of desiccation and as such is species/tissue dependent.

2. **Vitrification**

   Vitrification-based methods involve pretreatment of samples with concentrated cryoprotectant solutions, which on rapid cooling (e.g. direct immersion in LN) form a highly viscous solid 'glass', thus avoiding lethal ice formation. Figure 7.1d shows shoot growth from an incubated shoot tip after pretreatment in a vitrification solution and cryopreservation. The most widely used vitrification solution, for cryopreservation of a range of diverse tissues, is plant vitrification solution two (PVS2) developed by Sakai et al. (1990). PVS2 consists of 30% glycerol, 15% ethylene glycol and 15% and dimethylsulfoxide (DMSO). However, there are many other vitrification solutions (e.g. PVS1 and PVS3, Turner et al., 2001) that have proven to be just as effective for particular species.

3. **Encapsulation-dehydration**

   This method was developed by Fabre and Dereuddre (1990) and involves the encapsulation of tissue in a calcium alginate bead, followed by pregrowth (e.g. on high sucrose medium) and desiccation prior to direct immersion in LN (Benson, 1999). Figure 7.1c shows an alginate bead from which a shoot tip is growing after encapsulation/dehydration and culture incubation. This method has been applied to many species using various tissue types including embryonic axes, somatic embryos and shoot tips.

7.5.3 Freezing

Freezing can be achieved using either slow cooling with a programmable freezer (e.g. for suspension culture cells) or through rapid freezing by plunging tissue directly into LN. Cryopreservation techniques that rely on vitrification of water into a glassy state include any method combined with rapid freezing, for example desiccation, encapsulation-dehydration and vitrification-based methods. Vitrification of water involves the formation of an amorphous phase, a 'glassy state', on rapid freezing without the formation of lethal ice crystals (Fahy et al., 1984). Vitrification of water in the cells prevents injury via intracellular freezing (ice formation) and the lethal effects of excess dehydration. It is important to rapidly thaw the tissue to prevent devitrification events.

7.5.4 Storage

7.5.4.1 Storage vials

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.

7.5.4.2 Temperature

The storage temperature for cryopreservation is important to ensure longevity of germplasm. Germplasm must be maintained at the temperatures of LN (-196°C) or LN vapour (between -130°C and -196°C). Storage at temperatures higher than -130°C will lead to possible water movement and ice crystal formation.

7.5.4.3 Maintenance

Maintain long-term base collections under conditions of minimal disturbance, (i.e. dewars not used for other purposes, such as experimentation), as the constant warming, cooling and agitation may cause tissue damage, leading to a gradual decline in viability. Constantly monitor dewars, preferably fitted with alarms, so collections are not accidentally lost through loss of LN. Develop and maintain a regular LN replenishment schedule. Depending on the size of the dewar and how often it is accessed, dewars on average require topping up with LN once a week.

7.5.4.4 Liquid or vapour phase storage

Stored vials can be kept in either the vapour phase (between -130°C and -196°C) or in the liquid phase (lower than -196°C). Material stored in vapour phase can experience rapid fluctuations in temperature and if not monitored closely can quite easily warm to above -139°C leading to devitrification (lethal ice crystal formation), irreparable damage and loss of valuable germplasm. However, vials stored in vapour phase cannot explode upon removal and are not prone to LN mediated contamination.

Liquid phase storage on the other hand is cooler (lower than -196°C), and the maintenance temperature is more constant, however, vials may leak over extended periods of time, leading to possible explosions when removed (this danger can be negated by placing vials into the vapour phase for 24 – 48 hrs prior to removal). Leakages can also cause possible contamination of vials by bacteria, viruses or fungi which could destroy cultures upon warming and regeneration.

7.5.4.5 Storage quantity

Additional factors that need to be considered during storage are:

- The number of clones which should be stored.
- The number of shoot tips per vial and the number of vials to be stored in the entire collection.

- The number of clones needed should be enough to capture a representative sample of the genetic diversity of the population (see Chapter 3).
- The number of shoot tips per vial should be from 10 to 30 depending on the reliability of the techniques for that species, the survival rate, and the ease with which recovered shoot tips can be successfully regenerated into viable plantlets.
- The number of vials in storage should be sufficient for several regeneration attempts, to facilitate viability and genetic testing (if desired) and for indeterminate storage duration and possibly transfer to offsite backup collections.

7.5.5 Thawing

Thawing is generally achieved by rapid plunging of cryovials into a water bath adjusted to 40°C. Samples can also be directly plunged into a sterile 'thawing medium' at room temperature (around 23°C). It is important to remove any toxic cryoprotectants as rapidly as possible by washing samples in sterilised washing solutions which are typically high in sucrose.

7.5.6 Recovery and plant regeneration

Once thawed, recover and regenerate plant tissues in tissue culture media and under environmental conditions appropriate to the particular plant tissue. Develop these conditions prior to undertaking the establishment of base collections with a particular species or accession as this is critically important to maximise post-LN recovery. Post-LN survival does not necessarily need to be high (> 50%), more importantly it needs to be constant over several trials, and the technique reliably shown to be able to regenerate new plantlets e.g. stimulate rooting from recovering shoot tips post-LN. However, to achieve this, the appropriate recovery and post-recovery conditions need to be determined, as survival post-LN immersion does not necessarily equate with the ability to successfully regenerate an entire healthy plant. For species proving problematic to a given procedure, other factors may need to be considered, or adjusted during any of the key stages for successful cryopreservation.

7.5.7 Additional Considerations

7.5.7.1 Viability testing

To monitor the effects of different treatments, regular testing (via shoot regeneration) needs to be undertaken after critical stages in any protocol. For instance:

- after shoot extraction from mother plants;
- after preculture;
- after cryoprotection;
- after LN immersion;
- after the transfer of material to different dewars or institutions;
- for each batch of explants processed and cryopreserved, recover and grow a random sub-sample to test initial viability;

- evaluate viability and genetic fidelity for selected clones or species over many years to confirm maintenance of these traits under cryogenic conditions.

If viability is lower than the acceptable level, the entire batch should be removed.

7.5.7.2 Record keeping

As with tissue culture (see Chapter 6) and seed banking (see Chapter 4), a key feature for any cryopreservation program is efficient record keeping. To quickly and reliably find and track vials, record the location of all vials on a central database. Therefore, all boxes, stacks and vials need to be uniquely labelled using a permanent technique such as engraving, permanent marker or bar coding. Along with the details in Section 6.5.1 for tissue cultures, the following information at a minimum should also be recorded:

- Date put into LN;
- The person who put the material into LN;
- Type of material stored (e.g. shoot tip) as a code;
- Number of samples per vial;
- Number of vials put into LN;
- Batch viability;
- Number of accessions left in LN.

7.5.7.3 Risk management procedures

Before establishing a cryogenic collection, develop risk management plans to reduce risks to the collection and eliminate accidental losses. For example, in a pre-arranged exchange program, a small sub-sample of all accessions held may be sent to a similar facility either nationally or internationally. This may be done using dry shippers which have the capacity to hold cryogenically stored material for up to 21 days at LN temperatures but in a dry environment making them safe to transport on aircraft. This material can be easily transferred around the world via commercial airlines with minimal problems and usually arrive within one to two days.

