Seed Storage Behaviour of New Zealand's Threatened Vascular Plants

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ABSTRACT

At least 38% of New Zealand's approximately 2300 vascular plant taxa (species and subspecies) are threatened *in situ* and are vulnerable to extinction. It has become increasingly important that a number of different complementary conservation approaches and methods are used to insure against this loss of species. However, limitations exist for the development of *ex situ* storage techniques due to a lack of basic research on seed biology, including seed storage behaviour and germination requirement of many rare and threatened species.

Seven New Zealand native species, *Carmichaelia williamsii*, *Clianthus puniceus*, *Clianthus maximus*, *Hibiscus diversifolius*, *Myosotidium hortensium*, *Tecomanthe speciosa* and *Dysoxylum spectabile* are a priority for investigation for *ex situ* conservation strategies because of their conservation priority and lack of corresponding techniques for their long term *ex situ* conservation. This study reports on seed storage behaviour, dormancy mechanisms and potential *ex situ* conservation strategy including conventional seed storage and cryopreservation of these New Zealand indigenous species to facilitate germplasm storage.

C. williamsii, *C. puniceus*, *C. maximus*, *H. diversifolius*, *M. hortensium* and *T. speciosa* were found to tolerate low seed moisture content (<5%) and thus can potentially be stored for long periods under conventional seed bank conditions, whilst *Dysoxylum spectabile* displayed essentially recalcitrant seed storage behaviour i.e. loss of viability when desiccated below 25%. There is a need to consider alternative *ex situ* conservation strategies, such as cryopreservation, to conserve this species.

Both *C. maximus* and *M. hortensium* displayed 'essentially' orthodox seed storage behaviour i.e. tolerance to desiccation and low temperature. However, viability of *M. hortensium* seeds declined after 12 months in all storage treatments and may be defined as sub-orthodox.

This study demonstrated embryonic axis cryopreservation in *D. spectabile* using a method based on rapid desiccation, encapsulation-dehydration and vitrification. This is the first report of the successful cryopreservation of New Zealand native species. In *D. spectabile*, encapsulation-dehydration or vitrification-based cryopreservation gave higher levels of survival (20%) than rapid desiccation cryopreservation. However, further optimisation of the protocol for plantlet recovery is needed to improve efficiency before it can be considered suitable for conservation purposes.

It is essential to understand dormancy mechanisms and to have effective methods of seed germination so seed banks can increase their efficiency and enhance their role in biodiversity conservation. However, germination requirements for many native species are either incomplete or not available. Unlike *T. speciosa* and *D. spectabile*, seeds of *C. williamsii*, *C. puniceus*, *C. maximus*, *H. diversifolius* and *M. hortensium* were dormant and that this dormancy was found to be of function of the seed coat. Dormancy in *C. williamsii*, *C. puniceus*, *C. maximus* and *H. diversifolius* is a result of the seed coat preventing water uptake by the dry seed. While physical constraint of embryo growth by the seed coat is the cause of dormancy in *M. hortensium* seeds.

The findings of this study will facilitate the *ex situ* conservation of New Zealand native species, to complement *in situ* conservation and help secure the future of the New Zealand's native flowering plants.

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CHAPTER 1 INTRODUCTION

The unique and diverse flora of New Zealand is threatened *in situ* and is vulnerable to erosion of genetic diversity and extinction. Recently, 901 taxa (38% of the total vascular flora) were classified as threatened or naturally uncommon, with six believed to be extinct (de Lange, Heenan, Norton, Rolfe, & Sawyer, 2010; de Lange, et al., 2009). Although there has been considerable success in threatened species management including single-species recovery programmes (Craig, et al., 2000), the increase in the number of taxa listed as 'threatened' has continued, i.e. from 122 taxa (de Lange, et al., 2004) to 184 (de Lange, et al., 2010). In addition, large-scale environmental changes, such as global warming, could lead to the extinction of species at an increasing rate and even non-threatened taxa could become threatened in the future (Department of Conservation, 2000; Gitay, Suárez, Watson, & Dokken, 2002). In order to combat further species loss, a range of conservation solutions including both *in situ* protection and restoration and *ex situ* conservation is required.

Complementary to *in situ* conservation, some form of *ex situ* conservation is considered an important component to insure against loss of species. *Ex situ* approaches range from whole plants (e.g. field gene bank or botanical gardens) to seed, pollen or vegetative material (e.g. *in vitro* minimal growth or shoot tip cryopreservation) (Maunder, Havens, Guerrant, & Falk, 2004). The majority of the world's germplasm collections (about 90% of the 6.1 million accessions; FAO, 1996) are seed-based, as this is the simplest and safest option for desiccation tolerant seeds. Such long-term seed storage uses a standard method, i.e. storage of seed with 3-7% seed moisture content (fresh weight basis, depending on the species) at -18°C or cooler (FAO/IPGRI, 1994). Under these conditions, seed viability may be maintained for a long period, possibly centuries (Theilade & Petri, 2003). However, not all species are suitable for this storage method, and for such species, cryopreservation techniques have been suggested as an alternative strategy for long term storage.

Cryopreservation is the long-term storage of material at ultra low temperatures (e.g. liquid nitrogen, -196°C) at which metabolic activities cease (Kartha & Engelmann,

1994). There are various options to consider for cryopreservation techniques in terms of both type of explants (e.g. seed, somatic embryos, shoot tips) and technique. There are several reports that seed cryopreservation with intermediate seed or recalcitrant seed species is successful. Intermediate seed is seed that can be desiccated to low moisture but not to the low levels of orthodox seed. Intermediate seed is often often sensitive to chilling. Seeds of relatively small size and tolerant to desiccation have been cryopreserved directly after partial desiccation. With other species, where the entire seed is not amenable to cryostorage, embryos or embryonic axes are an alternative for long-term cryopreservation (Berjak & Dumet, 1996; Engelmann & Engels, 2002; FAO, FLD, & IPGRI, 2004).

Another option for *ex situ* storage of germplasm of non-orthodox seeded species is cultivaton in field collections. The field-based approach to maintaining germplasm has the adavantage of allowing easy access to conserved material when needed, but, field based collections are expensive to maintain and there is the risk of loss from , pests and disease, and natural disaster. Cryopreservation could potentially minimise such risks. Furthermore, compared with other storage techniques, cryopreservation requires very little space and maintenance requirements are low (Engelmann, 1997b; Padayachee, Watt, Edwards, & Mycock, 2009; Rao, 2004).

Effective methods for seed germination are essential for the conservation of threatened species, for the maintenance of a seed collection and for the use in restoration programs. The dormancy-breaking treatments must be able to alleviate dormancy in a large number of seeds relatively simply and/or quickly, and be repeatable and readily applicable to seeds (Godefroid, Van de Vyver, & Vanderborght, 2010; Merritt, Turner, Clarke, & Dixon, 2007; Walsh, Waldren, & Martin, 2003). However, germination requirements for native species are often unknown, particularly for rare and/or endemic species of which material is more difficult to obtain.

For many New Zealand threatened species, information on seed physiology, seed storage requirements and seed dormancy release mechanisms needed for the development of appropriate *ex situ* conservation strategy is either incomplete or not available.

The goal of this study is to determine seed storage behaviour and *ex situ* conservation strategies in selected New Zealand's threatened native species.

Seven New Zealand's native species, *Carmichaelia williamsii*, *Clianthus puniceus*, *Clianthus maximus*, *Hibiscus diversifolius*, *Myosotidium hortensium*, *Tecomanthe speciosa* and *Dysoxylum spectabile* are the focus of my investigations. These were selected based on their threat of extinction, availability of resources. The list includes species with lipid-rich orthodox seeds (*M. hortensia*) presumed to be problematic for long-term seed bank storage, and those with probable non-orthodox seed storage behaviour (*D. spectabile*) and lack of corresponding knowledge for their long term *ex situ* conservation. Seed of endangered species is difficult to obtain in sufficient quantities for the research into non-orthodox seed storage behaviour undertaken here. *D. spectabile* which has shown intolerance to dehydration and conventional long term storage was therefore selected to investigate cryopreservation storage options.

Specifically, the objectives of this study were to:

- Characterise the desiccation sensitivity and germination requirement of these native species
- Evaluate suitable conservation strategies, including dry storage and/or cryopreservation methods for these native species
- Determine the optimum seed moisture content and temperature for dry storage
- Develop cryopreservation storage options for non - orthodox species

CHAPTER 2 LITERATURE REVIEW

2.1 Rare and threatened vascular plants in New Zealand

New Zealand possesses an indigenous vascular plant flora of c.2,300-2,470 taxa (Department of Conservation, 2000; Dopson, et al., 1999; Wardle, 1991) with a very high rate of endemism (80%-85%) (Department of Conservation, 2000; Dopson, et al., 1999; Ministry for the Environment, 1997; Wardle, 1991). The New Zealand flora is internationally regarded as one of the world's biodiversity hotspots 'one of the richest and most threatened reservoirs of plant and animal life on earth' (Conservation International, 2007; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). New Zealand's plant genetic diversity, however, has been in decline and many species have become vulnerable to extinction. Many of the major threats to the New Zealand flora are result of the habitat loss, invasion of introduced species and the adverse effects of human activities (Department of Conservation, 2000; Halloy, 1995). Although 314 taxa were considered at risk in 1976 (Given, 1976), in a recent reappraisal of the conservation status of New Zealand indigenous flora, 901 taxa are classified as threatened or naturally uncommon, with six believed to be extinct (Table 2.1) (de Lange, et al., 2010; de Lange, et al., 2009).

Table 2.1 Summary statistics for threatened and uncommon vascular plant in New Zealand.

THREAT CATEGORY	NO. SPECIES
Nationally critical	94
Nationally endangered	46
Nationally vulnerable	44
Declining	83
Recovering	6
Relict	20
Naturally uncommon	542
Vagrant/Coloniser	25
Data deficient	35
Total threatened	895
Extinct (since 1840)	6

Modified from de Lange et al. (2009, 2010).

The increase in the species listed between 1976 and 2010 is most likely the result of an increased knowledge, but may also reflect an increase in risk. At present many taxa are threatened with extinction in their natural habitats, this is despite more than 30% of New Zealand's total land area being reserved and single-species recovery programmes having considerable success (Craig, et al., 2000; Halloy, 1995). In addition, large-scale environmental change, such as global warming, could render a wide range of species extinct in their natural habitat. As temperatures and climate patterns change, new tropical pests and diseases may potentially become established New Zealand (Department of Conservation, 2000; Ministry for the Environment, 2007). With impacts on habitat loss, climate change could lead to the extinction of species at an increasing rate (Gitay, et al., 2002). There is also a potential risk of an increasing number of introduced plants becoming naturalised and competing with native plants. New Zealand has more introduced plants than native plants species (Figure 2.1), i.e. 7.8% are native, 7.8% are introduced plants that have naturalised (become established in the wild) and 84.4% are introduced plants in cultivation or gardens, but not yet naturalised. In 2000, it was estimated at least 240 of these introduced plants have potential to displace native plants. This number is growing each year (Department of Conservation, 2000). In order to combat further species loss, a range of conservation solutions including both *in situ* protection and restoration and *ex situ* conservation is required.

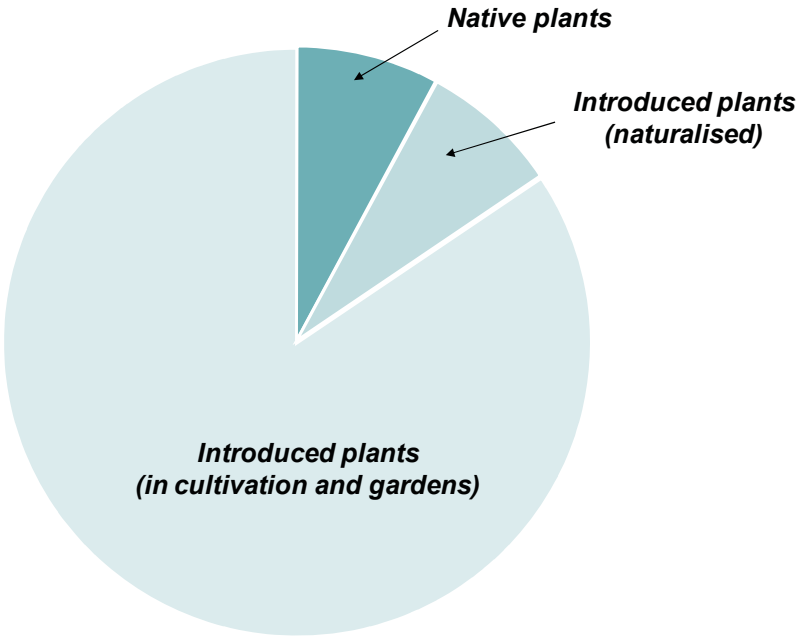

Figure 2.1 Composition of New Zealand flora (Department of Conservation, 2000).

The New Zealand Biodiversity Strategy 2000 (NZBS) has been prepared in response to an increasing rate of New Zealand's indigenous biodiversity decline. It also reflects New Zealand's 1993 ratification of the International Convention on Biological Diversity (CBD), to help prevent the loss of biodiversity on a worldwide scale (Department of Conservation, 2000; Ministry for the Environment, 2007). The main objectives of the CBD are to conserve, sustainably use and equitably share the benefits of biological diversity ("Convention on Biological Diversity," 1992).

The five key elements of the Convention for plant conservation work are ("Convention on Biological Diversity," 1992; New Zealand," 2001) ;

(a) Identifying status and trends

(b) Protecting areas to conserve

(c) Managing threats

(d) Promoting recovery activity

(e) Developing conversation strategy and action plans

The CBD adopted the Global Strategy for Plant Conservation (GSPC) to protect against the loss of plant biodiversity in 2002. Since the GSPC was completed after the NZBS was prepared, there is no specific national strategy for plant conservation. However New Zealand supports the general concept of the GSPC (Convention on Biological Diversity, 2002; Ministry of Foreign Affairs and Trade, 2007).

The GSPC prioritised threatened species for plant conservation using both *in situ* and *ex situ* measures (Convention on Biological Diversity, 2002). New Zealand is generally focusing on *in situ* conservation. Management is through protection of sites/ species, restoration programmes and controlling introduced species. Examples of legislation involved in plant biodiversity conservation (*in situ* approach) include The Native Plants Protection Act 1934, Reserves Act 1977, Conservation Act 1987, Resource Management Act 1991(Ministry for the Environment, 1997, 2007). However, there has

been recognition that *ex situ* conservation is an important complement *in situ* method. The NSBS (Department of Conservation, 2000) stated this in principle 8:

"Biodiversity is best conserved in situ by conserving ecosystems and ecological processes to maintain species in their natural habitats. Ex situ measures will be important to support the conservation of some species."

Several *ex-situ* collections where native plants are cultivated exist throughout New Zealand. These include The Auckland Regional Botanic Garden and Otari-Wilton's Bush Open Air Plant Museum. In 2007, a national seed bank has been developed by New Zealand Plant Conservation Network. This provides insurance against loss for species of acutely threatened indigenous plant that are able to be conserved in seed bank storage (Knightbridge, 2006; Ministry of Foreign Affairs and Trade, 2007).

2.2 Priority species

Research on the seed physiology and especially storage behaviour of a species is essential for the development of appropriate *ex situ* conservation strategies. This is especially important for species with intermediate and recalcitrant seeds. Several criteria for selection of wild species for *ex situ* conservation can be considered. This can include (Maxted & Guarino, 2003; Maxted, Hawkes, Guarino, & Sawkins, 1997; Reed, Engelmann, Dulloo, & Engels, 2004);

(1) Current conservation status

(2) Vulnerability to species extinction and/or genetic erosion

(3) Genetic distinctiveness

(4) Potential socio-economic value

(5) Availability of resources

For many of New Zealand's rare or threatened species, information on flora and seed physiology, regeneration system, genetic diversity and population dynamic needed for the development of appropriate *ex situ* conservation strategies is either incomplete or unavailable. After consideration of the above issues, seven New Zealand native species were chosen for further study in this PhD project. This was based on their threat of extinction, availability of resources, probable orthodox and non-orthodox seed storage behaviour and lack of corresponding knowledge required for their long term *ex situ* conservation. Seed of endangered species is difficult to obtain in sufficient quantities for the research into non-orthododx seed storage behaviour undertaken here. *D. spectabile* which has shown intolerance to dehydration and conventional long term storage was selected to investigate cryopreservation storage options. The protocols developed with *D. spectabile* should be applicable to endangered species.

2.2.1 *Carmichaelia williamsii* Kirk. (Williams broom, giant-flowered broom)

The genus *Carmichaelia* (Fabaceae) comprises about 23 species endemic to New Zealand (Heenan, 1998a), of which 15 species have been classified as nationally threatened or uncommon (de Lange, et al., 2009). One species, *Carmichaelia williamsii* Kirk. is unique in that it is the only yellow-flowered species of *Carmichaelia* in New Zealand (Heenan & De Lange, 1999; Wilson & Given, 1989).

C. williamsii is confined to coastal exposures of open forest, scrub, cliff faces and talus slopes (New Zealand Plant Conservation Network, 2013d; Wilson & Given, 1989). It forms an erect to sub-erect, spreading shrub (up to 4m). Flowering occurs sporadically throughout the year. This species features ornithophily, being pollinated by indigenous birds such as tui, bellbirds and stitchbirds (Heenan & De Lange, 1999; Morris & Ballance, 2008; Wardle, 2002). The plant can be also spread vegetatively by hardwood cuttings (Metcalf, 1995; Morris & Ballance, 2008).

Although recent research on *C. williamsii* has added considerably to knowledge of the biology of the species including taxonomy (Heenan, 1996), phylogeny (Heenan, 1998b; Wagstaff, Heenan, & Sanderson, 1999), wood anatomy (Heenan, 1997b), reproductive biology (Heenan & De Lange, 1999), leaf and habit heteroblasty (Heenan, 1997a), little is known about the biology of the seeds. According to Metcalf (1995), the seeds of *C. williamsii* can germinate in the seed pod. Other species of *Carmichaelia* are reported to exhibit physical dormancy which was alleviated by scarification (Gruner & Heenan, 2001). Although this species has been known to store well (Metcalf, 1995), Gruner & Heenan (2001) reported germination of 11 species of *Carmichaelia* varied from 0 to 100% after 23 years storage (Gruner & Heenan, 2001).

C. williamsii is restricted in its distribution to the northern offshore islands (particularly the Poor Knights and the Alderman Islands). The only known mainland location is now two small populations near East Cape (Kirk, 1896; Morris & Ballance, 2008; New Zealand Plant Conservation Network, 2013d). Previously this species ranked as 'nationally endangered' on the basis of the loss of mainland populations and the limited

extent of island populations (Hitchmough, Bull, & Cromarty, 2007), it is now ranked as 'relict' on the basis that the loss of (most) mainland populations was historical and the island populations appear stable (de Lange, et al., 2009; New Zealand Plant Conservation Network, 2013d)

However, within the remaining populations there continue to be population declines and loss due to browsing by introduced mammals and coastal erosion. The loss of native pollinators and attack from the lemon tree borer (*Oeomona hirta*) is another threat (Morris & Ballance, 2008; New Zealand Plant Conservation Network, 2013d; Wilson & Given, 1989). The East Coast Hawke's Bay Conservancy has established as a high priority action the protection of the *C. williamsii* historic site and to support a planting programme at Whangaokena (Thorsen, 2003).

2.2.2 *Clianthus spp.* (Kakabeak, kowhai ngutu kaka, kaka beak)

The genus *Clianthus* (Fabaceae) is endemic to the North Island, New Zealand (de Lange, et al., 2010). There are only two species in the genus (Morris & Ballance, 2008). *Clianthus* is recorded as one of the first endemic plants to be cultivated by Maori around their settlements and is used for horticultural plantings throughout New Zealand (and worldwide) (Colenso, 1885; Metcalf, 1987).

The two species are distinctly different. *Clianthus puniceus* (G.Don) Sol. ex Lindl. is a small shrub with small, dull, grey-green to olive-green leaves, while *Clianthus maximus* Colenso is a larger shrub (up to 4m tall), with larger, glossy green to dark green leaflets (de Lange, et al., 2010; Heenan, 2000; Morris & Ballance, 2008). The main flowering period is September to December but, on rare occasions, flowering can occur all year round (Shaw & Burns, 1997). Flowers are a typical papilionaceous flag blossom and are thought to be predominantly outcrossing because the stigma is covered with a cuticle from early bud through to full flower (Heenan, 1998c). The species may be ornithophilic; some indigenous birds have been observed visiting cultivated sites, but none have been observed in the wild (Shaw & Burns, 1997). Plants can also be propagated vegetatively through semi-hardwood cuttings and stem layering (Metcalf, 1995; Shaw & Burns, 1997).

Despite their popularity in cultivation, wild populations of both species of *Clianthus* are classified as 'nationally critical' (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013a, 2013b). *C. puniceus* has been identified recently at only one location on the Kaipara Harbour (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013a). At this site plants are threatened by summer droughts, browsing animals and competition from weeds (Auckland Conservancy, 2006; de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013a). The Department of Conservation has prepared and initiated implementation of a recovery plan for the species. This plan will support of *C. puniceus* in the wild and the maintenance of its genetic diversity (Shaw, 1993).

Though more widespread than *C. puniceus*, less than 200 mature plants of *C. maximus* are known at several sites on the East Cape (near Te Araroa), Te Urewera National Park, near Wairoa, and on Boundary Stream Mainland Island in Hawke's Bay. All of the sites comprise only a few individuals (typically <10 per population) (Auckland Conservancy, 2006; de Lange, et al., 2010; Morris & Ballance, 2008; New Zealand Plant Conservation Network, 2013b; Song, et al., 2008). At these sites plants are threatened by fire, weed control operations, natural succession, and the unstable, erosion prone nature of the habitats in which they grow (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013b)

The East Coast Hawke's Bay Conservancy has identified as high priority actions the protection of all remaining of *C. maximus* populations (particularly from deer and goats) and continued replanting into suitable sites and surveying of historic and potential sites (Thorsen, 2003).

2.2.3 *Hibiscus diversifolius* Jacq. (Native hibiscus, swamp hibiscus, prickly hibiscus)

The genus *Hibiscus* (Malvaceae) is large, containing some 200 species. Species in the genus are found throughout the tropics, except the United States, and extend into the northern and southern temperate zones (Fisher & Forde, 1994). However, there are only two species in the New Zealand flora (New Zealand Plant Conservation Network, 2013c, 2013e). One species *Hibiscus diversifolius* Jacq, which persists in the north possibly arrived with the Maori from tropical Polynesia (Wardle, 2002). This species, however, is now very rare in New Zealand and is classified as 'nationally endangered'(de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013e).

H. diversifolius is confined to coastal wetlands and streamsides. It typically forms dense intertangled thickets growing up to 2 x 3 m. Flowering occurs sporadically throughout the year (de Lange, et al., 2010; Wilson & Given, 1989). Very little is known about the floral biology or seeds of *H. diversifolius*. According to Walters et al. (2005) seed of this species retained 70% germination after 39.5 years storage at -18°C indicating orthodox storage behaviour. Similarly, other species in the *Hibiscus* genus, e.g. *H. micranthus* (Walters, Wheeler, & Grotenhuis, 2005) and *H. cameronii* (Royal Botanic Gardens Kew, 2008) have been reported to have long lived seeds.

This species has apparently always been restricted to the northern most extremity of the North Island (from about Reef Point and Doubtless Bay north). The largest populations known occur on the eastern side of Te Paki (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013e). These remaining populations continue to decline due to browsing by feral animals and loss of habitat from encroaching housing development (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013e)

2.2.4 *Myosotidium hortensium* (Decne.) Baill. (Chatham Island Forget-me-not, Kopakopa, Kopukapuka)

Myosotidium hortensium (Decne.) Baill. (Boraginaceae) is endemic to the Chatham Islands (Wilson & Given, 1989). The Boraginaceae family is large and widely distributed throughout the temperate regions. Plants are valued as ornamental subjects (Fisher & Forde, 1994). The genus *Myosotidium* however, has only one species, *M. hortensium*, and is found nowhere else in the world (Wilson & Given, 1989). Although this species is relatively widespread and is available commercially, the natural range has been significantly reduced (Fisher & Forde, 1994; Wilson & Given, 1989).

M. hortensium is found on coastal cliffs, rock outcrops and sandy and/or rocky beaches. It is a perennial herb that can grow up to 1 x 1-1.5m (New Zealand Plant Conservation Network, 2013f). Seeds of *M. hortensium* were thought to be recalcitrant (Fountain & Outred, 1991), however they have been found to retain viability for up to 21 months when stored at 7.5% moisture content (McGill, McIntosh, Outred, & Fountain, 2002). A very small percentage of fresh seeds will germinate but the vast majority are dormant and tend to have slow, erratic germination (McGill, Outred, Hill, Hill, & Fountain, 2000; Metcalf, 1995; Wilson & Given, 1989). Dormancy can be alleviated by scarification (McGill, et al., 2000). Plants can also spread vegetatively from root stock.

M. hortensium is vulnerable and facing the possibility of extinction in the wild. It is found on Chatham (Rekohu), Pitt, South East, Mangere and most of the smaller islands, islets and some rock stacks (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013f). It is now classified as 'nationally vulnerable' (de Lange, et al., 2009). The population decline has been attributed to competition from exotic weeds, browsing by introduced and feral mammals and direct encroachment from human activities. Removal of wild plants for sale as garden specimens is another threat (de Lange, et al., 2010; Dopson, et al., 1999; Morris & Ballance, 2008; Wilson & Given, 1989).

An active management plan for the species has developed by the Department of Conservation to reduce the risk of extinction. As a result some plant populations are well on the path to full recovery (Dopson, et al., 1999; Morris & Ballance, 2008).

2.2.5 *Tecomanthe speciosa* W. R. B. Oliv. (Tecomanthe)

Tecomanthe speciosa W.R.B. Oliv. is the only member of the Bignoniaceae family found in New Zealand. It exists as a single genotype on Great King, in the Three Kings Islands. Until recently, only one plant had been found in the wild (Allan, 1982; de Lange, et al., 2010; Given, 1975; Wilson & Given, 1989). Due to its attractiveness as a garden ornamental, *T. speciosa* is increasing in popularity and has increased public interest in conserving native flora of New Zealand (reviewed in Edson, Wenny, Leegebrusven, Everett, & Henderson, 1994; Wilson & Given, 1989). Despite its popularity in cultivation, this species in threatened with extinction in the wild and listed as 'nationally critical' (de Lange, et al., 2010; de Lange, et al., 2009).

The sole *T. speciosa* remaining in the wild is growing in a stony stream bed within mixed coastal forest (Wilson & Given, 1989). *T. speciosa* is a woody vine that grows up to 10m (Allan, 1982). Very little is known about the biology of *T. speciosa* floral and seeds. Although it is known to be self-fertile, *T. speciosa* had not been known to flower and set fruit in the wild since 1946. Many cultivated *Tecomanthe* vines also rarely set fruit (Godley, 1980; Morris & Ballance, 2008; Wilson & Given, 1989). Bats are thought to be the principal pollinators in the wild, but this has never been observed. Cultivated plants are pollinated by native and exotic birds (New Zealand Plant Conservation Network, 2013g; Wilson & Given, 1989). *T. speciosa* can propagate from stem cuttings, aerial and ground layering and from seed. The seeds must be sown fresh after harvest, they will not keep for very long, especially if they have dried (Metcalf, 1995; Morris & Ballance, 2008).

Population loss and decline has been attributed to competition from surrounding forest and browsing by introduced goats (de Lange, et al., 2010; New Zealand Plant Conservation Network, 2013g; Wilson & Given, 1989). Lack of genetic diversity in the population of cultivated plants is also a possible threat to this species (reviewed in Edson, et al., 1994). A *T. speciosa* Recovery Plan through monitoring has been prepared by the Department of Conservation (Dopson, et al., 1999).

2.2.6 *Dysoxylum spectabile* Hook. (Kohekohe, New Zealand mahogany)

Dysoxylum spectabile Hook. is the only member of the Meliaceae found in New Zealand (Braggins, Large, & Mabberley, 1999). This species is the most southerly member of a genus of about 150 species found in tropical Asia, Malaysia, Australia and the Pacific Islands (Fisher & Forde, 1994). *D. spectabile* is largely confined to coastal forest of the North Island. It is foundin the South Island but does not extend far beyond the Marlborough Sounds, reaching a southern limit near the Hurunui River (Napenape) (Brooker, 1960; Fisher & Forde, 1994; New Zealand Plant Conservation Network, 2013h; Wardle, 2002).

The tree grows up to 15m and is easily recognized by the large compound green leaves and cauliflorous flowering habit (Allan, 1982; Moore & Irwin, 1978). The species is dioecious, flowering varies across the years with heavy flowering seasons usually followed by lighter ones (Braggins, et al., 1999). The most common method of propagation is from seeds. The seeds are not dormant and must be sown freshly, as they will not withstand drying out (Burrows, 1996b; Court & Mitchell, 1988).

Currently, this species is not threatened with extinction. However, if numbers of possum and rats are uncontrolled, the population may decline due to browsing of the inflorescences and seeds (New Zealand Plant Conservation Network, 2013h).

2.3 Seed storage behaviour

Storage of seeds is widely used for plant genetic resource conservation, and is considered the most effective and efficient *ex situ* conservation strategy (Hong & Ellis, 1996; Pritchard, Tompsett, Manger, & Smidt, 1995; Roberts, 1973). Seed storage behaviour varies from one species to another. Thus, knowledge of the seed storage behaviour of a species is essential for the development of appropriate *ex situ* conservation strategies.