7.6 Protocols

7.6.1 Orthodox seed (desiccation tolerant)

Seeds in this category (see Section 2.4.1.1 for determination) have naturally low moisture content and can be stored in LN without adjustment of moisture or chemical cryoprotection, although ideally the moisture content should be reduced in line with current recommendations of equilibration at 15% RH and 15°C for several weeks then placed into the LN vessel without any other pretreatments. It is advisable that once a sample of seeds has been placed into LN then a small sub-sample be removed for viability/germinability testing within a few days to make sure that the temperature changes experienced by the seeds during LN cooling and warming are not deleterious to the seeds. An appropriate quantity of seed (depending on seed availability, viability and size) is placed in a vial and immersed in LN and when required, seed should be thawed slowly for approximately 20 minutes at ambient room temperature.

7.6.2 Non-orthodox seed (desiccation sensitive and/or short-lived)

This category includes those seeds with recalcitrant qualities (i.e. desiccation sensitive) and those that display other storage constraints (e.g. intermediate seeded species, see Section 2.4.1.1). For such species cryopreservation of seed, embryos and embryonic axes can be used. Steps may include:

- Determination of original seed viability (see Section 4.5.2).
- Desiccation. Desiccate seeds over silica gel or a saturated salt solution at a specific relative humidity for a few days to 1–3 weeks. Care must be taken however to make sure that the seeds can survive the selected desiccation process.
- Chemical cryoprotection. Cryoprotection will vary between species, but in general media supplemented with 15% dimethylsulfoxide (DMSO) may be used. Seeds can be placed in the cryogenic solution for a period of time to facilitate cryoprotection prior to LN immersion.
- Freezing of seeds may be possible by direct immersion in LN for some species. However, slow cooling procedures may need to be employed for difficult-to-store species. This is done with a programmable controlled-rate freezer. Optimal cooling rates vary between species, but a cooling rate of 0.5°C/min is generally applicable to a range of species.
- Seeds must be frozen in a cryoprotective medium if slow freezing procedures are used. The freezing medium may contain a number of cryoprotectants at varying concentrations. One containing 15% DMSO is recommended.

7.6.3 Seed embryos and embryonic axes

This technique is used mainly for those seeds with non-orthodox qualities (i.e. high moisture content and desiccation-sensitive, see Section 2.4.1.1) and those that are relatively short-lived but do not display true recalcitrance. The cryopreservation of seed embryos or embryonic axes for these types of species involves a number of key steps, summarised as follows:

- Excise embryos or axes from seeds and test for viability.
- Desiccation. Desiccate embryos over silica gel or a saturated salt solution at low relative humidity. For larger embryos, desiccate rapidly in a sterile air flow for 4-5 hours.
- Cryoprotection. Embryos can be placed for up to 24 hours in medium containing 15% DMSO and 0.4-1.2 M glucose prior to LN immersion. Alternatively, vitrification techniques using PVS2 can also be readily used and indeed may be preferable for some species.
- Freezing is usually performed by direct immersion of cryo-vials containing the embryos/zygotic axes in LN. Alternatively, slow cooling may need to be employed where direct immersion proves to be deleterious to survival.
- Thawing and recovery. Embryos are thawed in a 40°C water bath. The cryoprotective medium is removed 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 (see Meney and Dixon, 1995).

7.6.4 Shoot tips or other somatic organised tissues

The three main procedures for cryopreservation of shoot tips are vitrification, encapsulation/dehydration and slow cooling. The most successful for Australian species has been a vitrification method modified from Yamada et al. (1991). These procedures are based on using PVS2 as the cryoprotectant and involve:

- 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 in vitro grown plants. These are typically 1–2 mm long and consist of only several leaf primordia and the apical meristem.
- For preculture, shoot tips are placed onto a tissue culture based medium containing 0.4–1.2 M sorbitol, sucrose or glycerol for 24–72 hours under standard growth conditions to begin cell desiccation.
- Loading phase. Shoot tips are removed from the preculture medium and exposed to a 'loading solution' of 2 M glycerol, plus half-strength MS liquid medium supplemented with 0.4 M sucrose for 20 mins at room temperature (usually done in a sterile petri dish).
- Cryoprotection. This critical step involves incubation in 100% PVS2 (15% DMSO, 15% ethylene glycol and 30% glycerol) for 10–30 mins (depending on species tolerance) at either room temperature or 0°C, which involves transferring shoot tips to cryovials filled with PVS2.
- Freezing is done via direct immersion of shoot tips (inside cryovials) in LN (dewar).
- Thawing. Shoot tips are thawed rapidly in a 40°C water bath for 1–2 mins.
- The highly concentrated cryoprotective solution must be removed and shoot tips washed with a 1 M sucrose solution to minimise toxicity resulting from over exposure to the vitrification solution. Shoot tips should then be recovered on an optimised in vitro recovery medium under low light conditions (Touchell et al., 2002). Signs of recovery growth should begin within 7–14 days if the technique has been successful.

7.7 Cryopreservation of Australian germplasm

The use of cryopreservation for the conservation of native Australian plant diversity is still in its infancy. Therefore, until generalised protocols are established, cryopreservation of a diverse range of species should proceed cautiously and may require some experimentation before a protocol can be readily implemented.

Cryopreservation of seeds after drying to low moisture contents has been demonstrated as a potential strategy for long-term storage of many species. Pritchard (2007) reviewed seed

cryopreservation studies, over ten years (1995-2005), and found considerable interest, with over 60 socio-economically important species being researched. In Australia, cryopreservation of vegetative (shoot tips) and seed material from many endangered Australian species has been undertaken since the early 1990's in Kings Park and Botanic Garden in Perth, Western Australia. In this cryogenic program an integrated ex situ conservation strategy has been developed for threatened taxa, incorporating genetic assaying to determine which genotypes are most desirable, in vitro micropropagation and storage (cryopreservation) techniques utilising diverse tissues such as embryogenic callus cultures and shoot tips for rapid large-scale production and protection of germplasm for species recovery efforts (Touchell et al. 2002; Cochrane, 2004). However, the majority of Australian species, especially non-orthodox seeded species, are still poorly understood and cryopreservation protocols need to be developed and optimised. Figure 7.3 shows examples of Australian subtropical fruits that are of conservation priority and have potential horticultural value (as 'bush foods') for which cryopreservation techniques are a priority (Ashmore et al., 2007a). Table 7.2 provides a list of reported methods for Australian native and crop species, based on a range of techniques and type of storage material.