Seeds can be classified into three different categories depending on their storage behaviour. These categories are based on their desiccation tolerance, longevity and tolerance to low temperatures during storage (Ellis, Hong, & Roberts, 1990; Hong & Ellis, 1996; Roberts, 1973). In this respect, desiccation tolerant seeds (so-called orthodox seeds) can be dried without damage and survive in the dried state for periods that are predictable depending on storage conditions (the main influence being seed moisture content and temperature) (Hong & Ellis, 1996; Roberts, 1973). Seed tolerance to desiccation (e.g. for storage purposes) is acquired during seed development at completion of morphological development, around mass maturity (completion of reserve accumulation) and is often dependent on the developmental status of the seed (Golovina, Hoekstra, & Van Aelst, 2001; Hay & Smith, 2003; Kermode, Finch-Savage, & Black, 2002; Pritchard, 2004). However, it is difficult to identify the exact point of acquisition of desiccation tolerance. Seeds that are capable of a high level of germination prior to maturity may not necessarily have attained their maximum tolerance to desiccation and storage (Pritchard, 2004). If developing seeds are artificially dried, they may tolerate desiccation, but the effect of drying rate differs with development stage. Immature orthodox seeds which have reached a certain level of maturity will survive slow drying but not rapid drying, whereas more mature seeds survive rapid water loss (Kermode, 1995; Kermode, et al., 2002), e.g. *Ceiba pentandra* (Lima, Ellis, Hong, & Ferraz, 2005), *Vicia sativa* (Samarah, 2007). This is suggested to be because immature seeds need time to develop tolerance mechanisms, which slow drying facilitates, but rapid drying does not (Berjak, Farrant, & Pammenter, 2007).

During maturation, seed water content declines. As water is lost, a number of potentially damaging processes can occur. Seeds must have or have acquired mechanisms to protect against these processes (Kermode, et al., 2002; Nedeva & Nikolova, 1997; Pammenter & Berjak, 1999; Vertucci & Farrant, 1995). These mechanisms can involve;

1) Accumulation of proteins, particularly the late embryogenesis abundant (LEA) proteins and small heat-shock proteins (HSPs) that are associated with orthodox seed maturation. Their appearance is concomitant with abscisic acid (ABA) regulation of *lea* gene transcription, suggested to be the basis of the formation and maintenance of the intracellular glassy state in desiccated cells (Berjak, 2006; Berjak, et al., 2007; Kermode, et al., 2002)

2) The occurrence of high sucrose concentrations was suggested to be important in prevention of sucrose crystallization at low water concentrations or in stabilizing dry membranes (Berjak, et al., 2007; Leprince, Hendry, & McKersie, 1993; Obendorf, 1997)

3) Intracellular glass formation (vitrification) imposes highly restricted molecular mobility including the mobility of damaging free radicals and is also held to confer stability on macromolecules and membranes (Berjak, 2006; Berjak, et al., 2007; Leopold, Sun, & Bernal-Lugo, 1994)

4) The use of reactive oxygen species (ROS) and antioxidants, in both signalling and intracellular damage. ROS are formed when high-energy-state electrons are transferred to molecular oxygen (O_2). ROS include $^1O_2$ (single oxygen), H_2O_2 (hydrogen peroxide), $O_2^{\bullet-}$ (the superoxide radical), and $OH^{\bullet}$ (the hydroxyl radical) (e.g. Berjak, et al., 2007; Franca, Panek, & Eleutherio, 2007; Hendry, 1993). They have long been considered as toxic species that can cause oxidative damage to lipids, proteins and nucleic acids (Berjak, et al., 2007; Berjak & Pammenter, 2008; Hendry, 1993; Suzuki & Mittler, 2006). The activity of a spectrum of enzymatic and non-enzymatic antioxidants is considered to be of

prime importance in quenching ROS activity, i.e. in intracellular protection (Berjak, et al., 2007; Berjak & Pammenter, 2008).

Bonner (1990) suggested a more refined classification of orthodox seeds into true orthodox and sub-orthodox. Whereas true orthodox seeds can be stored for relatively long periods at subfreezing temperatures with about 5 to 10% MC (fresh weight basis), sub-orthodox seeds can be stored for much shorter periods under the same conditions. The reasons for the decreased longevity are not completely known. However, indirect evidence suggests that some causes are high lipid contents such as in some species of *Carya* Nutt., *Fagus* L., *Juglans* L., *Pinus* L. and *Arachis* L. and thin fruits or seedcoats, including some species of *Acer* L., *Populus* L., and *Salix* L. (Bonner, 1990; Gagliardi, Pacheco, Valls, & Mansur, 2002; Pritchard & Dickie, 2003; Yilmaz, 2008). Retention of viability for more than 10 years would be rare for sub-orthodox species with current seed storage conditions (Bonner, 2008).

However, a relationship between high oil content and short storage life-span has not been supported by recent studies (Pritchard & Dickie, 2003; Probert, Daws, & Hay, 2009; Walters, et al., 2005). Nonetheless, it is possible that lipid composition is important in determining longevity since increases of lipid peroxidation and phospholipid de-esterification have been considered to be probable reasons for seed viability loss during dry storage (Benson, 1990; Goel & Sheoran, 2003; Sattler, Gilliland, Magallanes-Lundback, Pollard, & DellaPenna, 2004), but, this remains to be confirmed (Probert, et al., 2009).

In contrast, recalcitrant seeds lose viability with dehydration (below about 15% or higher moisture content) and may also be chilling sensitive (Hong & Ellis, 1996; Roberts, 1973). Generally, recalcitrant seeds cannot be stored for long periods and their viability is short, even if stored under moist conditions (Kermode, et al., 2002). Low moisture content and sub-zero temperatures storage will not reduce, and in most cases will accelerate, loss of viability of recalcitrant seeds (Pritchard, 2004; Roberts, 1973). The long-term preservation of such seeds is still an unsolved problem for most species.

Recalcitrant seeds undergo no maturation drying, have high water content when they are shed, and remain metabolically active throughout development. The lack of maturation drying and continuing metabolic activity of recalcitrant seeds means they are highly susceptible to desiccation (Hong & Ellis, 1996; Roberts, 1973; Vertucci & Farrant, 1995). In addition most of these seeds, especially those originating from tropical regions (Tweddle, Dickie, Baskin, & Baskin, 2003), are sensitive to chilling and thus cannot be stored below approximately 15°C. Recalcitrant seeds tend to be produced by trees, are relatively large in size and shed during the rainy season (Pritchard, et al., 2004). They germinate relatively rapidly, unhindered by the presence of a relatively thin seed coat (Daws, Garwood, & Pritchard, 2005, 2006; Pritchard, et al., 2004). Sufficient evidence has now accumulated to show that tolerance of desiccation depends not only on the inherent characteristics of the species, but also on the developmental status of the seeds and the conditions under which they are dried, particularly the rate of drying (Pammenter & Berjak, 1999).

Flash-drying can improve the desiccation tolerance of desiccation-sensitive (recalcitrant) seeds. This is particularly so when excised axes are dried (Berjak, Vertucci, & Pammenter, 1993; Pammenter, Vertucci, & Berjak, 1991). Species to which flash-drying has been successfully applied include *Artocarpus heterophyllus* (Wesley-Smith, Pammenter, Berjak, & Walters, 2001), *Hopea hainanensis* (Lan, Jiang, Song, Lei, & Yin, 2007), *Mangifera persiciformis* (Tang, Tian, & Long, 2008). However, there are contradictory reports on the effect of drying rate on survival of axes excised from recalcitrant seed. In *Shorea chinensis* (Yan, Cao, & Xu, 2007), *Aquilaria agallocha* (Kundu & Kachari, 2000) and *Theobroma cacao* (Liang & Sun, 2000) rapid drying did not necessaorly improve survival of the axes. Liang and Sun (2002) in their study of embryonic axes of *Theobroma cacao* and *Ginkgo biloba*, found both faster and slower drying were detrimental, and increased the sensitivity of embryonic axes to desiccation. They suggest the there is an optimal drying rate at which maximum desiccation tolerance could be achieved.

For recalcitrant species, the effect of developmental status on seed desiccation tolerance has been well reported. For example, desiccation tolerance has been shown to increase with development, in seeds of *Hopea hainanensis* (Lan, et al., 2007), *Acer*

pseudoplatanus (Hong & Ellis, 1990), *Aesculus hippocastanum* ((Farrant & Walters, 1998; Tompsett & Pritchard, 1993), *Camellia sinensis* (Berjak, et al., 1993) and *Landolphia kirkii* (Pammenter, et al., 1991), albeit not to the same extent as orthodox species.

In several species there is uncertainty over their desiccation tolerance. *Azadiracta indica* seeds have been variously classified as orthodox, intermediate and recalcitrant (Hong & Ellis, 1998; Sacande, Buitink, & Hoekstra, 2000). Daws, et al. (2004) suggest that environmental conditions during seed development affect the level of seed desiccation tolerance in *Aesculus hippocastanum* seeds, i.e. there is increasing desiccation tolerance in seeds that developed under warmer conditions. Daws, et al. (2004) suggest this is the result of development progressing further before seed shed. Similarly, provenance has been reported to have an effect on desiccation tolerance of *Coffea arabica* seeds (Ellis, Hong, & Roberts, 1991b). These authors proposed the possibility of 'phenotypic recalcitrance' which requires further investigation, particularly for species that are spread across a number of climate zones. The more recent work of Daws, et al. (2004) further supports the need for investigation of 'phenotypic recalcitrance'.

A third, intermediate, category of storage behaviour was recognized by Ellis, Hong, & Roberts (1990, 1991a) in which are placed seed that are relatively desiccation-tolerant, but cannot withstand water loss to a moisture content as low as orthodox seeds. Intermediate seeds may be also damaged during dry storage at low temperature (0°C and -20°C) (Ellis, Hong, & Roberts, 1990; Hong & Ellis, 1996). Seed viability of intermediate seeds stored at cool and sub-zero temperatures and at low moisture contents deteriorated more rapidly than at either warmer temperatures or higher moisture contents. In other words, dry seeds are more easily injured by low temperatures (Yang, Kuo, & Lin, 2007). Tolerance of low temperature in intermediate seeds is dependent on the ecological conditions the seeds have developed in. Intermediate seeds native to temperate regions are usually tolerant to sub-zero temperatures. The optimal storage temperature for these seed is usually lower than 5°C (Hong & Ellis, 1996), for example, *Champereia manillana* (Chen, Kuo, Chien, Baskin, & Baskin, 2007), *Neolitsea aciculate* (Chen, Kuo, & Chien, 2007) *Fagus sylvatica*, *F. crenata* (León-Lobos & Ellis, 2002) all tolerate storage at temperatures below 5°C. In

contrast, seeds of many species originating from tropical regions are sensitive to low temperatures and need to be stored at temperatures higher than 10°C (Hong & Ellis, 1996). Species in this category include *Cordeauxia edulis* (Andersson, Yahya, Johansson, & Liew, 2007)vand *Mimusops elengi* (Truong Mai, et al., 2006).

Seeds classified as intermediate usually survive exposure to either low water contents or low temperatures, but do not survive when exposed to both stresses simultaneously (Ellis, Hong, & Roberts, 1990; Ellis, et al., 1991b). Damage induced by a combination of low water content and low temperature has been explained by phase changes in water and water-soluble components of the cells (Koster, Lei, Anderson, Martin, & Bryant, 2000; Steponkus, Uemura, & Webb, 1995; Sun, Irving, & Leopold, 1994). However, the water contents and temperatures that induce damage in intermediate seeds (Ellis, et al., 1991a; Sacande, Golovina, van Aelst, & Hoekstra, 2001) do not correspond to those that result in phase changes of water or water-soluble components (Crane, Kovach, Gardner, & Walters, 2006). An alternative explanation has been suggested by Crane et al. (2006) based on their work *Cuphea* seeds. Their suggestion is that *Cuphea* seeds express sensitivity to temperature rather than desiccation, and that the temperature range and accumulation of damage correspond to triacylglycerol phase behaviour and crystallization rate.

Seeds with recalcitrant and intermediate storage behaviour are collectively referred to as non-orthodox seeds.

It is not straightforward to classify seeds into one of these categories. There is uncertainty in the classification or contradictory reports on the storage behaviour of a number of species (Wood, Pritchard, & Amritphale, 2000). Pammenter & Berjak (1999) and Vertucci & Farrant (1995) suggested that it is more appropriate to consider storage behaviour to be a continuum from recalcitrant species through to these species sharing orthodox storage behaviours rather than as three discrete categories. The continuum concept is considered pertinent and is supported by recent evidence (e.g. Daws, Cleland, et al., 2006; Daws, et al., 2004; Dussert, et al., 2000); however categorizing seeds into the three distinct groupings is useful to describe the seed storage behaviour of most species and will be used here.

2.4 Seed dormancy

Though *in situ* conservation of plant species is the preferred conservation strategy, *ex situ* conservation of threatened species can also play an effective role in preventing extinction. *Ex situ* conservation supports habitat (*in situ*) conservation and also provides resources to re-establish wild populations if or when a suitable site exists (Cochrane, Crawford, & Monks, 2007; FAO, 1996; Havens, Vitt, Maunder, Guerrant, & Dixon, 2006; Heywood & Iriondo, 2003). The *ex situ* conservation of a target species involves the collection of material, its storage and maintenance. Loss of plant material in store means that regeneration in order to ensure its continued availability is also critical (Lawrence, 2002). It is important that *ex situ* collection materials are representative of the genetic diversity of the *in situ* population (Brown & Briggs, 1991). *Ex situ* conservation material should be collected without disturbing or damaging natural populations, especially rare and endangered species (Menges, 1991). *Ex situ* conservation of plant species in seed banks has a number of advantages in terms of efficiency and economy of long-term storage. It allows for the preservation of large populations with minimal genetic erosion (Fay, 1992; Roberts, 1991) and collection of seed has less impact on a population than removal of whole plants or plant parts (Menges, 1991).

Seeds cannot be stored indefinitely. Checking viability prior to storage and monitoring of viability in storage is needed to ensure seed material is available for future conservation utilisation (Rice, Smith, Mitchell, & Kresovich, 2006). The germination test is commonly used to assess seed viability ((Phartyal, Thapliyal, Koedam, & Godefroid, 2002). However, in some species seeds fail to germinate even under favourable germination conditions as a result of dormancy (Baskin & Baskin, 2004b; Hilhorst, 1995; Li & Foley, 1997). Thus, knowledge of the optimum germination and dormancy breaking conditions of a species is essential for testing seed viability during long term storage. To be of value the germination results must be both accurate and reproducible (Phartyal, et al., 2002). Failure to alleviate dormancy can result in both inaccurate and non-reproducible germination results. Investigation of seed germination behaviour and the identification of appropriate pre-treatments to alleviate dormancy are therefore critical for successful *ex-situ* conservation.

2.4.1 Classification of dormancy

Several forms of dormancy are found in seeds (Bewley & Black, 1994; Li & Foley, 1997) and a number of schemes for classifying seed dormancy have been published. Harper (1977) differentiated between innate, enforced (=quiescence, also could include conditional dormancy) and induced dormancy (=secondary dormancy). Quiescence can also be used to describe the state where the seed is not dormant but is only waiting for one or more of the three factors required (water, oxygen and an appropriate temperature) for germination (Harper, 1977).

The Lang 'universal' system of endodormancy, paradormancy and ecodormancy, is intended to be used with all types of plant dormancy, not just seeds (Lang, 1987; Lang, et al., 1985; Lang, Early, Martin, & Darnell, 1987). Simpson (1990) listed three criteria for classifying dormancy: the apparent origin of the control of dormancy, e.g. genetic, structural or environmental, the depth of the dormancy, e.g. shallow, deep or relative, and the timing of the dormancy, e.g. primary, or secondary. Nikolaeva (1977) devised a dormancy classification system reflecting that dormancy is determined by both morphological and physiological properties of the seed.

Recently, Baskin & Baskin (2003, 2004b) proposed a modified version of the Nikolaeva classification scheme which included three kinds of dormancy; classes, levels and types. The modified system classified dormancy into the five classes responsible for the endogenous and/or exogenous factor(s) that maintain each classification. In their classification scheme, mechanical or chemical dormancy are not recognized as dormancy types *per se*, thus differing from that Nikolaeva (1977). Instead mechanical dormancy is a component of physiological dormancy and chemical dormancy is combined with physiological dormancy (Baskin & Baskin, 2003, 2004b; 2008). Although this scheme may need further refinements/ updates to be made from time to time to accommodate new kinds of seed dormancy, it is the most comprehensive classification system, and it can cover the diversity of kinds of dormancy known to occur in seeds, and will be used here.

2.4.2 Dormancy classes

Physical dormancy occurs if one or more of the enclosing seed tissues (for example, endosperm, perisperm, megagametophyte, pericarp, or testa) impose a block to prevent water uptake (Baskin & Baskin, 2003, 2004b). Physical dormancy can be alleviated under both natural and artificial conditions. This is assumed to involve the formation of an opening "water gap" in a specialized anatomical structure on the seed (or fruit) coat through which water moves to the embryo (Baskin, Baskin, & Li, 2000). Several types of specialised structures ("water gaps") have been found in 12 of the 17 families that have physical dormancy; for example, the carpellary micropyle in Anacardiaceae; the bixoide chalazal plug in Bixaceae, Cistaceae, Cochlospermaceae, Dipterocarpaceae and Sarcolaenaceae; the imbibition lid in Cannaceae; the chalazal plug in Malvaceae (Baskin, et al., 2000) and the micropyle-water gap complex in Geraniaceae (Gama-Arachchige, Baskin, Geneve, & Baskin, 2011). In Fabaceae, water entry in the region of the lens[1] has been reported by several authors (de Souza, Voltolini, Santos, & Silveira Paulilo, 2012; Dell, 1980; Hanna, 1984; Morrison, McClay, Porter, & Rish, 1998; Serrato-Valenti, Devries, & Cornara, 1995). The hilum[2] and micropyle[3] also have been reported to allow water entry into seeds after physical dormancy is broken (Hu, Wang, Wu, & Baskin, 2009; Hu, Wang, Wu, Nan, & Baskin, 2008; Hyde, 1954; Zeng, Cocks, Kailis, & Kuo, 2005), as well as cracks develop in the extrahilar region (Hu, et al., 2009; Morrison, et al., 1998). Water entry through the strophiole[4] has also been reported for other Fabaceae seeds, e.g. *Trifolium arvense* (Dickie & Stuppy, 2003) and *Leucaena leucocepha* (Serrato-Valenti, et al., 1995). Fire, high or fluctuating temperatures, or biological actions such as passage through the gut of a bird or animal or both or micro-organisms are possible means by which physical dormancy is overcome naturally (Baskin & Baskin, 1998; Bewley & Black, 1994). A variety of pre-

[1] The the lens is the spezialized structure localized in the hilum region that controls water-impermeability (Leubner, 2013).

[2] The hilum is a scar or mark left on a seed coat by the former attachment to the ovary wall or to the funiculus (which in turn attaches to the ovary wall) (Leubner, 2013).

[3] The micropyle is a canal or hole in the coverings (seed coat) of the nucellus through which the pollen tube usually passes during fertilization (Leubner, 2013).

[4] The strophiole is an outgrowth of the hilum region which restricts water movement into and out of seeds (Leubner, 2013).

sowing treatments such as hot water, sulphuric acid and mechanical scarification have successfully been used to alleviate physical dormancy (Baskin & Baskin, 1998; Funes & Venier, 2006). However, the optimal level of the various scarification treatments, e.g. the length of time seed is exposed to sulphuric acid, varies among species and/or seed lots and needs to be determined empirically for any given species.

Physiological dormancy prevents germination until a chemical change takes place in the seed. However, low growth potential of the embryo, which cannot overcome mechanical constraint imposed by the seed (or fruit) coat may play a role in preventing germination (Baskin & Baskin, 2003, 2004b). Following Nikolaeva (1977), three levels of physiological dormancy have been recognised: deep, intermediate, and non-deep (Baskin & Baskin, 2003, 2004b; Baskin & Baskin, 2008). Characteristics of each level are summarised in Table 2.2.

Table 2.2 Characteristics of seeds with deep, intermediate and non-deep physiological dormancy.

Deep
Excised embryo produces an abnormal seedling
Gibberellic acid does not promote germination
Seeds require 3-4 months of cold stratification to germinate
Intermediate
Excised embryo produces a normal seedling
Gibberellic acid promotes germination in some (but not all) species
Seeds require 2-3 months of cold stratification for dormancy break
Dry storage can shorten the cold stratification period
Non deep
Excised embryo produces a normal seedling
Gibberellic acid promotes germination
Depending on species, cold or warm stratification breaks dormancy
Scarification may promote germination

(adapted from Baskin & Baskin, 2004b).

In seeds with morphological dormancy, the embryo is either small (underdeveloped) and undifferentiated or small (underdeveloped) and differentiated, i.e., cotyledon(s) and radicle can be distinguished. In such seeds, the embryos simply need time to grow to full size and then germinate (radical protrusion) (Baskin & Baskin, 2003, 2004b).

Seeds with morpho-physiological dormancy have an underdeveloped embryo that also has a physiological component of dormancy. These seeds therefore require a dormancy-breaking treatment, e.g. a defined combination of warm and/or cold stratification which in some cases, can be replaced by gibberellic acid (GA) application. In morphophysiological dormancy, embryo growth/emergence requires a considerably longer period of time than in morphological dormancy (Baskin & Baskin, 2003, 2004b).

In seeds with combinational dormancy, the seed (or fruit) coat is water-impermeable and the embryo is physiologically dormant. Baskin and Baskin (1998)comment that the physiological component appears to be at the non-deep level. In this case physical dormancy is released first and the subsequent water uptake during imbibition is followed by the release of the embryo dormancy (physiological dormancy non-deep) during a few weeks of cold stratification (Baskin & Baskin, 2003, 2004b).

2.4.3 Dormancy mechanisms

Seeds of many species exhibit some level of dormancy that can be either coat-imposed (physical dormancy) or a function of the embryo (physiological dormancy), or both (Bewley & Black, 1994; Li & Foley, 1997). The last is essentially the combinational dormancy of Baskin and Baskin (1998). A number of mechanisms have been proposed by which the coat imposes dormancy on a seed (Bewley & Black, 1994). These include: (1) mechanical restriction of germination of the embryo - the `mechanical barrier' model; (2) prevention of the exit of inhibitors from the embryo - the `inhibitor exit' model; (3) presence of chemical inhibitors of germination in the seed coat - the `seed coat inhibitor' model; (4) restriction of water uptake - the `water-impermeable' model; and (5) restriction of oxygen uptake - the `oxygen diffusion' model (Morris, Tieu, &

Dixon, 2000). Where the coat imposes dormancy, removal of the coat will allow the embryonic axis to elongate, assuming no embryo dormancy. In contrast, embryo dormancy prevents elongation of the embryonic axis even if the embryo is excised from its enclosing seed tissues and moistened.

Embryo dormancy can be caused by structural immaturity of the embryo or the presence of germination inhibitors such as ABA as well as the absence of growth promoters, such as GA. In addition, the sensitivity of the seed tissue to these hormones can be a cause of embryo dormancy (Baskin & Baskin, 1998; Baskin & Baskin, 2004b; Bewley & Black, 1994).

Dormancy can vary even within a species, depending on the differences between individuals, location, climatic conditions, time of collection, as well as the nature and duration of seed storage after collection (Phartyal, et al., 2002). Seed rehydration characteristics or germination requirements may change during drying and/or dry storage, e.g. *Carica papaya* (Wood, et al., 2000).

2.5 *Ex situ* conservation approaches

Complementary to *in situ* conservation, some form of *ex situ* storage is an important component of conservation of genetic diversity of threatened species (Cochrane, et al., 2007; FAO, 1996). Principle 8 of the New Zealand Biodiversity Strategy (Department of Conservation, 2000) recognises this for New Zealand in stating that:

"Biodiversity is best conserved in situ by conserving ecosystems and ecological processes to maintain species in their natural habitats. Ex situ measures will be important to support the conservation of some species."

An *ex situ* approach is most appropriate where populations and/or plant numbers are small or are threatened by disease, human activities or habitat change. *Ex situ* storage provides a resource that can be used for revegetation if *in situ* storage fails (Cochrane, et al., 2007; Crawford, Steadman, Plummer, Cochrane, & Probert, 2007).

The appropriate *ex situ* strategy depends on factors such as the biological characteristics of the plants, the purpose of the conservation effort and the availability of germplasm (FAO, 1996; Guerrant, Havens, & Maunder, 2004). For many plant species, *ex situ* seed storage has been considered as a safe, effective and relatively inexpensive method of plant conservation (Fay, 1992; Roberts, 1991). However, these methods can only be applied to seeds of orthodox species (Roberts, 1973), i.e. those which can be dehydrated without loss of viability and stored dry at low temperature.

As already discussed, not all species produce orthodox seeds. Dickie & Pritchard (2002) state that species with recalcitrant storage behaviour comprise approximately 7% of the world's seeds. Around 2% are considered to produce seed with intermediate storage behaviour (Ellis, Hong, & Roberts, 1990; Roberts, 1973). It should be noted that this information is based on less than 2.5% of all plant species (Dickie & Pritchard, 2002). Conventional seed storage approaches are not suitable for non-orthodox seeds and as a result, alternative methods of storage of seeds have been investigated. These include maintaining seeds in storage in an imbibed state and storage of seeds in liquid nitrogen. The vigour and viability of recalcitrant seeds can be maintained under wet-storage, but

it is strictly a short- to medium-term option (Berjak, 2000; Berjak & Pammenter, 2008). For long-term storage, it is clear that cryopreservation, often coupled with *in vitro* culture, represents the only option (Engelmann & Engels, 2002). Efficient and robust cryopreservation protocols are unavailable for many plant species and the diverse germplasm types likely to be encountered within one species. Additionally, the methods require sophisticated *in vitro* procedures and facilities (Berjak, 2000; Panis & Swennen, 2008).

Thus, knowledge of the seed storage behaviour, in particular desiccation tolerance of a species, is essential for the development of appropriate *ex situ* conservation strategies. Several approaches to predict seed storage behaviour have been suggested, including estimations based upon taxonomy, plant ecology, fruit characteristics and seed characteristics (Hong & Ellis, 1996) or the use of probabilistic model based on two seed traits (seed mass and seed coat ratio). This latter approach was used by Daws, Garwood, & Pritchard (2006) to determine seed storage behaviour in 104 species of different families/genera. However, these approaches only assist in prediction of seed response to storage and experimental data on seed responses to storage are still required for an understanding of the seed storage physiology of individual species (Hong & Ellis, 1996).

2.5.1 Seed bank storage for orthodox seeds

The majority of germplasm collections worldwide are seed-based (about 90% of the 6.1 millions accessions; FAO, 1996), as this is the simplest and safest option for storage of desiccation tolerant (orthodox) seeds (Roberts, 1973). For long-term seed storage of orthodox seed a standard method is used *viz*. seed is stored at 3-7% seed moisture content (on a fresh weight basis with the actual moisture content depending on the species) at -18°C or lower (FAO/IPGRI, 1994). Seeds of many orthodox species conserved this way are likely to retain viability for long periods, possibly centuries (Theilade & Petri, 2003). But even for seed stored under these controlled conditions, viability may decrease as a result of a deterioration process. Consequently, studies into

the long term viability of seeds are needed to determine the potential storage life of seed in germplasm collections and identify the conditions that will maintain the viability of each accession for as long as possible above a minimum value (FAO/IPGRI, 1994). The survival of orthodox seeds improves in a predictable way in response to decreasing temperature and moisture (Ellis & Roberts, 1980) and so this can be used to model seed storage life using viability equations (Ellis & Hong, 2007; León-Lobos & Ellis, 2003; Mead & Gray, 1999; Usberti, Roberts, & Ellis, 2006).

Recently, an attempt has been made to develop a 'low-input' alternative to the conventional cold storage of seed. The technique is called ultra-dry storage which involves drying seed to very low moisture contents, usually below 5% seed moisture content, and storing at room temperature (Ellis, Hong, & Roberts, 1988; Vertucci & Roos, 1993; Walters & Engels, 1998). Recent studies have supported the possibility of using ultra-dry methods. These include studies by Perez-García et al. (2007) and Demir & Ozcoban (2007) who found that ultra-dry storage at ambient temperatures can be successful. No loss in viability was detected during 60 months storage period in okra, melon, bean, winter squash, summer squash, cowpea, radish, cabbage at -20°C and 20°C with either moisture content (Demir & Ozcoban, 2007). According to Ellis (1998) and Hong et al. (2005) maximum longevity occurs when desiccation takes places at 10-12% RH at 20ºC. Seeds of *Daucus carota*, *Arachis hypogaea*, *Lactuca sativa*, *Brassica napus* and *Allium cepa* had 2.0 - 3.7% MC which was close to that suggested for ultra-dry storage. However, Vertucci and Roos (1993) suggested that optimal seed moisture content for conservation depends on storage temperature. It has been argued that drying seeds beyond a critical moisture content may provide no additional benefit to longevity and may even accelerate seed ageing rates (Ellis, et al., 1988; Vertucci & Roos, 1993; Walters & Engels, 1998). Therefore further research on various aspects of ultra-dry seed storage, including drying techniques and their applicability to a broader number of species is required (FAO, et al., 2004).