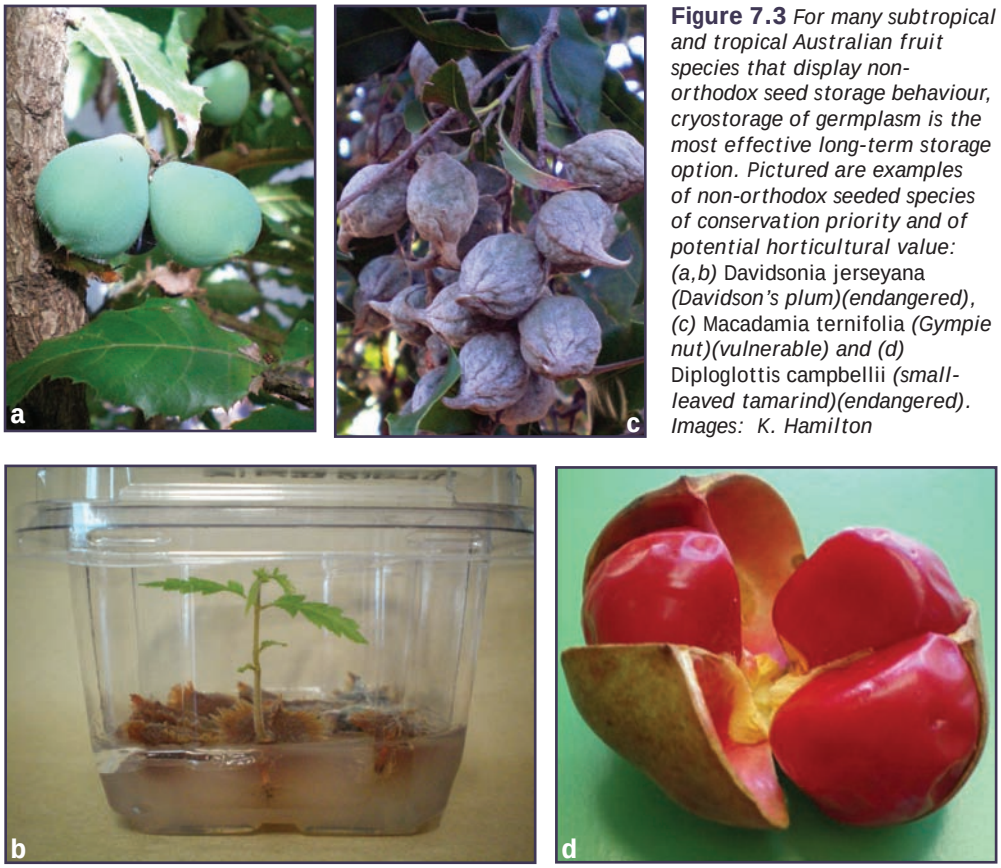


Figure 7.3 *For many subtropical and tropical Australian fruit species that display non-orthodox seed storage behaviour, cryostorage of germplasm is the most effective long-term storage option. Pictured are examples of non-orthodox seeded species of conservation priority and of potential horticultural value: (a,b)* Davidsonia jerseyana *(Davidson's plum)(endangered), (c)* Macadamia ternifolia *(Gympie nut)(vulnerable) and (d)* Diploglottis campbellii *(small-leaved tamarind)(endangered). Images: K. Hamilton*

Table 7.2 *Examples of the application of cryopreservation techniques to Australian native and crop species*

Family	Species	Tissue type	Technique	Reference
Caricaceae	*Carica papaya* L.	Shoot tip	Vitrification (PVS2)	Ashmore et al. (2007b)
Haemodoraceae	*Anigozanthos humilis* Lindl.	Shoot tip	Vitrification (modified PVS2)	Turner et al. (2001)
Haemodoraceae	*Macropidia fuliginosa* (Hook.) Druce	Embryogenic Callus	Vitrification (PVS2)	Turner et al. (2000a)
Myrtaceae	*Eucalyptus graniticola* Brooker & Hopper ms	Shoot tip	Vitrification (PVS2)	Touchell et al. (2002)
Restionaceae	*Loxocarya gigas* B.G.Briggs & L.A.S.Johnson	Excised embryo	Preculture (2 d 0.75M sucrose)	Touchell and Dixon (1994)
Rutaceae	*Citrus australasica* F. Muell.	Seed	Desiccation	Hamilton et al. (2005)
Rutaceae	*Citrus inodora* F.M. Bailey	Embryogenic Callus	Encapsulation/ dehydration	Hamilton (2007)
Sapindaceae	*Dodonaea hackettiana* W.Fitzg.	Seed	Vitrification (35% DMSO)	Touchell and Dixon (1994)

Case Study 7.1

Seed cryopreservation of Australian wild citrus - Australia's unique wild citrus diversity

Australia has six native species of citrus (family Rutaceae), the largest number of indigenous citrus species of any country worldwide and these represent an important source of untapped genetic diversity for this economically important genus. Five of these species are found in Eastern Australia and two are listed as rare and threatened. The only Australian wild lime not found in Eastern Australia is the Humpty Doo lime (*Citrus gracilis*), a newly discovered species found only in the Northern Territory. Australian wild limes have breeding compatibility with commercial cultivars and some species, such as finger limes (*C. australasica*), are eaten as a popular 'bushfood' and are currently being commercialised.

Case Study 7.1 continued

Citrus is a crop of worldwide economic importance that has been historically vulnerable to genetic erosion from hybridisation (domestication over hundreds of years), serious diseases, pests and pathogens, as well as land clearing. The urgent need for in situ and ex situ conservation of existing wild biodiversity is widely recognized as important for both conservation and development of crop diversity (FAO, 2005). *Citrus* germplasm has traditionally been conserved ex situ in field collections of botanic gardens and research stations because of non-orthodox seed storage behaviour (see Section 2.4.1.1). In oily-seeded species such as citrus, variation in seed responses and other storage constraints makes cryopreservation the safest storage option to prevent seed deterioration (Hor et al., 2005; Pritchard, 2007; Hamilton, 2007). Cryopreservation of seeds after drying has been demonstrated as a potential strategy for long-term ex situ conservation for many cultivated *Citrus* species, but tolerance to drying and cryopreservation has varied in the citrus species so far studied (Hamilton, 2007). Cryopreservation of Australian wild citrus has been demonstrated using a simple desiccation protocol. In this method, seeds are dried over silica gel or at 15% relative humidity until the moisture content is reduced to about 5% and seeds are then directly immersed in LN (Hamilton, 2008; Hamilton et al., in press).

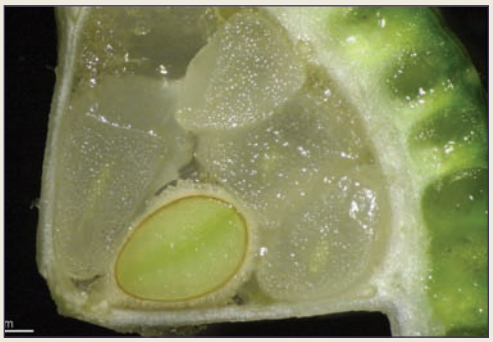


Figure 7.4 *Whole (top) and cut fruit (above) of rare listed* Citrus garrawayi.

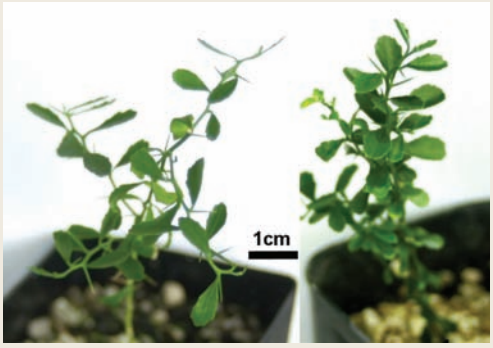


Figure 7.5 *Seedlings of* Citrus garrawayi *growing from seed without (above left) and after (above right) cryopreservation. Plant growing from cryopreserved seed in the field (left).*

Case Study 7.2

Shoot tip cryopreservation of Australian Haemodoraceae

The Kangaroo Paw family (Haemodoraceae) reaches its maximum diversity in the south-west of Western Australia, with roughly 77 species not found anywhere else in the world. Some of the genera include well-recognised taxa such as *Macropidia* (Black Kangaroo Paw), *Anigozanthos* (common Kangaroo Paws), and *Conostylis* (Cotton Heads) (Hopper, 1993) many of which are used in horticulture and as cut flowers. Due to land clearing and salinity, many are now classified as rare and threatened (Brown et al., 1998). Several species of Haemodoraceae have been cryogenically preserved by Kings Park and Botanic Garden as a way to ensure their ex situ persistence in the face of continuing in situ threats.