2.5.2 Cryopreservation for non-orthodox seeds

Cryopreservation is the long-term storage of material at ultra-low temperatures (e.g. liquid nitrogen, -196°C). At these ultra-low temperatures metabolic activity is stopped. Coupled with *in vitro* culture, this technique is often the only safe and cost-effective option for storage of non-orthodox species (FAO, et al., 2004). Seeds of species with relatively small size and tolerance to desiccation have been cryopreserved intact after partial desiccation, e.g. *Azadirachta indica* (Varghese & Naithani, 2008) and *Swietenia macrophylla* (Marzalina & Normah, 2002). In cases where whole seeds are not amenable to cryostorage, embryos or embryonic axes have been used, e.g. *Quercus* spp. (Gonzalez-Benito & Perez-Ruiz, 1992), *Citrus macroptera* and *C. latipes* (Malik & Chaudhury, 2006). In such cases, selecting embryos at the right developmental stage is critical to the success of the cryopreservation (Engelmann & Engels, 2002; FAO, et al., 2004). With species for which attempts to freeze whole embryos or embryonic axes have proven unsuccessful, various authors (e.g. N'Nan, et al., 2008; Scocchi, Vila, Mroginski, & Engelmann, 2007) have suggested using shoot apices taken from the embryos, adventitious buds or somatic embryos induced from embryonic tissues (FAO, et al., 2004). However, in this case, more advanced tissue culture procedures have to be developed to obtain these materials. In many cases, the disadvantage of cryopreservation is the overall difficulty of regeneration of whole plants (FAO, et al., 2004).

Cryopreservation of recalcitrant seeds is still experimental and only limited success has been achieved on a few species such as *Ilex* spp. (Mroginski, Sansberro, Scocchi, Luna, & Rey, 2008), *Ekebergia capensis* (Peran, Berjak, Pammenter, & Kioko, 2006) and *Castanea sativa* (Corredoira, San-Jose, Ballester, & Vieitez, 2004). This technique still remains unfeasible for most recalcitrant seeds (Wen, 2009). Cryopreservation techniques vary considerably between species in terms of both choice of explants (e.g. seed, somatic embryo, shoot tip) and technique. Generally, the following components are included *in vitro* culture: pregrowth, cryoprotection, freezing, thawing, recovery and plant regeneration (Reed, et al., 2004). The following is a summary of some of the main techniques.

2.5.2.1 Desiccation methods

Desiccation methods involve simple dehydration of an explant prior to immersion in liquid nitrogen (Berjak, et al., 1998). This is to remove 'free water' to prevent cryo-injury by lethal ice formation. The reduction of ice formation is dependent on the extent of water removal (Kartha & Engelmann, 1994). Moisture contents of 10 to 20% (fresh weight basis) are usually optimal for survival of freezing (Engelmann, 2004). Thus, this technique is only viable for somatic tissue (e.g. somatic embryos), seeds and embryonic axes that will tolerate this level of desiccation and as such is species/tissue dependent (Engelmann, 1997a). Desiccation techniques for cryopreservation have been mainly used for desiccation tolerant seeds (orthodox and intermediate) such as *Passiflora* species (Gonzalez-Benito, Aguilar, & Avila, 2009), *Citrus garrawayae* (Hamilton, Ashmore, & Drew, 2008) and *Champereia manillana* (Chen, Kuo, Chien, et al., 2007) and embryonic axes of recalcitrant seeded species, for example *Archontophoenix alexandrae* (Shao, Yin, & Wang, 2009), *Ekebergia capensis* (Peran, et al., 2006) and *Castanea sativa* (Corredoira, et al., 2004).

2.5.2.2 Vitrification-based methods

Vitrification-based methods involve pretreatment of samples with concentrated cryoprotectant solutions. On rapid freezing (direct immersion in liquid nitrogen) a highly viscous solid 'glass' forms, thus avoiding lethal ice injury (Sakai, Kobayashi, & Oiyama, 1990; Yamada, Sakai, Matsumura, & Higuchi, 1991). This technique has been employed successfully to cryopreserve a wide range of plant species (Hong, Yin, Shao, Wang, & Xu, 2009; Ishikawa, et al., 1997; Tsai, Yeh, Chan, & Liaw, 2009; Yamada, et al., 1991). Examples of the application of the vitrification-based techniques to embryonic axes of non-orthodox species include: *Citrus* species (Cho, Hor, Kim, Rao, & Engelmann, 2002b; Malik & Chaudhury, 2006), *Sechium edule* (Abdelnour-Esquivel & Engelmann, 2002) and *Artocarpus heterophyllus* (Thammasiri, 1998).

Many of the cryoprotectant solutions are potentially toxic to living tissue. In the vitrification procedure, the optimal treatment with a vitrification solution requires

finding the correct balance between toxicity and providing enough dehydration (Thinh, Takagi, & Yashima, 1999; Touchell, Chiang, & Tsai, 2002). Many different vitrification solutions are used for cryopreservation of a range of diverse tissues including Plant Vitrification Solution one (PVS1, Uragami, Sakai, Nagai, & Takahashi, 1989); PVS2 (Sakai, et al., 1990); PVS3 (Nishizawa, Sakai, Amano, & Matsuzawa, 1993); PVS4 (Sakai, 2000). PVS2 is the most widely used vitrification solution consists of 30% glycerol, 15% ethylene and15% dimethyl sulfoxide (DMSO).

2.5.2.3 Encapsulation-dehydration methods

The encapsulation-dehydration method was developed by Fabre & Dereuddre (1990) and involves the encapsulation of an explant in a calcium alginate bead, followed by pregrowth (e.g. on high sucrose medium) and desiccation prior to direct immersion in liquid nitrogen (Matsumoto & Sakai, 1995; Sakai, Matsumoto, Hirai, & Niino, 2000). All these steps aim at the induction or modification of the tolerance of plant tissues to dehydration (Dereuddre, Blandin, & Hassen, 1991). Research (Engelmann, 2000; Jekkel, Gyulai, Kiss, Kiss, & Heszky, 1998; Martínez, Tames, & Revilla, 1999; Steinmacher, Saldanha, Clement, & Guerra, 2007) has indicated that 15-25% moisture content in the sample is usually optimal for survival after liquid nitrogen exposure. Techniques involving alginate encapsulation and dehydration have been used on a range of plant tissues but mainly shoot tips from *in vitro* plantlets (e.g. Al-Ababneh, Karam, & Shibli, 2002; Kami, Uenohata, Suzuki, & Oosawa, 2008; Niino & Sakai, 1992; Uchendu & Reed, 2008) and somatic embryos (e.g. Gale, John, Harding, & Benson, 2008; Gonzalez-Arnao, Juarez, Ortega, Navarro, & Duran-Vila, 2003; Shibli, 2000). This technique has also been tested on zygotic embryos or embryonic axes of many non-orthodox seeded species, including *Bactris gasipaes* (Steinmacher, et al., 2007), *Citrus* spp. (Cho, Hor, Kim, Rao, & Engelmann, 2002a; Malik & Chaudhury, 2006) and *Hevea brasiliensis* (Yap, Hor, & Normah, 1998).

CHAPTER 3 DESICCATION SENSITIVITY AND DORMANCY

3.1 Desiccation sensitivity

3.1.1 Introduction

New Zealand has relatively small flora comprising over 2,400 taxa, but most species (80%) are endemic (de Lange, et al., 2010; Department of Conservation, 2000). Currently New Zealand has 901 taxa listed as threatened or naturally unknown, with a further six taxa believed to be extinct. Of those 184 taxa classified as being endangered (de Lange, et al., 2010). The high numbers of threatened species in combination with high species endemism has resulted in New Zealand being listed as one of the world's 34 biodiversity hotspots (Conservation International, 2007). Preventing further biodiversity decline through the loss of rare and threatened plant species in New Zealand is a high priority for conservation. In order to combat the loss of so many species several recovery actions are being undertaken. These include a species recovery plan (Department of Conservation, 2000; Dopson, et al., 1999). The NZ Endangered Species Seed Bank, established in 2007 by New Zealand Plant Conservation Network, and AgResearch, with sponsorship from MWH New Zealand, is the facility primarily responsible for the storage of seeds of New Zealand native flora. This facility is insurance against species loss. The seed bank utilises recommended standard procedures for seed storage of 3-7% moisture content and −20°C (FAO/IPGRI, 1994; Knightbridge, 2006). Storage of seed under these conditions is only possible if the seeds exhibit orthodox storage behaviour (Roberts, 1973). Thus before attempting storage, the tolerance of seeds to desiccation and low temperatures needs to be determined. This is most essential for non-orthodox seeded species. Identification of non-orthodox species means that for these species alternative strategies for long-term *ex situ* germplasm conservation can be considered. These alternatives include cryopreservation of embryonic axes (Berjak & Pammenter, 1994; Phartyal, et al., 2002).

This chapter investigates the response of seeds of seven species from the New Zealand flora to desiccation. The aim is to provide the fundamental information necessary for the development of long term conservation strategies for endangered species.

3.1.2 Materials and methods

3.1.2.1 Seed material

Seed materials (i.e. fruits, nutlets or seed pods) were collected from a range of locations in 2007 and 2008 (Table 3.1). Following collection, all seed materials were transported to Massey University (Palmerston North Campus). On receipt, the seed were extracted from the reproductive structures by hand and the initial moisture content and germination determined. Also, the dimension (length, breadth) of 100 seeds was measured to the nearest 0.1 mm using vernier callipers and the 1000-seed weight was measured using a digital balance accurate to 0.0001g.

3.1.2.2 Determination of moisture content

Seed moisture content (MC) of either fresh or dried seeds was determined using the low-constant-temperature oven method described in the International Rules for Seed Testing (ISTA, 2007). Four replicates of 10-25 seeds (Table 3.1) were cut in half and weighed with a digital balance accurate to 0.001 g before and after drying for 17 hours in a 103°C ± 2°C oven. MC was calculated as the percentage of water on a fresh weight basis.

3.1.2.3 Determination of germination capacity

Seed germination was determined using four replicates of 20-50 seeds (Table 3.1). Seeds of all species except *Dysoxylum spectabile* were placed between moist folded 38 lb regular weight seed germination paper (Anchor Paper Company, St. Paul, Minnesota) held in closed plastic boxes and incubated at 20°C ± 2°C, under a 16 hr light/8 hr dark photoperiod. *D. spectabile* was germinated in plastic boxes (220×120×40mm), seeds were placed upright in moist, heat-sterilized sand, which was previously sterilized at 103°C for 24 hr. Seeds were pressed into the surface of the sand until half covered. The remaining exposed seed was then covered with a layer of sterilized sand. Sand was

remoistened when necessary to maintain moist conditions during germination. A seed was considered to have germinated when a normal seedling had developed; a seedling was classified as normal when it had a well-developed primary root and intact hypocotyl and cotyledons (ISTA, 2007).

To achieve full germination, seeds of *Carmichaelia williamsii, Clianthus puniceus, Clianthus maximus, Hibiscus diversifolius* and *Myosotidium hortensium* were scarified by cutting the seed coat in the cotyledon area with a scalpel.

3.1.2.4 Seed desiccation experiment

Seed lots were dried to a number of target moisture contents (Table 3.1) chosen according to the IPGRI screening protocol (IPGRI-DFSC, 2004). Seed from each sample was mixed with an equal weight of silica gel in polythene bags and placed in a desiccator and held at 20°C. Seed samples were monitored daily for target weights. The target weight that corresponded to each target MC was calculated using the following formula (IPGRI-DFSC, 2004):

$$\text{Weight of seed (g) at target moisture content} = \left(\frac{100 - IMC}{100 - TMC}\right) \times \text{initial seed weight (g)}$$

where IMC = initial seed moisture content
TMC = target seed moisture content

As a control for the desiccation experiment of *Tecomanthe speciosa* and *Dysoxylum spectabile*, samples of seeds were held at as close as possible to their collection moisture content by mixing them with moist vermiculite in polythene bag and holding them at 20°C; bags were regularly vented. Once the target weight was reached seed MC and germination were determined as described above for both the samples that had been desiccated and the samples held in vermiculite.

To avoid imbibition damage of seed at 10% moisture content and below, seeds were humidified before germination by placing them above water in a closed container at 20°C for 24hr (IPGRI-DFSC, 2004).

3.1.2.5 Data analysis

Data were analysed for statistical significance by analysis of variance (ANOVA). Fisher's least significant difference ($p \leq 0.05$) was used to determine whether there were significant differences between treatments. Prior to analysis, data were checked for normality using Shapiro–Wilk tests. Data was transformed by arcsine if necessary. SAS for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

Table 3.1 Seed material, number of seeds and chosen target and actual (in brackets) moisture content reached for species used in the desiccation experiment.

Species	Collection Location	Collection date	Arrival date	No. of seeds per replicate used for:		Target moisture content (Actual moisture content) (%)		
				Moisture content	Germination			
Carmichaelia williamsii	Auckland Botanic Garden	20 Dec. 2007	27 Dec. 2007	10	20	10 (8.4)	5 (3.7)	
Clianthus puniceus	Auckland Botanic Garden	20 Dec. 2007	27 Dec. 2007	25	50	10 (10.3)	5 (5.3)	3 (3.7)
Clianthus maximus	Massey University	Dec. 2008	Jan. 2009	25	50	10 (10.7)	5 (5.4)	3 (3.7)
Hibiscus diversifolius	Auckland Botanic Garden	31 Aug. 2007	12 Sep. 2007	25	25	10 (10.8)	3 (2.9)	
Myosotidium hortensium	Otari Wilton Bush, Wellington	Jan. 2008	05 Feb. 2008	25	50	10 (9.2)	7 (5.9)	5 (4.5)
Tecomanthe speciosa	Auckland Zoo	Jan. 2008	27 Feb. 2008	10	50	50 (49.5) 20 (20.7)	40 (39.2) 10 (11.7)	30 (30.7) 5 (5.1)
Dysoxylum spectabile	Horseshoe Bend Reserve, Tokomaru	31 May 2008	31 May 2008	10	50	40 (38.6) 10 (10.4)	30 (29.2) 5 (6.3)	20 (21.3)

3.1.3 Results

3.1.3.1 Initial MC and viability

The characteristics at receipt of the seeds of the seven species used are shown in Table 3.2. The moisture content of *C. williamsii*, *C. puniceus* and C. *maximus* seeds on receipt was around 20% and germination over 95%. Seeds of *H. diversifolius* and *M. hortensium* had around 10% MC with 46% and 86% germination, respectively. The initial moisture content of *T. speciosa* was 53% and seed germination 88%. Freshly harvested mature seeds of *D. spectabile* were at 50% MC and had a germination of 93%.

Table 3.2 Characteristics of *Carmichaelia williamsii, Clianthus puniceus, Clianthus maximus, Hibiscus diversifolius, Myosotidium hortensium, Tecomanthe speciosa* and *Dysoxylum spectabile* on receipt.