At the beginning of the experimental program nothing was known about cryogenic storage of any Haemodoraceae species. Shoot tips were initially selected as the targeted tissue type for they allow for the conservation of genetically stable germplasm from key genotypes which are essential for maintaining genetic diversity. The program has also focused on vitrification, adapted and modified from Sakai et al. (1990). By concentrating on a model species (*Anigozanthos viridis*), post-LN survival was significantly increased (Turner et al., 2000b, 2001) from 36% to over 80%, with the modified protocol then successfully applied to six other threatened Haemodoraceae.

Due to the approach used (i.e. focussing on the one species and then applying key outcomes to other closely related species) rapid progress was made in the development of a robust cryogenic technique for a group of threatened species.

Given that around 10% of Australia's flora (approximately 2,500 species) are currently threatened and need ex situ conservation, relatively simple and generic cryogenic protocols are urgently required to deal with this large number in a relatively quick and efficient manner.

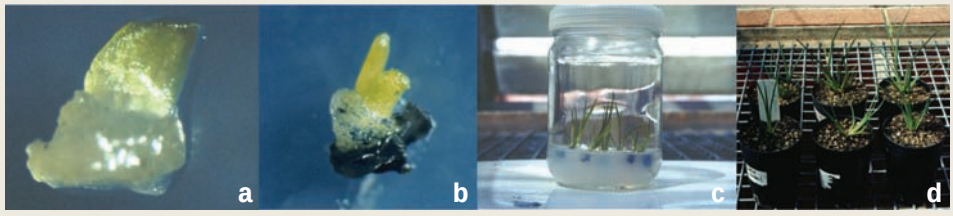


Figure 7.6 *(a) Freshly excised shoot tip of* Anigozanthos viridis *prior to LN storage. (b) Cryopreserved shoot tip of* Anigozanthos humilis *two weeks after removal from LN and placement on recovery medium. (c) Re-established cultures of* A. viridis *derived from LN stored shoot tips several months after removal from LN. (d) Deflasked* A. viridis *plants 6 months after removal from LN.*

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Chapter 8

Living plant collections

Catherine A. Offord and Thomas G. North

8.1 Introduction

Living plant collections for conservation are collections of plants maintained primarily for the purpose of reproducing seeds and/or vegetative material for ready use in restoration, research, education, horticultural development and display. Such collections can be maintained in pots, gardens, plantations or in field genebanks. Living plant collections are increasingly used to bridge the gap between ex situ conservation, and recovery in situ. They are especially useful when continuing access to in situ populations would place the species under additional pressure.

Living plant collections are similar to zoo animal collections in that they support the management of threatened species and contribute to habitat conservation (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 material of economic potential and provide material for other scientific investigations. Establishing a living plant collection that is genetically representative requires considerably more time and effort than some other forms of ex situ conservation, such as seed banking. The questions in Box 8.1 must be very carefully considered when contemplating the need for living plants as an ex situ conservation option. Current practice emphasises that living collections should be used with one or more other conservation techniques such as seed storage ex situ. There are circumstances, however, particularly with very rare species and species for which seed storage is not feasible, where it is the sole conservation option (see Case Study 8.1).

A plant conservation strategy might include cultivation of living plants for the following reasons:

- To produce material to aid recovery planning for reintroduction, population reinforcement, habitat restoration or management;
- To conserve species with desiccation sensitive seeds that cannot be maintained in a seed bank;
- To conserve species that produce non-viable, few or no seed;
- To ensure clonal replicates are maintained, where unique or elite genotypes need conservation;
- 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;
- 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 make material available for reference, education and display;
- To conserve species that are threatened but are easily cultivated.

(After www.bioversityinternational.org and www.bgci.org).

Prior to establishing a living collection, it is important to carefully consider the reasons for the collection, how it will be produced and maintained, and the end-use of the material. A guide to the most important issues to be considered is found in Box 8.1.

Box 8.1

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 of the collection?
- Is living plant conservation appropriate?
- 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 3.1 Checklist for Plant Material Collection?

8.2 Benefits and risks of living plant collections

A major benefit of cultivated collections is that living plants of key genotypes can be conserved with ready access. Material for further propagation and cultivation can be harvested from these plants, thus alleviating the need to collect from wild populations. This is especially important for rare species or those from remote locations. Special or rare genotypes can be conserved and distributed (see Case Study 8.1) to protect against the likely loss of the species in situ.

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

Case Study 8.1

The lonely King's Lomatia

Lomatia tasmanica (King's Lomatia, Proteaceae) is a Critically Endangered Tasmanian endemic (Threatened Species Section 2006). This species is thought to be a sterile hybrid which propagates by suckering and may be the oldest known living flowering plant in the world (Lynch et al., 1998).

The sole extant population is at risk from the root rot pathogen, *Phytophthora cinnamomi*, 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 Royal Tasmanian Botanical Gardens, the Australian National Botanic Gardens and Royal Botanic Gardens, Kew (UK).

Figure 8.1 Lomatia tasmanica. *Image: W.Potts.*

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, and this, as well as 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.

— Wendy Potts

sensitive seeds (Chapter 2.4.1.1) can be germinated and grown-on, in turn providing a source of seeds or vegetative material.

Living collections are available for conservation or biological research and plant breeders often use these collections as sources of desirable genotypes. Germplasm conservation can also be integrated with public or other horticultural collections, giving opportunities for public appreciation.

Living plant collections allow successive harvests of plant material for propagation without impacting the original populations. In some cases e.g. tree species, collecting can occur for many years. 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 harvesting (see Case Study 8.4).

Despite the many benefits of living collections, they are not without their limitations and risk. Major limitations are usually the costs involved in providing facilities for specialist propagation, establishing a physical space for the growth and maintenance of plants and facilities. Adding to cost is the consideration that, for trees in particular, the growing area required needs to be large enough to represent the genotypic range of a taxon.

Special attention is required to ensure maintenance of genetic integrity and continuity of curation if living collections are to be maintained over long periods, especially for trees and shrubs. 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. The result is that the collection may not reflect the genetic background of the original collection, or natural population.

In designing the layout for a living collection from which seeds might be collected, the possibility of unwanted hybridisation needs to be considered. Plantings in close proximity to closely-related taxa could potentially hybridise. For species requiring cross fertilisation, natural pollinators may be absent or in low numbers, and appropriate artificial pollination strategies need to be developed, requiring an understanding of the reproductive biology of the species (Cochrane and Barrett, 2009).

8.3 Types of living collections

8.3.1 Botanic and specialist horticultural gardens

Traditionally, botanic gardens are the main living germplasm repositories for threatened species, although the genetic diversity within species represented is often low. Living collections, in these circumstances, are either displayed in horticulturally attractive settings or as part of nursery stock and can be categorised as follows:

- Species collections – where species are planted according to a certain taxonomic grouping, such as by family.
- Clonal collections – which consist of genetically identical collections propagated asexually (see Case Studies 2.2 and 8.1)
- Conservation collections – consisting of threatened species or species from threatened ecosystems (see Case Study 8.2).

An advantage of a botanic garden, over many other types of gardens, 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. As a result, propagation material can be used to assist in the recovery planning for that species.

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,

Case Study 8.2

Conservation collections at Royal Botanic Gardens Melbourne

Five 'conservation beds' have been established at the Royal Botanic Gardens Melbourne (RBGM) 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. 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 subjected to 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 RBGM. Source material was vouchered by herbarium 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 RBGM 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 chances of successful local adaptation to the very different growing conditions at RBGM 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. 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. Planting commenced in April 2008, and despite the horror Melbourne summer of 2008/09, most plants have flourished with minimal irrigation.

— Neville Walsh

Figure 8.2 *Conservation beds at RBG Melbourne. Image: Neville Walsh.*

living plant collections are deliberately made to study the biology of a species. 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) 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.

8.3.2 Field genebanks

A field genebank is a collection of genotypes of a taxon. The term has often been used interchangeably with: 'living collection', 'plantation', 'clonal repository' or 'orchard' (IBPGR, 1991); but see this section for the terminology that should be used in a given situation. The term 'field' here may be taken to mean pots in a nursery, planted rows in the open or managed wild stands. Field genebanks provide material for a variety of purposes, from specific genotypes for horticultural purposes (Said and Rao, 2001; Reed et al., 2004) through to a wide range of taxa for revegetation (Florabank Guidelines at <www.florabank.org>) and, to a lesser extent, for threatened species conservation. For restoration of degraded habitats, especially at the broad-scale, field genebanks offer increasing opportunities for plant germplasm conservation.

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. A field genebank for the production of 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 avoided.

Over recent years there has been a change in Australia in the terminology applied to field genebanks for conservation. For many years, the term 'seed orchards' 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 on the basis of desired traits or characteristics. These are purpose-driven collections and may be sources of material with conservation value. However, although seed orchards are usually well-documented in terms of the source of material, plants growing in seed orchards may have been selected for particular characteristics and are likely to be genetic outliers of natural populations. Under this definition, therefore, 'seed orcharding' is not usually appropriate for the restoration of threatened species as the genetic composition may not reflect the wild population.

Field genebanking of native flora for conservation purposes is increasingly practiced by managing genetically diverse species collections in wild, semi-wild or cultivated situations. The level of management in field genebanking varies from minimal, such as the introduction of a species into a wild location (see Case Study 8.3), to intensive cultivation for high volume seed production (see Case Study 8.4).

Case Study 8.3

Conservation introductions of three WA threatened species

Banksia montana A.S.George (Proteaceae), *Persoonia micranthera* P.H.Wilson (Proteaceae) and *Leucopogon gnaphalioides* Stschegl. (Ericaceae) are threatened, narrow-range endemics that inhabit the low but richly diverse mountain peaks of the Stirling Range National Park in south-west Western Australia. These species have faced declines in population size and health in recent years due to a combination of frequent fire, grazing of seedlings and *Phytophthora cinnamomi* dieback, and are now facing the threat of extinction.

Although in situ conservation of wild plants is considered the most essential component of a biodiversity conservation program, the ability to adequately conserve natural populations jeopardised by disease is sometimes unachievable in the short term. Application of the fungicide Phosphite® is an effective short to mid-term strategy but establishment of new plants in a Phytophthora-free site was considered crucial to the long-term viability and recovery of each species.

In order to successfully implement recovery of these species in the wild, a number of challenges had to be overcome. In the case of *B. montana*, a limited number of seeds were available from ex situ seed storage. Seed was not available at all for *L. gnaphalioides* or *P. micranthera* due to a number of factors including grazing, low seed output from only a few mature plants, and difficulty in accessing populations.

These species were vegetatively propagated using cuttings in order to create a field genebank to act primarily as a seed production site. The field genebank could not be sited close to the original sites due to the lack of 'critical habitat' that was deemed disease-free.

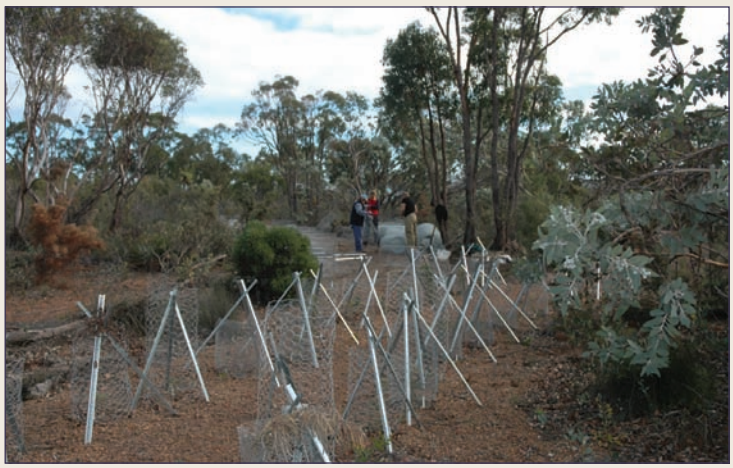

Figure 8.3 *Inter situs conservation planting southern Western Australia. Image: A.Cochrane.*

Case Study 8.3 continued

Therefore, a site was selected at considerable distance from the natural populations, in a lowland area considered 'safe' from disease. A section of the well-drained gravely loams of this remnant patch of revegetated bushland was deep ripped prior to planting, which was carried out in a number of stages, commencing with the planting of 14 individuals of *B. montana* in 2003. In 2004, a further 90 plants were added to the site and, in 2005, 100 plants each of the other two species were planted. Transplants were protected from herbivores and a watering system was installed.

By 2007 survival for all species was 70% or greater. Flowering of both *B. montana* and *L. gnaphalioides* commenced earlier than in the wild, possibly due to the more mesic environmental conditions at the new site. These inter situ populations will provide easy access for research and monitoring of the species' biology (e.g. reproductive biology). It will also provide representative genetic material for other ex situ conservation efforts, and as the source of material for potential translocation of the natural populations in the future, once the threat of disease has been alleviated.

— Anne Cochrane

8.4.2.1 Introduced populations grown inter situ

When new populations of a species are established under field conditions similar to the parent in situ populations, they are sometimes referred to as 'conservation introductions' which are grown 'inter situ' (at multiple sites) or 'inter situs' (at one site). Generally, these populations are grown in a wild or semi-wild situation analogous to the original population and constitute a type of 'translocation'. Access is managed and genetic diversity can be maintained as a self sustaining population and manipulated to produce material for other conservation purposes.

Conservation introductions provide an appropriate methodology for the rescue and recovery of threatened species with the opportunity to recreate 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 (see Case Study 8.3). 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.

8.3.2.2 Seed Production Areas

Seed Production Areas (SPA) is a term used by the revegetation industry to refer to plant populations established under field or nursery conditions with the primary or secondary objective of seed production. SPA offer potential for low cost, high volume production of material, mainly seeds, for large scale restoration purposes and translocation of threatened species. Ideally, the seed produced under these systems is more closely related to the

Case Study 8.4

Seed production of herbaceous Victorian species

The 'Grassy Groundcover Research Project' was initiated by Greening Australia and the University of Melbourne in November 2004 to investigate techniques required to assemble species-rich grassland on agricultural land by direct-seeding. Since then, there have been three annual sowings at each of thirteen 1 ha experimental sites across south-western Victoria (39 separate sowing in total) involving a diverse range of grassland species (approx. 200). The seed used in these sowings originated from either local grassland remnants (therefore taking provenance into account), or from plants propagated from the wild seed, in controlled seed production facilities.

Seed collection protocols for wild seed aimed to ensure genetic characteristics present in the field populations were adequately captured in production populations (e.g. collection from large numbers of individuals, over distance and time).