Species	Length (mm)	Breadth (mm)	Fresh weight/ 1000 seeds (g)	Dry weight/ 1000 seeds (g)	Initial MC (%)	Germination (%)
Carmichaelia williamsii	4.17 ± 0.026	3.36 ± 0.029	19.8 ± 0.48	15.7 ± 0.82	19.2 ± 1.36	97.5 ± 1.44
Clianthus puniceus	3.26 ± 0.021	2.40 ± 0.022	12.4 ± 0.32	9.5 ± 0.25	22.8 ± 0.29	93.5 ± 0.96
Clianthus maximus	3.08 ± 0.023	2.29 ± 0.023	8.8 ± 0.17	7.1 ± 0.09	20.0 ± 0.54	99.0 ± 0.58
Hibiscus diversifolius	4.12 ± 0.017	3.01 ± 0.014	11.3 ± 0.12	8.9 ± 0.34	12.4 ± 0.45	46.0 ± 6.22
Myosotidium hortensium	8.16 ± 0.081	4.67 ± 0.062	37.0 ± 1.60	33.4 ± 1.46	9.6 ± 0.23	86.2 ± 3.35*
Tecomanthe speciosa	16.12 ± 0.203	36.41 ± 0.334	38.5 ± 2.21	18.1 ± 1.36	52.5 ± 1.10	88.0 ± 3.68
Dysoxylum spectabile	11.82 ± 0.094	8.05 ± 0.101	485.9 ± 3.76	245.6 ± 2.27	49.5 ±0.41	92.5 ± 0.96

Data points are mean of four replicates (± standard error).

* Radicle emergence was taken as the criterion of germination.

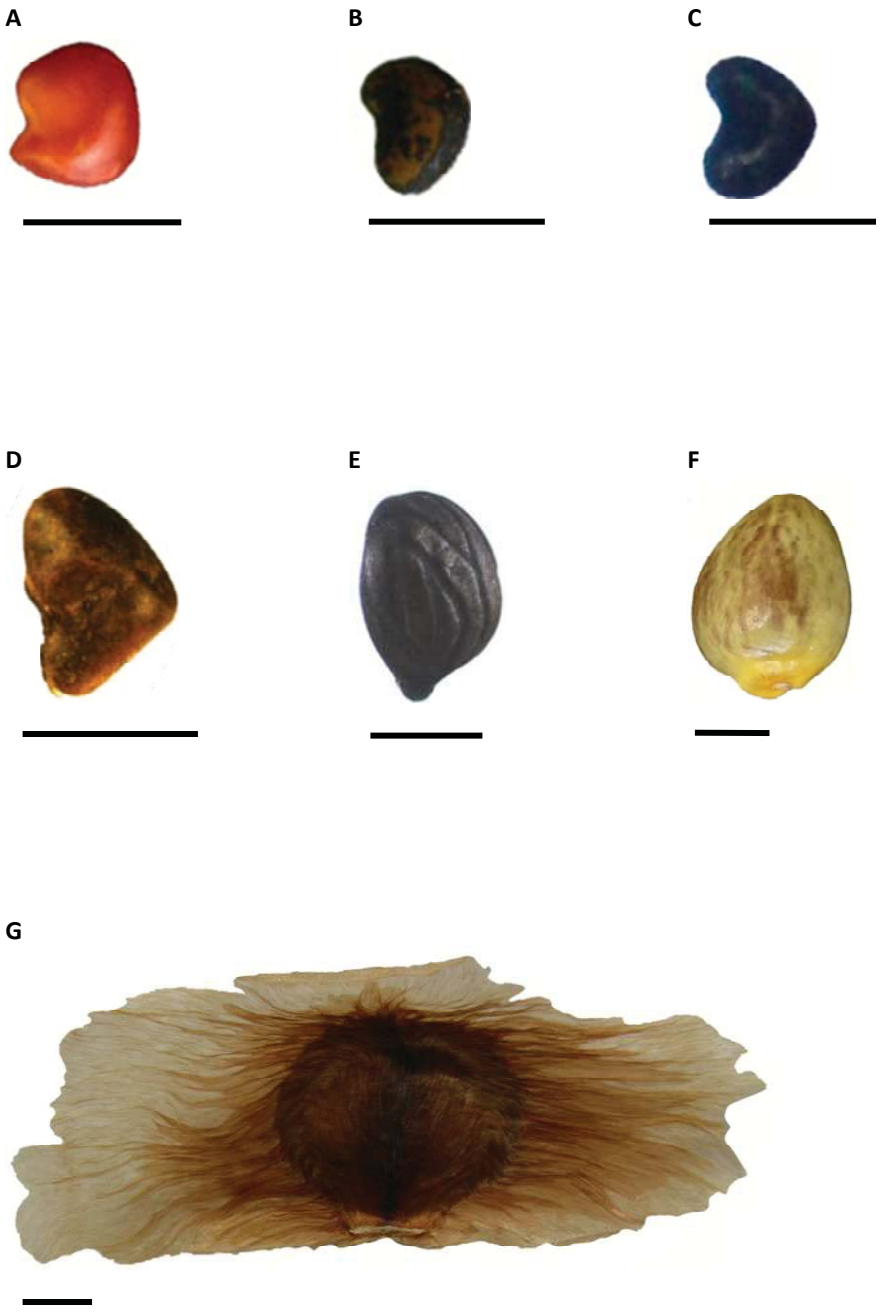

Figure 3.1 A single seed of *Carmichaelia williamsii* (A), *Clianthus puniceus* (B), *Clianthus maximus* (C), *Hibiscus diversifolius* (D), *Myosotidium hortensium* (E), *Dysoxylum spectabile* (F) and *Tecomanthe speciosa* (G). Bars indicate 0.5 cm.

3.1.3.2 Desiccation sensitivity

Seeds of *C. williamsii*, *C. puniceus, C. maximus, H. diversifolius* and *M. hortensium* tolerated desiccation over silica gel to 2.9-4.5% MC while maintaining their initial germination. *C. williamsii* seed germination of 96-98% was maintained as MC declined from 19.8% to 3.7% (Figure 3.2, A). Both *C. puniceus* and *C. maximus* seeds tolerated desiccation to about 3.7% MC without loss of initial germination (Figure 3.2, B). *H. diversifolius* seed had poor initial germination (46%), but germination did not decline further with desiccation to 2.9% (Figure 3.2, C). For *Hibiscus diversifolius* the dead seed percentage at 12.4, 10.8 and 2.9% MC was 50%, 37% and 41%, respectively. Seeds of *M. hortensium* survived desiccation to MC of 4.5% without loss of viability (Figure 3.2, D).

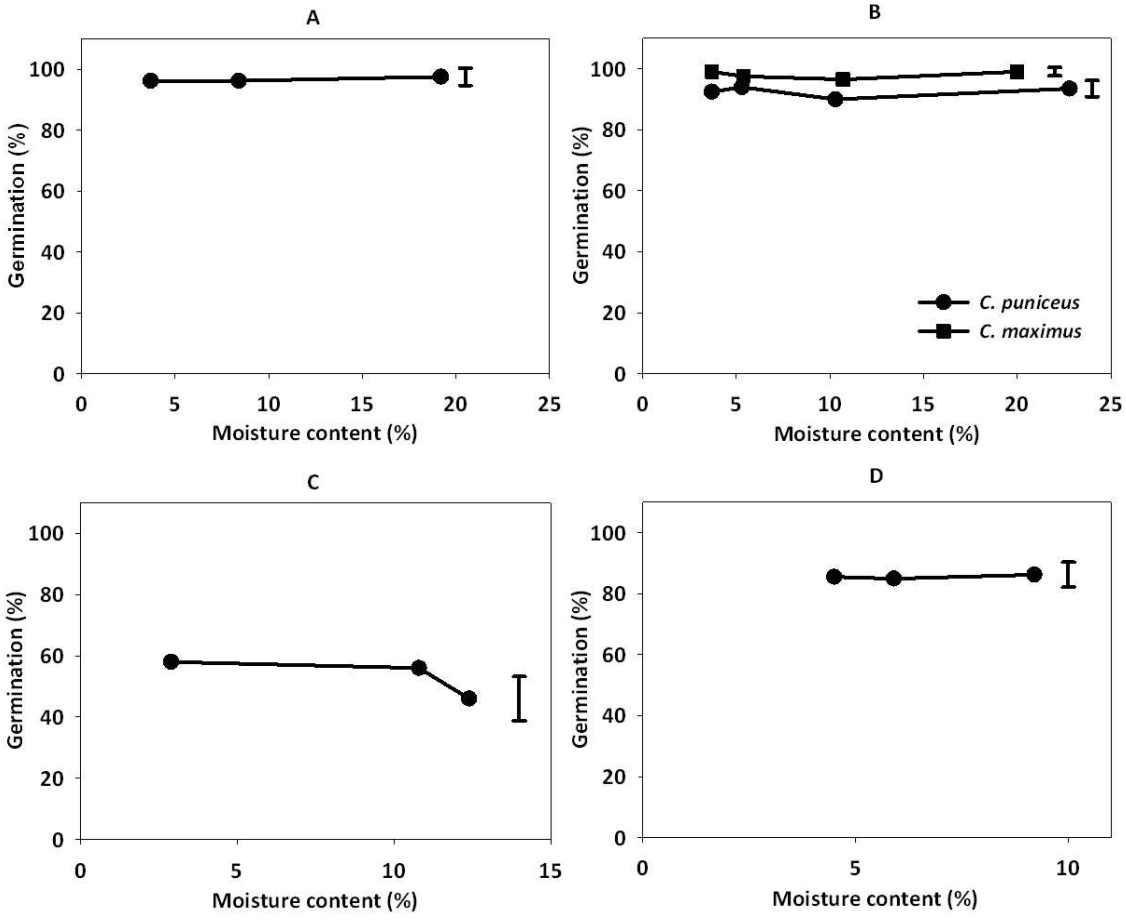

Figure 3.2 Germination (%, normal seedlings) of seeds during desiccation: (A) *Carmichaelia williamsii*, (B) *Clianthus puniceus* and *Clianthus maximus*, (C) *Hibiscus diversifolius*, (D) *Myosotidium hortensium*. Vertical bars indicated the least significant difference between mean germination percentages for each species ($p \leq 0.05$). Radicle emergence was taken as the criterion of germination of *M. hortensium*.

The majority of seed of *T. speciosa* germinated after desiccation to the lowest MC studied, 5.1%. Nevertheless, progressive reduction in germination with desiccation was detected ($p \leq 0.05$). The moisture content of seeds in the control treatments increased slightly throughout the experiment but there was no significant difference in the germination of control seeds at any moisture content (Table 3.3).

Table 3.3 Change in germination (normal seedlings [arcsin value]) in *Tecomanthe speciosa* as seed moisture declined compared with control germination.

Drying time (hr)	Target MC (%)	Drying		Control	
		SMC (%)	Normal seedlings (%)	SMC (%)	Normal seedlings (%)
0	Initial	52.5 a	88.0 [70.8] a	52.5 c	88.0 [70.8] a
3	50	49.6 b	89.1 [72.0] a	55.2 bc	89.5 [72.9] a
17	40	39.2 c	92.6 [75.1] a	55.4 bc	91.5 [73.8] a
34	30	30.7 d	89.7 [74.3] a	56.4 abc	88.7 [71.6] a
51	20	20.7 e	85.5 [68.7] a	57.4 ab	79.9 [64.0] a
81	10	11.7 f	73.1 [60.1] ab	57.6 ab	89.5 [72.2] a
136	5	5.1 g	60.9 [51.4] b	60.4 a	89.3 [70.9] a

* Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Viability of *D. spectabile* was reduced considerably by desiccation. The germination of *D. spectabile* decreased from 93% to 58% after drying to 29% MC. Only 3% of the seed population survived desiccation to 21% moisture content and no seed germinated below this moisture level (Table 3.4). The moisture content of the control treatments showed a slight increase after 140 hours from 50% to 53% at 140 hours. The germination of control seeds declined over time form 93% at 0 hour to 43% after 83 hours before increasing to 59% at 140 hours (Table 3.4).

Table 3.4 Change in germination (normal seedlings) in *Dysoxylum spectabile* as seed moisture declined compared with control germination.

Drying time (hr)	Target MC (%)	Drying		Control	
		SMC (%)	Normal seedlings (%)	SMC (%)	Normal seedlings (%)
0	Initial	49.5 a	93 a	49.5 b	93 a
12	40	38.6 b	96 a	50.5 b	80 ab
31	30	29.2 c	58 b	50.3 b	67 bc
53	20	21.3 d	3 c	50.0 b	51 de
83	10	10.4 e	0 c	50.1 b	43 e
140	5	6.3 f	0 c	52.9 a	59 cd

* Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

3.1.4 Discussion

3.1.4.1 *C. williamsii and C. puniceus spp.*

The desiccation tolerance of *C. williamsii, C. puniceus* and *C. maximus* seeds is typical of orthodox seed. The desiccation tolerance of *C. williamsii,* and the two *Clianthus* spp. seeds is consistent with that widely reported in other Fabaceae of which 99% were classified as orthodox (Dickie & Pritchard, 2002; Royal Botanic Gardens Kew, 2008). For example, Ellis et al. (2007) determined seed storage behaviour of 17 Fabaceae species. Seeds of all 17 species tolerated drying to moisture contents to 5-6% and survived 3-120 months' hermetic storage at -18°C and so are probably orthodox, as defined by Roberts (1973). However, seeds which tolerate desiccation to low moisture content do not necessarily show orthodox storage behaviour. For example, seeds of *Anadenanthera colubrine* (Fabaceae) tolerated desiccation to 4% MC (98% germination), but only retained 20% germination after 23 months of hermetic storage at –18°C and 4% MC (Rojas Espinoza, 2005). To confirm that the seed behaviour of species assessed in this work are orthodox, retention of germination in storage at these low moisture contents and low temperature (for example -18°C) will need to be determined as suggested by Hong & Ellis (1996).

3.1.4.2 *H. diversifolius*

Hibiscus diversifolius also appears to show orthodox seed storage behaviour. The response to desiccation is similar to seeds of other *Hibiscus* species of which 98% were classified as orthodox (Royal Botanic Gardens Kew, 2008). *H. cannabinus* has been reported as desiccation tolerant, retaining 86% germination at 7.2 and 1.1% MC ((Hu, Zhang, Hu, Tao, & Chen, 1998). Also this species retained 66% germination after 39.5 years storage at -18°C indicating orthodox storage behaviour (Walters, et al., 2005).

In this study, the germination percentage of *H. diversifolius* was low. Angelini et al. (1998) commented that low germination percentage (40.8%) of *H. cannabinus* grown

in central Italy was due to unfavourable temperature and moisture conditions during seed filling, resulting in rapid seed deterioration and increased seed coat susceptibility to fungal pathogens. This presence of fungi on dead *H. diversifolius* seeds observed in this study may suggest a similar problem, but in the absence of data on reserve accumulation in the seed lot, this is speculative. Nevertheless germination did not decline further with desiccation suggesting *H. diversifolius* seeds are likely to be orthodox in their storage behaviour.

3.1.4.3 *M. hortensium*

McGill et al. (2002) reported that *M. hortensium* seeds can be stored for 21 months at 7.5% moisture content and 5°C suggesting *M. hortensium* seeds are orthodox in storage behaviour. However, Hong and Ellis (1996) use desiccation tolerance to 5% or below as the criterion for confirmation that storage behaviour is likely to be likely orthodox. This work has established that *M. hortensium* seeds will tolerate desiccation to 4.5% moisture content confirming the suggestion by McGill et al. (2002) that *M. hortensium* seed appears to be orthodox in storage behaviour.

McGill et al. (2002) also suggested that evaluation of the storability of *M. hortensium* at lower seed moisture contents be undertaken, in particular, because of the high lipid content of *M. hortensium* seeds (24% of the dry weight). The authors reported that an increase in seed moisture content to 9.5% resulted in a complete loss of germination by nine months of storage. It has been suggested that ultra-dry storage at very low seed moisture content could be more appropriate for seeds with high lipid contents (Ellis, Hong, & Roberts, 1989; Vertucci & Roos, 1990).

3.1.4.4 *T. speciosa*

Germination of *T. speciosa* was not affected by desiccation until MC reduced to 5.1% when germination was significantly lower than that at all other seed moisture contents except 11.7%. Nonetheless, even at 5.1% seed moisture, 60% of the seed population retained germination. *T. speciosa* has a high level of desiccation tolerance and this is consistent with other Bignoniaceae being classified as orthodox (Royal Botanic Gardens Kew, 2008). For example, seeds of *Kigelia africana* tolerated desiccation to 3-7% seed moisture and survived in storage for at least 18 months at low temperatures (4°C) (Dudley, Wood, & Pritchard, 2001; Sanon, Gamene, Sacande, & Neya, 2005). This study has shown that that *T. speciosa* seeds are not recalcitrant, but the loss of some viability between 5.1 and 20.7% may indicate that seed storage behaviour is not orthodox. Metcalf (1995) and Morris & Ballance (2008) suggest that *T. speciosa* cannot be stored for long periods, especially if it has dried out. Further research will be required, including long-term storage trials, to determine if the seed storage behaviour of *T. speciosa* is orthodox as defined by Hong & Ellis (1996).

3.1.4.5 *D. spectabile*

Seeds of *D. spectabile* appear to be desiccation sensitive, as none of the seeds dried in silica gel to moisture contents below 21.3% remained viable. The level of desiccation sensitivity displayed by *D. spectabile* seeds is typical of many recalcitrant, seeds i.e., viability is lost at 20–30% moisture content (Pritchard, 1991, 2004). Moreover seeds of most other *Dysoxylum* species are classified as recalcitrant (Royal Botanic Gardens Kew, 2008). Hong and Ellis (1998) investigated patterns of response to seed desiccation in 40 Meliaceae species. The authors found that species with desiccation-sensitive seeds typically occur in moist areas, particularly rainforest, and produce large (>1 g), round seeds, which are shed at high moisture contents. *D. spectabile* also produce almost round seeds and shed at high moisture contents around 50% moisture content.

For *D. spectabile* seeds maintained in vermiculite under ambient conditions for 140 hours, germination fluctuated despite no significant change in seed moisture content over the first 83 hours storage and an increase, rather than a decrease, in moisture at 140 hours (Table 3.4). The fluctuation in germination was most likely a function of fungal infection. This is typical at many recalcitrant seeds which usually harbour a wide spectrum of fungi and bacteria that even when they have been newly harvested (Berjak, 1996; Mycock & Berjak, 1990). Other studies on recalcitrant seeds (e.g. Berjak, 1996; Motete, Pammenter, Berjak, & Frédéric, 1997) also report that rapid decline in viability during storage was accompanied by fungal proliferation. For future trials to produce unequivocal results in terms of desiccation sensitivity, it is essential that the mycoflora be inactivated or eliminated. However, even when seeds are treated with antimicrobial agents, the sustained stress of hydrated-storage on seeds promotes fungal proliferation while mechanisms to counteract the proliferation of fungi become increasingly ineffective (Anguelova-Merhar, Calistru, & Berjak, 2003; Calistru, McLean, Pammenter, & Berjak, 2000; Mycock & Berjak, 1990).

3.2 Seed germination

3.2.1 Introduction

Ex situ conservation of threatened species is regarded as a possible tool for supporting habitats and for re-establishing self-sustaining wild populations if or when a suitable site is located (Cochrane, et al., 2007; FAO, 1996; Havens, et al., 2006; Heywood & Iriondo, 2003). The *ex situ* conservation of a target species involves the collection of material, its storage of maintenance and its regeneration in order to ensure the continued availability of viable material (Lawrence, 2002). It is important that *ex situ* collection materials represent as closely as possible the genetic diversity of the population (Brown & Briggs, 1991). Plant materials that can be used for *ex situ* conservation range from whole plants through to seeds, pollen, vegetative material or DNA (Maxted, Ford-Lloyd, & Hawkes, 1997). Storage of seeds is considered an efficient and cost-effective method of conserving variation within and among individual species (Roberts, 1991), minimizing the loss of genetic diversity (Fay, 1992). Seed collection has less impact on a population than removal of whole plants or plant parts (Menges, 1991). Seeds cannot be stored indefinitely and viability must be monitored to ensure that the seeds do not die before they can be regenerated (Rice, et al., 2006). The germination test is commonly used for assessing seed viability (Phartyal, et al., 2002). However, in some species seeds fail to germinate even under favourable germination conditions as a result of dormancy (Bewley & Black, 1994). There are a number of different forms of dormancy found in seeds (reviewed in section 2.4). Identification of any germination requirements, including dormancy-breaking strategies is important for efficient *ex situ* conservation. Moreover, the choice of suitable techniques, based on the requirements of each species, may reduce waste of seeds (Brusa, Ceriani, & Cerabolini, 2007), especially for threatened species where seed may be difficult to obtain regularly and/or in large quantities (Baskin & Baskin, 2004a; Lawrence, 2002).

For many threatened New Zealand native species, however, basic knowledge on seed dormancy and germination requirements is either incomplete or unavailable. Thus, the objectives of this study are to confirm the presence of dormancy in the seeds of seven native species and determine the possible mechanisms by which any dormancy is

imposed. Previous studies had shown that *M. hortensium* had coat-imposed dormancy but that the seed appeared to be water-permeable. To confirm the water-permeability of the coat, water absorption by *M. hortensium* seed was determined and to determine the mechanism by which the coat imposes dormancy, a number of dormancy-breaking techniques were applied.

3.2.2 Materials and methods

3.2.2.1 Seed material

Seeds of *C. williamsii, C. puniceus, C. maximus, H. diversifolius, M. hortensium, T. speciosa* and *D. spectabile* were sourced and processed as described in 3.1.2.1.

3.2.2.2 Determination of moisture content and germination

Seed moisture content determination and germination was as described in 3.1.2.2.

3.2.2.3 Determination of dormancy mechanisms

3.2.2.3.1 Determining hardseededness

Seeds were set to germinate as described in 3.1.2.3. Seeds that had not germinated after 3 weeks on the germination blotter were counted and scarified with a scalpel by cutting the seed coat in the cotyledon area. The following species required scarification: *C. williamsii, C. puniceus, C. maximus, H. diversifolius*. Scarified seeds were returned to 20°C to continue the germination process for another 6 days after which ungerminated seeds were classified as viable when they showed no sign of infection and were firm when pressed. Normal seedlings were assessed as described in 3.1.2.3.

3.2.2.3.2 Assessment of water absorption by M. hortensium seeds

Seeds were put into transparent plastic boxes (175×113×45mm) lined with two soaked filter papers (10 ml of distilled water). The boxes were kept at 20°C in an incubation room. All seeds were dried with a paper towel at 1-4 hr intervals and then weighed with a digital balance accurate to 0.001 g. After weighing, seeds were returned to their respective plastic boxes in the incubation room. Four replicates of 50 seeds were used for the assessment.

3.2.2.3.3 Determining coat-imposed dormancy mechanisms in M. hortensium seeds

M. hortensium seeds had a water-permeable coat but showed no germination after 3 weeks. *M. hortensium* seed was therefore subjected to a number of dormancy-breaking treatments and germination and the respiration rate of treated seed measured to determine whether the seed coat prevents germination by restricting O_2 uptake, by mechanically preventing embryo expansion or the seed coat contains chemical inhibitors that inhibit germination.

Prior to the application of the dormancy-breaking treatments seeds were preconditioned by rolling them in moistened 38lb regular weight seed germination paper (Anchor Paper Company, St. Paul, Minnesota). The roll was placed in a beaker with approximately 2 cm of water in the bottom. The beaker and roll were covered with a plastic bag and left at 20°C for 24 hours. Seeds were then surface dried by pressing them between two sheets of Whatman Number One filter paper, and manipulated in the following ways.

1) The seed coat was cut with a scalpel taking care to apply gentle pressure. The seed coat was then removed by hand – "decoated seeds"

2) The seed coat was removed as described above and then the decoated seed was then loosely re-covered with the seed coat

3) The seed was pricked with a fine (~0.1 to 0.2mm thick) needle at the centre of the seed (in the cotyledon area) – "pricked seeds"

4) The seed was pricked and the pricked area covered with Vaseline®

Manipulated seeds, plus control seed, was then split into two lots. Fungicide was applied to one seed lot. These seeds were dusted with the fungicide Thiram 80W (800g/kg thiram) as a powder, by placing seed in a conical flask with an amount of thiram equal in volume to one seed for every fifty seeds. Seeds were then swirled until evenly coated with Thiram 80W. Excess Thiram 80W was removed by sieving the seeds.

Four replicates of 50 seeds with or without the application of fungicide were set to germinate as described in 3.1.2.3.

The second part of the dormancy study was conducted to determine the effect of the seed coat on oxygen diffusion to the embryo. The rate of oxygen uptake and CO_2 evolution by intact seeds, decoated seeds, pricked seeds and seeds with Vaseline® applied to pricked area was measured using a Gilson differential respirometer. Seeds of each four replicate from each treatment were split into two flasks. One flask used for the measurement of oxygen uptake. A single paper wick (Whatman Number One filter paper) with saturated with 0.5 ml of 20% KOH (w/v) solution was placed in the centre well of each flask for CO_2 absorption. Thus, changes in volume of gas represented oxygen uptake. The other flask for the measurement of net O_2 and CO_2 exchange did not contain KOH on a filter paper wick in the centre well. A series of 12mL flasks was used for each measurement, each flask containing four seeds. The system was allowed to equilibrate for 30 min prior to measurements. Oxygen uptake and net O_2 and CO_2 exchange was measured daily for one hour after equilibration. CO_2 evolution was calculated by subtraction of O_2 consumption from the net O_2 and CO_2 exchange. The experiment was repeated six times with two replications.

3.2.2.4 Data analysis

Data were analysed for statistical significance by analysis of variance (ANOVA). Fisher's least significant difference ($p \leq 0.05$) was used to determine whether there were significant differences between treatments. Prior to analysis, data were checked for normality using Shapiro–Wilk tests. No transformations were necessary. SAS for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

3.2.3 Results

3.2.3.1 Germination of *C. williamsii*

Freshly collected seeds of *C. williamsii* reached 95% germination without any pre-treatment. However, the ability of dried seeds to germinate declined subsequently to 14 and 13% at moisture contents of 8.4% and 3.7%, respectively (Table 3.5). Seeds that did not imbibe during the 3-week germination test were classified as impermeable. The proportion of seeds of *C. williamsii* having an impermeable seed coat increased as moisture content declined to 3.7%. A consequence of the development of hardseedness was that seed required scarification for germination to occur. After scarification, germination percentage increased to 96-98% compared to 13-14% in untreated seeds (Table 3.5).

Table 3.5 Hardseededness percentages of fresh and after drying on silica gel and changes of germination of without scarification and after scarification *Carmichaelia williamsii.*

Drying time (days)	Moisture Content (%)	Hardseededness (%)	Germination (% of viable seed)	
			Without scarification	After scarification
0	19.8	3 B	95 Aa	98 Aa
5	8.4	86 A	14 Bb	96 Aa
35	3.7	82 A	13 Bb	97 Aa

Numbers following the same uppercase letters are not significantly different;
Numbers within rows sharing the same lowercase letters are not significantly different (LSD, $p \leq 0.05$).

3.2.3.2 Germination of *C. puniceus and C. maximus*

Freshly collected seeds of *C. puniceus* and *C. maximus* were able to germinate (73-98%) without any pre-treatment. However, the ability of dried seeds to germinate declined to 5% and 0%, respectively for each species, at approximately 10% moisture content. This decline in germination was accompanied by an increase in hardseededness (Table 3.6). However, hardseededness in *C. puniceus* and *C. maximus* decreased markedly when seeds were further dried to 5.4% and 3.7% MC.

Seeds with a water impermeable seed coat required scarification for germination to proceed. After scarification, germination percentage increased to 96-100% for both species compared to 0-5% in untreated seeds (Table 3.6).

Table 3.6 Hardseededness percentages of fresh and after drying on silica gel and changes of germination of without scarification and after scarification the native species, *Clianthus puniceus* and *C. maximus*.

Drying time (days)	Moisture Content (%)	Hardseededness (%)	Germination (% of viable seed)	
			Without scarification	After scarification
C. puniceus				
0	22.8	25 B	73 Bb	96 Aa
2	10.3	92 A	5 Cb	96 Aa
10	5.3	12 C	85 Ab	96 Aa
35	3.7	9 C	84 Ab	96 Aa
C. maximus				
0	20.0	2 C	98 Aa	99 Aa
2	10.7	100 A	0 Cb	97 Aa
9	5. 4	21 B	74 Bb	98 Aa
35	3.7	3 C	97 Aa	99 Aa

Numbers following the same uppercase letters are not significantly different within each species;
Numbers within rows sharing the same lowercase letters are not significantly different (LSD, $p \leq 0.05$).

3.2.3.3 Germination of *H. diversifolius*

Only 5-20% of fresh and dried seeds in *H. diversifolius* germinated without scarification. Scarification improved germination of *H. diversifolius* to 98-100%. There was a small but insignificant decrease in the percentage of seed with an impermeable seed coat of *H. diversifolius* during desiccation (Table 3.7).

Table 3.7 Hardseededness percentages of fresh and after drying on silica gel and changes of germination of without scarification and after scarification *Hibiscus diversifolius*.

Drying time (days)	Moisture Content (%)	Hardseededness (%)	Germination (% of viable seed)	
			Without scarification	After scarification
0	12.4	95 A	5 Ab	100 Aa
1	10.8	89 A	11 Ab	97 Aa
19	2.9	80 A	20 Ab	98 Aa

Numbers following the same uppercase letters are not significantly different;
Numbers within rows sharing the same lowercase letters are not significantly different (LSD, $p \leq 0.05$).

3.2.3.4 Germination of *M. hortensium*

3.2.3.4.1 Assessment of water absorption

M. hortensium seeds absorbed water rapidly during the first five hours of imbibition (Figure 3.3). Uptake then slowed, but continued for about 36-48 hr.