'Production plants' were only harvested for two seasons to guard against genetic bottlenecking in production crops. Following this, new plants were propagated and grown for production from wild collected seed. Over two years, six production sites (each linked to specific sowing areas) produced seed that supplemented field collections. These production facilities produced approximately 90kg of seed in two harvest seasons from a wide range of rare and threatened forbs and (to a lesser extent) grasses for direct sowing. Most of the approximately 200 grassland species (many rare or threatened) grown in these production facilities propagated readily from seed and were well suited to the intensive, (mostly) above-ground (in pots or raised beds) seed production system used.

Ex situ seed production (rather than wild collection), has simplified seed harvest and production of reliable quantities of high quality, weed-free seed at times when field production was severely restricted from the effects of drought.

— Paul Gibson-Roy

Figure 8.4 *(a) Mixed species for a box/ironbark herbaceous community restoration. (b) In-ground production of seed from a common grassland species* Chrysocephalum apiculatum. *(c) Containerised system for growing the nationally threatened daisy* Leucochrysum albicans *ssp.* albicans *var.* tricolour. *Images: P. Gibson-Roy.*

parent population than from that produced in a seed orchard system. For herbaceous species, plant 'crops' might be grown for seed on an annual or biannual basis, after which new stock is propagated from wild seed for the living collection. For more information refer to Florabank Guideline 7 (www.florabank.org).

SPAs fall into three categories:

1. **Commercial cultivation** – where the SPA is managed to provide seed/plant material to supply the restoration industry. These SPAs are mostly established using common colonising woody perennials e.g. Acacias and Eucalypts. However, commercial SPAs have been established to grow seed for restoration of a large range of herbaceous and grassland species e.g. grasses, daisies, lilies; such ecosystems often contain threatened species which may benefit from ex situ seed production (see Case Study 8.4).
2. **Community cultivation** – commonly used by Landcare and Field Naturalist 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** – collections that 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.

8.4 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. 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 - depends on the capabilities of staff and availability of technology);
- the likely genetic erosion of the collection over time;
- the ability of the taxon to adapt to cultivation; and,
- the resources available to establish and manage the collection.

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, avoiding skewing of the ex situ population towards extremes, and therefore reflecting the broad wild population diversity (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 could

be sampled. 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 (see Case Study 2.2). Where resources are limiting, an alternative strategy is to disseminate individual plants to other organisations where accession information is retained (multi-site collection). This strategy ensures that collections have a greater chance of survival in the case of unanticipated unfavourable events e.g. watering failure.

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 prior to translocation in a potted nursery collection than existed in the natural population (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 (Chapter 3), and to ensure that this diversity is maintained for the required life of the collection.

8.5 Propagation and cultivation

The major propagation types for living collection establishment are:

1. Seed (or extracted embryos).
2. Cutting or division.
3. Tissue culture.
4. Grafting or budding.
5. Transplanting.

Cultivated collections can be derived from one or a number of these techniques, from ex situ collections, or direct from wild populations. To maximise genetic diversity, propagation from seed is preferable. 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.

Where seed is unavailable, ungerminable or the genotype of the individual needs to be replicated, vegetative propagation through cuttings or tissue culture (Chapter 6) may be appropriate. An ex situ population established using vegetatively propagated material should represent as many parent plants as possible (see Chapter 3), being mindful of the constraints imposed by the plants interaction with the cultivation environment as well as physical and resource limitations e.g. staff to maintain large numbers of plants. While the techniques for vegetative cutting propagation are not treated as a separate chapter in these guidelines (cf. seed germination and tissue culture) it is a valid and widely used technique.

Many references detail the principles and practices of propagation, e.g. Hartmann et al. (2002) for general principles, Bowes (1999) for conservation collections or Stewart (1999) for Australian plants.

Living plants can be cultivated in soil or artificial media, in garden beds or in pots, large or small. Special attention should be paid to the soil or potting mix, fertiliser and watering regimes for many species, and the reader is referred to Handreck and Black (2002) for consideration of growing conditions for Australia.

8.6 Management of living collections

High conservation-value living plant collections should be considered separately from collections for other purposes such as horticultural display. Living collections are one of the most vulnerable means of maintaining germplasm and they require intensive management to prevent loss of material through disease, watering failures etc. 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). Meticulous record keeping is vital in the management of living collections. As for other types of collections, as much of the information about the original collection must be maintained (see Chapter 3), 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. Good database systems should aid in managing record keeping.

Various manuals for management of living collections for conservation and related purposes are available from Botanic Gardens Conservation International (BGCI), e.g. Leadlay and Greene (1995), and Bioversity International. Reference should also be made to Australian Network for Plant Conservation publications, especially '*Guidelines for the Translocation of Threatened Plants in Australia*' (Vallee et al., 2004) where issues dealing with the management of translocation sites can also be applied to field genebank sites. Greening Australia Capital Region has published two brochures dealing with the establishment of SPAs, '*Introducing… Seed Production Areas, An Answer to Native Seed Shortages*' and '*Sex in SPAs, Genetic Issues in Seed Production Areas*' that provide basic coverage of the topic (<http://www.greeningaustralia.org.au/community/capital-region>).

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Glossary

abiotic	non-living components of the natural world such as soils and climate.
accession	unique identifying code; in the context of ex situ collections, usually refers to material collected from a single population on a particular date.
active collection	the portion of an ex situ collection for general use (cf. base collection, which is used only in defined circumstances).
after-ripening	the progressive loss of dormancy in mature, dry seeds; controlled by factors such as temperature and seed moisture content.
allele	one of two or more alternative forms of a gene.
angiosperm	a flowering plant; a division of Spermatophyta that produce ovules and seeds in closed megasporophylls (carpels) cf. gymnosperm.
anthropogenic	resulting or produced by human beings.
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.
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.
base collection	the portion of an ex situ collection kept separately from the active collection, and only used only in defined circumstances.
biodiversity	the variety of life forms, the genes they contain and the ecosystems they form.
biodiversity hotspot	areas that support natural ecosystems that are largely intact and where native species and communities associated with these ecosystems are well represented; areas with a high diversity of locally endemic species, which are species that are not found or are rarely found outside the hotspot; they are also areas subject to threatening processes rendering species within them prone to extinction.
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 and followed by increased inbreeding.

Glossary

bradysporous	(species) store seeds in the tree canopy; syn. serotinous (preferred).
bryophytes	a taxonomic division of non seed-bearing vascular plants which produce spores; mosses and liverworts.
caespitose	growing in tufts or patches.
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	seedbank formed on the plant by serotinous (bradysporous) species; many such species shed their seeds after fire.
chromosome	the structure that carries genetic information.
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, which in horticulture are known as a cultivar or variety; 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.
co-culture	culture of two or more organisms together resulting in benefit to one or more organisms e.g. culture of symbiotic orchids and fungi.
cohort	a group of individuals of the same age, recruited into a population at the same time.
conservation (plant)	management actions that allow the taxon to follow an evolutionary path in its natural environment cf. preservation
cryopreservation	preservation of cells, tissue, organs, etc., through storage at very low temperatures in or over liquid nitrogen (usually <-130°C).
cryovials	vials used to store material for cryopreservation.
cut-test	a technique used to determine the viability of a seed; when cut (usually in half), a viable seed should have firm 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 promote tissue growth and budding; commonly used in tissue culture to promote cell division.
dehiscent fruit	any fruit in which the fruit wall splits open at maturity releasing seeds.
desiccation sensitive species	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
desiccation tolerant species	(of a seed) see orthodox species.
dewar	a 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 smallest unit of seed dispersal in plants (e.g. fruit, seedling or seed).
dioecious	species that have male and female flowers on separate plants.
dispersal	(of a seed) can occur through gravity, wind, water or other vectors such as animals.
dispersal mechanism	(of a plant) the distribution of seeds following release from the parent plant; most commonly Australian species are geosporous, but serotinous species occur in many fire-prone areas.
dispersal unit	see diaspore.
DNA	the genetic material of most living organisms; deoxyribonucleic acid.
DNA fingerprinting	a method employed to determine differences in DNA characteristics among individuals; these can be used to identify single individuals from the rest of the population.
dormancy	property of a seed that prevents its germination even under environmental conditions that normally favour germination; usually operates to prevent the seed germinating at a time that is not conducive to good seedling growth and establishment.
ecosystem	a unit comprising a community of living organisms and their environment.
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.
edaphic	of the soil, substrate or topography.
embryo	the result of fertilization, 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).
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 and with a limited distribution; a high level of endemism often occurs in floras that have been fragmented 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. drupes.

endosperm	the storage tissue in the seeds of most angiosperms.
ephemeral	a plant with a short life cycle.
epigenetic	environmentally or developmentally induced variations in the phenotype that persist once induced and are perpetuated by cloning, but which are not reflected in the genotype.
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).
ex situ conservation	conservation of an organism away from its original habitat; the maintenance of germplasm away from the wild.
exocarp	the outermost layer of some fruits e.g. orange 'skin'; the additional layer(s) beyond the pericarp of anthocarpous fruits (which have attached floral parts that have undergone a marked development during post-fertilisation to aid in the dissemination of the seed).
explant	the excised portion of a plant used to initiate and perpetuate a tissue culture.
extant	existing or living now.
extinct	of taxa, not located in the wild during the past 50 years, or not been found in recent years despite thorough searching.
fecundity	the rate of reproduction, productivity, fruitfulness, abundance; low fecundity results in a low number of seed produced.
fruit	the fruit is the ripened ovary that encloses the seed or seeds.
gene	the functional unit of heredity; the part of the DNA molecule that encodes a single enzyme or structural protein unit.
gene flow	exchange of gene diversity among individuals or populations.
generation length	average age of all breeding individuals.
genet	genetically distinct individual (cf. ramet).
genetic diversity	the sum total of all genetic variation for a population, species or other taxonomic rank.
genetic drift	the random change in allele frequencies due to chance; 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.
genotype	the genetic constitution of an individual, fixed except under certain conditions (mutation).
geosporous	species have a dispersal mechanism that releases their annual production of seeds into the soil seedbank.
germination	the events occurring in a seed between imbibition and emergence, usually of the radicle (root).
germplasm	1. living plant material; 2. the genetic material that carries the heritable characteristics of an organism e.g. cells, seed, plants etc.
gibberellic acid	a family of plant growth regulating compounds that can regulate seed germination and other aspects of plant growth; often used as a germination promoter.
gymnosperms	a group of plants - conifers, cycads & Gnetatae - 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.
heterozygous	having two different alleles at a given locus of a pair of chromosomes (cf. of homozygous).
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.
hygrometer	used for non destructive measurement of eRH.
imbibition	the uptake of water by a seed from the surrounding medium.
inbreeding	the mating of individuals related by descent, usually causing a reduction in gene heterozygosity and diversity.
inbreeding depression	a reduction in vigour and fitness due to inbreeding.
indehiscent fruit	a fruit that does not open at maturity to expose or release the seeds.
intermediate species	have seeds that can withstand desiccation to around 10-12%, or in some species as low as 8% moisture content; often maintain viability best at temperatures above freezing; sometimes classed with 'orthodox' seeds in terms of storage capability.
inter situ conservation	plants cultivated in near-natural conditions for conservation reasons.
introgression	the incorporation of genes of one species or subspecies into the gene pool of another which usually occurs

	through hybridisation i.e. pollen of one taxon fertilising the ovule of another, producing fertile seed.
in situ	the original place; pertaining to the maintenance of plants in the wild.
in vitro	an artificial environment (such as a test tube).
longevity (seed)	the life span of a seed or batch of seeds.
masting	an event during which a large crop of seeds is produced by trees; often synchronised within a population.
mesocarp	the fleshy middle layer of the fruit wall (pericarp).
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 dry weight x 100 (%).
morphology	the form of the external structure cf. anatomy, which refers to the internal structure.
mutation	a change within the genetic system (by either a gene or chromosome) which produces in the mutant (or variant) a slight or profound effect.
mycorrhiza	a non-pathogenic association of a fungus with a vascular plant or bryophyte.
orthodox species	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).
outbreeding	production of progeny by the fusion of distantly related gametes (cf. inbreeding).
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.
phenology	the study of the influence of seasonal and other environmental conditions on the recurrence of flowering, seed production and other life cycle events.
phenotype	the expressed characteristics of an organism, determined by the interaction of the genotype with the environment.
pollination vector (pollinator)	the mode by which pollen is successfully transferred from the anther to the stigma of the same flower or another flower.
population	a local community of potentially interbreeding organisms.
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.
propagule	a unit of reproduction (includes seed, spores, or vegetative matter capable of independent growth).
provenance (genetic)	the place of origin of a collection; area containing a population of a species that is genetically distinct from other populations; usually thought to represent genetic adaptation to local environmental conditions.
pteridophytes	a taxonomic division of non seed-bearing vascular plants which produce spores; ferns and fern-allies
ramet	plant formed by asexual reproduction; a physically but not genetically distinct individual (cf. genet).
recalcitrant seeds	are intolerant of desiccation and rapidly lose viability when bulk 'free' water is removed by drying; such seeds cannot therefore be stored at sub-zero temperatures (cf. orthodox seeds); typically found in wetter environments e.g. species from the wet-tropics; often have large-seeds, thin seed coats and fleshy-fruits.
rehabilitation	the process of improving specific ecosystem factors in a degraded habitat.
restoration	the return of a degraded habitat to its original species composition, structure and function.
revegetation	to provide a habitat with vegetation; may not necessarily include original provenance or species composition.
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 fertilization and some growth within the mother plant.
self-sustaining (population)	a population of plants that maintains itself without external assistance.
serotinous	species that retain their non-dormant 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).
soil seedbank	seeds that are stored in 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 on callus (indirect embryogenesis).
somatic tissue	tissue derived from a body cell i.e. non-sexual origin.
sporophyll	a modified leaf that bears sporangia, the structures within which spores (reproductive bodies) are formed.
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.
stratification	chilling or warm-temperature treatments (of moist seeds) used to break dormancy in some species.
symbionts	1. (narrow sense) two organisms living together to their mutual benefit (syn. mutualism); 2. (wide sense) other relationships such as commensalism – which benefit only one partner.
taxon (taxa)	the named classification unit to which individuals are assigned e.g. genus, species, subspecies, variety etc.
testa	part of the seed coat derived from the outer or single integument (layers covering the nucellus).
tetrazolium test	a staining method used to test seed viability; a healthy embryo usually stains red, but staining patterns vary from species to species.
tissue culture	the culture 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.
viable seed	a living seed, although often apparently acquiescent, capable of eventual germination; seeds may appear non-viable if they are dormant and/or not given suitable germination conditions at a point in time.
viable population	a group of plants of the same species that possesses the ecological, demographic and genetic attributes required to persist in both the short and long term.
voucher specimen	a herbarium voucher specimen is a pressed and dried plant sample that can be stored for future reference; should include key plant parts used in formal identification.
zygotic embryo	embryo formed following fertilization or union of haploid male (sperm) and female (ovum) sex cells, inheriting traits from both parents.

Appendix 1: Contacts

Government Conservation Agencies

National

Department of Environment, Water, Heritage and the Arts
PO Box 787
Canberra ACT 2601
Tel: 02 6274 1111
Fax: 02 6274 1666
www.environment.gov.au/

Australian Capital Territory

Department of the Environment, Climate Change, Energy and Water
GPO Box 158
Canberra City ACT 2601
Tel: 13 22 81
Fax: 02 6207 6084
Email: environment@act.gov.au
www.environment.act.gov.au

New South Wales

Department of Environment and Climate Change
PO Box A290
Sydney South NSW 1232
Tel: 02 9995 5000
TTY: 02 9211 4723
Fax: 02 9995 5999
Email: info@environment.nsw.gov.au
www.environment.nsw.gov.au

Northern Territory

Natural Resources, Environment, The Arts and Sport
PO Box 496
Palmerston NT 0831
Tel: 08 8999 5511
www.nt.gov.au/nreta/

Queensland

Department of Environment and Resource Management
PO Box 15155
City East Qld 4002
Tel: 07 3227 7111
www.derm.qld.gov.au

South Australia

Department of Environment and Heritage
GPO Box 1047
Adelaide SA 5001
Tel: 08 8204 1910
Fax: 08 8124 4939
www.environment.sa.gov.au

Tasmania

Department of Environment, Parks, Heritage and the Arts
GPO Box 771
Hobart Tas 7001
Tel: 03 6233 5512
Fax: 03 6233 5905
www.depha.tas.gov.au

Victoria

Department of Sustainability and Environment
PO Box 500
East Melbourne Vic 3002
Tel: 03 9637 8000
TTY: 1800 122 969
Fax: 03 9637 8100
Email: customer.service@dse.vic.gov.au
www.dse.vic.gov.au

Western Australia

Department of Environment and Conservation
Locked Bag 104
Bentley Delivery Centre WA 6983
Tel: 08 6467 5000
Fax: 08 6467 5562
www.dec.wa.gov.au

State Herbaria

Contact details for the major Australian Herbaria can be found at the Council of Heads of Australian Herbaria (CHAH) web page:
www.chah.gov.au/chah/index.html

State Botanic Gardens
Contact details for the major Australian Botanic Gardens can be found at the Council of Heads of Australian Botanic Gardens (CHABG) web page: www.chabg.gov.au/chabg/

Plant-related resources

Atlas of Living Australia
A five-year project funded under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS). Its mission is to develop a biodiversity data management system which will link Australia's biological knowledge with its scientific and agricultural reference collections and other custodians of biological information.
http://www.ala.org.au/

Australian Association of Bush Regenerators
c/- Total Environment Centre
PO Box A176
Sydney South NSW 1235
Tel: 0407 002 921
Email: enquiries@aabr.org.au
www.aabr.org.au

Australian Bureau of Meteorology
Head Office
GPO Box 1289
Melbourne Vic 3001
Tel: 03 96694000
http://www.bom.gov.au/index.shtml

Australian Network for Plant Conservation
ANPC National Office
GPO Box 1777
Canberra, ACT 2601
Tel: 02 6250 9509
Email: anpc@deh.gov.au
http://www.anbg.gov.au/anpc

Australian Seed Conservation & Research

Contact details for the Australian partners in the Millennium Seed Bank Project, Kew can be found on the Kew web site listed below.
www.kew.org/msbp/where/Australia.htm

Australian Tree Seed Centre (CSIRO)

The Australian Tree Seed Centre is part of CSIRO Forest Biosciences, a division of CSIRO.
Officer in Charge
The Australian Tree Seed Centre
CSIRO Forest Biosciences
PO Box E4008
Kingston ACT 2604
AUSTRALIA
Tel: 02 6281 8211
Fax: 02 6281 8266
Email: atsc@csiro.au
www.csiro.au/content/pt6.html

Australian Virtual Herbarium (AVH) (portal to Australian plant databases)

http://www.anbg.gov.au/avh/

Florabank

Florabank is an initiative of the Australian Government, Greening Australia and CSIRO
C/o Greening Australia
PO Box 74
Yarralumla ACT 2600
Tel: 02 6202 1600
www.florabank.org.au

Millennium Seed Bank Project

Wakehurst Place
Ardingly
Haywards Heath
West Sussex
RH17 6TN
United Kingdom
Tel: +44 (0)1444 894100
http://www.kew.org/msbp/index.htm

Nursery and Garden Industry Australia
Contact details for the State and Territory Associations can be found by contacting the National Office or by visiting the web site listed below.
PO Box 907
Epping, NSW 1710
Tele: 02 9876 5200
Fax: 02 9876 6360
www.ngia.com.au/contact_us/contact_us.asp

Seed Information Database (SID)
Millennium Seedbank Project,
Royal Botanic Garden Kew.
http://data.kew.org/sid/

Information on key threats to Australian plants

Phytophthora cinnamomi
Information on *Phytophthora cinnamomi* is available on the Botanic Gardens Trust Sydney web site at
http://www.rbgsyd.nsw.gov.au/science/Research_programs/phythophthora_in_national_parks, which includes a Fact Sheet on controlling the spread of the disease developed by plant pathologists for the disease in NSW
http://www.rbgsyd.nsw.gov.au/plant_info/pests_diseases/fact_sheets/phytophthora_root_rot
The Western Australian Dieback Working Group maintains an excellent website providing information on the management of Phytophthora dieback at http://www.dwg.org.au/
The National Strategy for managing Phytophthora can be found at:
http://www.environment.gov.au/biodiversity/invasive/publications/p-cinnamomi.html

Invasive weeds
http://www.weeds.gov.au/

Salinity
http://www.environment.gov.au/land/pressures/salinity/index.html

Climate change
http://www.anbg.gov.au/climate-change/botanic-gardens-resources.html

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Anne Cochrane manages the Western Australian Department of Environment and Conservation's Threatened Flora Seed Centre, a seed bank for the conservation of endemic and conservation-listed flora. Her work includes research into seed ecology and biology, as well as the collection of seeds for use in recovery.

Andrew Crawford is the Principal Technical Officer at the Western Australian Department of Environment and Conservation's Threatened Flora Seed Centre. Andrew's work encompasses all stages of the seed conservation process from collection, storage and viability testing, to the utilisation of seeds for reintroductions. Andrew is currently undertaking a PhD examining aspects of seed longevity.

Kim Hamilton has worked on international and local projects on conservation and use of tropical and rainforest plant diversity in support of sustainable food production and natural ecosystem conservation. Kim is currently based at Mount Annan Botanic Garden (Royal Botanic Gardens and Domain Trust, Sydney) where she is coordinator and postdoctoral scientist of the Rainforest Seed Project which is investigating the seed biology of Eastern Australian rainforest species.

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Contributors

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David Merritt is a Research Scientist at Kings Park and Botanic Garden, Perth. David manages the seed science programs at Kings Park and his research is focussed primarily on the use of seeds in conservation and restoration. David's research encompasses seed storage behaviour and low-temperature storage physiology, and the ecophysiology of seed dormancy and germination.

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Shane Turner is currently a post-doctoral researcher employed by the University of Queensland but based in Kings Park and Botanic Garden where he has been since 2002. Dr Turner works mainly on the seed biology of Australian native species focusing on problematic taxa required for mine site and urban bushland restoration. In addition, he also works on the tissue culture and cryostorage of native plants.

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