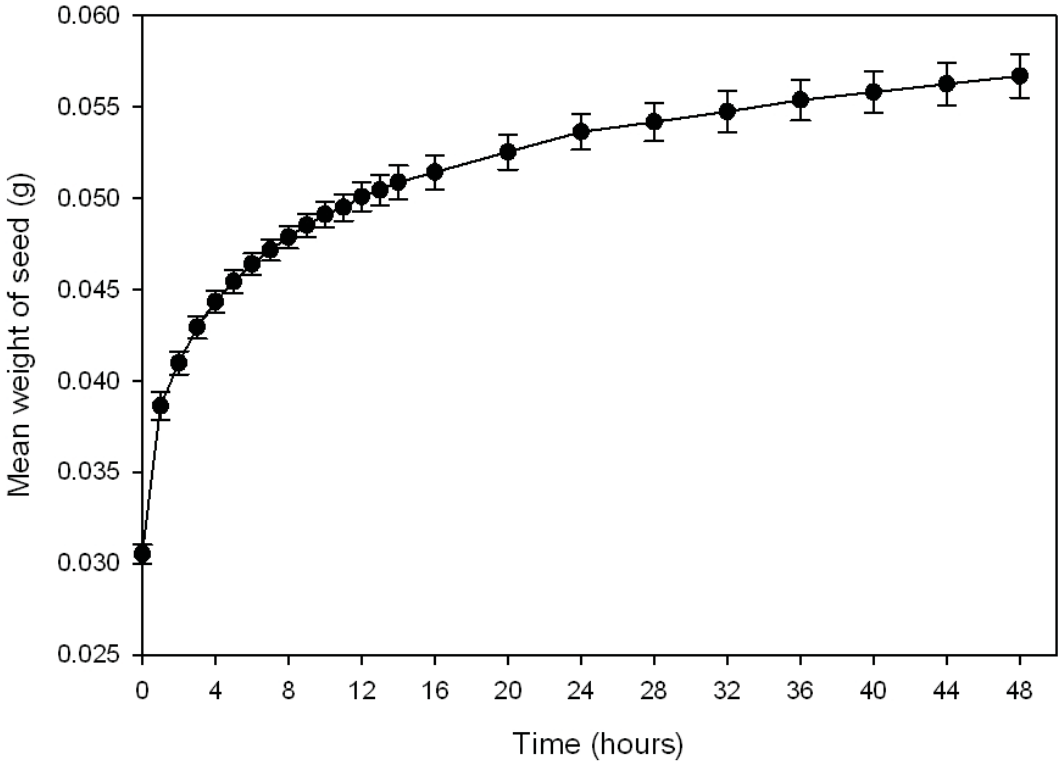

Figure 3.3 Water uptake of *Myosotidium hortensium* seed over time. n=4; vertical bars are standard errors.

3.2.3.4.2 M. hortensium response to the different dormancy breaking treatments

After 63 days, only 20.8% seeds of *M. hortensium* had germinated. Removal of the seed coat increased germination but whether the seed coat was removed (88.2%) or the decoated seed loosely replaced in the coat (81.5%) did not significantly affect germination (Figure 3.4). Pricking of seeds with or without the application of Vaseline® increased germination to 50.4% and 43.7%, respectively and the effect of Vaseline® was not significant (Figure 3.4).

The percentage of fresh ungerminated seed in the pricking treatments (15.5 - 22.5%) was significantly higher than for decoated treatments (0%) (Table 3.8).

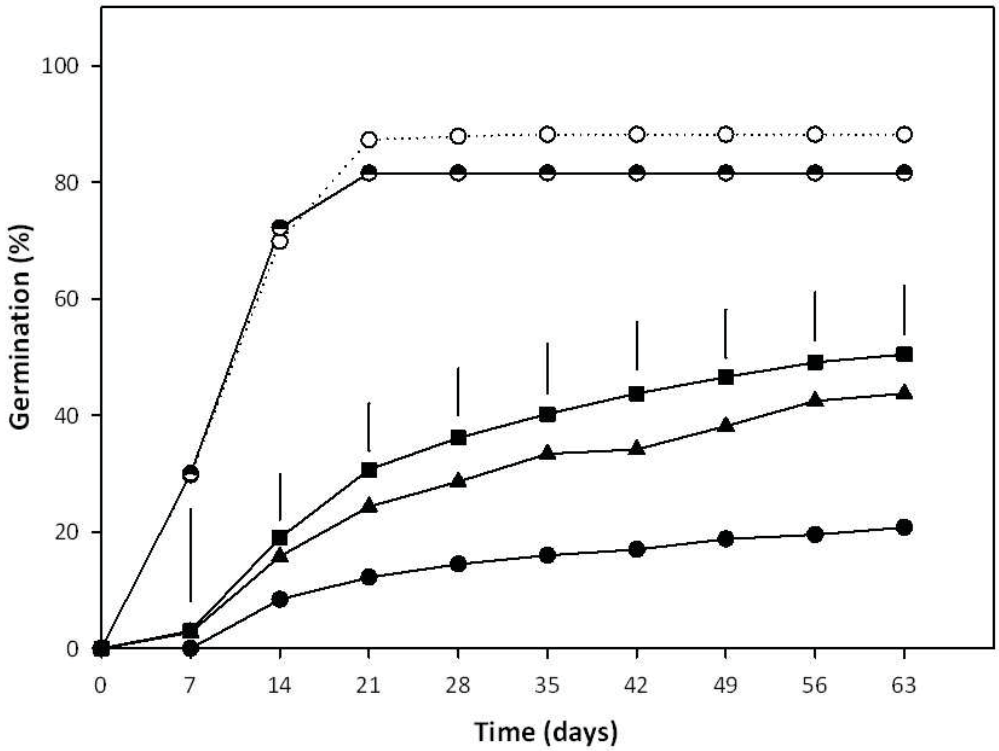

Figure 3.4 Cumulative germination (%,final radicle emergence) of control (●), decoated seeds without a seed coat (○), with their seed coat returned (◓), seeds that were pricked with a fine needle at the centre (■), or with Vaseline® applied to pricked area (▲) of *Myosotidium hortensium* seeds. Vertical bars indicated the least significant difference between mean germination percentages for each day ($p \leq 0.05$).

Fungicide reduced fungal growth on seeds in the control treatment and reduced the percentage of dead seeds and abnormal seedlings between the different dormancy breaking treatments. However, the percentage of dead seeds was significantly increased by the combined application of fungicide and Vaseline®. (Table 3.8).

Table 3.8 Percentage of normal and abnormal seedlings, fresh ungerminated and dead seeds of *Myosotidium hortensium* after 63 days.

Dormancy breaking treatments	Normal seedlings		Abnormal seedlings		Fresh ungerminated seeds		Dead seeds		Radicle emergence	
	Non fungicide	Fungicide	Non fungicide	Fungicide	Non fungicide	Fungicide	Non fungicide	Fungicide	Non fungicide	Fungicide
Control	16.5 d	22.0 c	3.0 c	0.0 b†*	43.0 a	61.5 a†**	37.5 a	16.5 b†**	19.5 d	22.0 c
Decoated	80.5 a	84.5 a	7.0 b	4.3 a	0.0 c	0.0 c	12.5 d	11.2 b	87.5 a	88.9 a
Decoated with seedcoat	67.5 b	81.2 a†**	11.2 a	2.8 a†**	0.0 c	0.0 c	20.9 cd	16.0 b	79.1 a	84.0 a
Pricked	44.0 c	45.3 b	9.5 ab	2.1 ab†*	15.5 b	15.6 b	31.0 ab	37.0 a	53.5 c	47.4 b
Pricked with Vaseline®	42.5 c	35.9 b	8.5 ab	0.5 b†**	22.5b	20.7 b	26.5 bc	42.9 a†**	51.0 c	36.4 b

Numbers following the same letters are not significantly different (LSD, $p \leq 0.05$).
†Statistical analysis was done with a two-tailed, unpaired, Student's t test. Compared to the non-fungicide treatments within each dormancy breaking treatments, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

3.2.3.4.3 The effect of the dormancy breaking treatments on oxygen uptake by the seeds

Removal of seed coats increased seed oxygen uptake (Figure 3.5). During the first 2 hours, oxygen uptake by decoated seeds increased 58-81% compared with other treatments and by approximately 450-570% after 72 hours. However, O_2 uptake by pricked seeds and seeds that had Vaseline® applied to the pricked area did not differ significantly ($p \leq 0.05$) from each other or from intact (control) seeds.

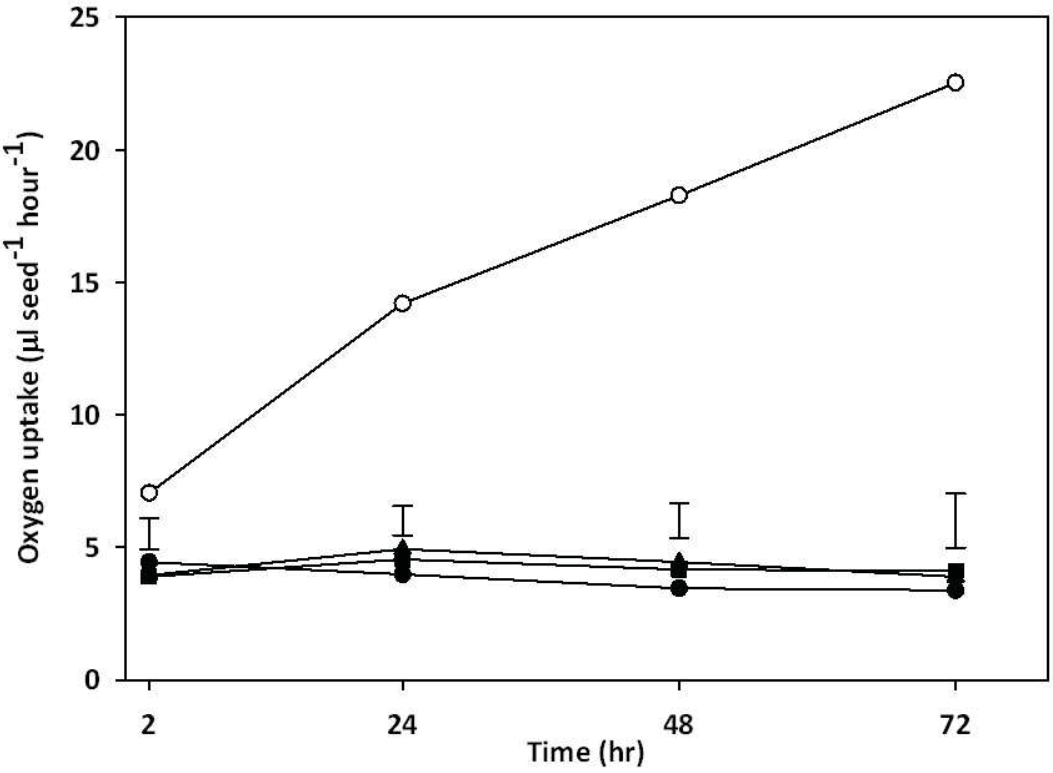

Figure 3.5 The rate of oxygen uptake of intact seeds (●), decoated seeds without a seed coat (○), seeds that were pricked with a fine needle at the centre (■), and Vaseline® applied to pricked area (▲) of *Myosotidium hortensium*. Data are the means of six separate assessments using the Gilson Respirometer with two replications per assessment. Vertical bars indicated the least significant difference between mean oxygen uptake for each time ($p \leq 0.05$).

3.2.3.5 Germination of *T. speciosa*

The germination percentage of fresh harvested *T. speciosa* seeds was 88%. The time to 50% germination (T_{50}) was 19 ± 3 days (Figure 3.6).

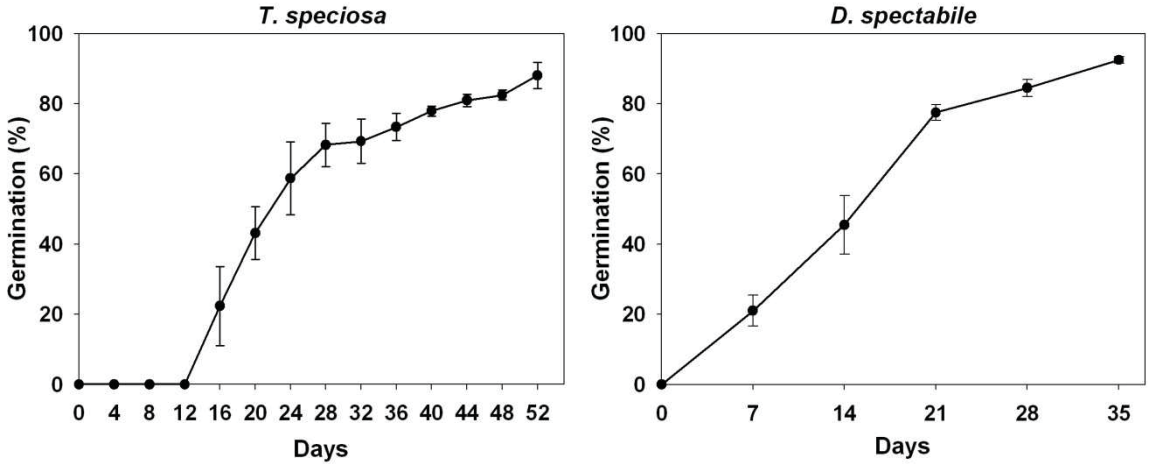

Figure 3.6 Cumulative germination percentages of *Tecomanthe speciosa* and *Dysoxylum spectabile* seeds. n=4; vertical bars are standard errors.

3.2.3.6 Germination of *D. Spectabile*

For *D. spectabile*, fresh mature seeds showed 93% germination and a T_{50} of 14 ± 1 days (Figure 3.6). Some of the seeds of *D. spectabile* germinated as soon as they were shed from the fruits.

3.2.4 Discussion

3.2.4.1 *C. williamsii, Clianthus* spp. and *H. diversifolius*

Seeds of *C. williamsii*, *Clianthus* spp., and *H. diversifolius* clearly have impermeable coats. Non-scarified dried seeds do not imbibe water, whereas manually scarified seeds took up water and germinated rapidly. These results confirm that dormancy in these species is a function of the seed coat preventing water uptake by the dry seed. Hardseedness is typical of many species of Fabaceae and Malvaceae (Mai-Hong, Hong, Hien, & Ellis, 2003; Michael, Steadman, & Plummer, 2007; Van Assche & Vandelook, 2006). At the species level, Shaw & Burns (1997), Gruner & Heenan (2001) and Westra et al. (1996) have reported the scarification is required for germination of *C. puniceus*, *Carmichaelia* spp. and *Hibiscus trionum* respectively.

In species exhibiting hardseededness, water impermeability of the seed coat usually develops at moisture contents between 54% and 15% during maturation drying of the seed (Michael, et al., 2007). If seeds are removed from the plant after embryo maturity, but before any impermeability can develop, seeds should be capable of germination (Mai-Hong, et al., 2003; Michael, et al., 2007; Usberti, et al., 2006). This response has been observed in Fabaceae (Mai-Hong, et al., 2003; Usberti, et al., 2006) and was observed in the Fabaceae studied here where desiccation also caused an increase in the impermeability of the seed coat.

A water-impermeable seed coat has been suggested as a factor in the ability of seed of some species to survive long-term storage (e.g. *Liparia villosa* is reported to have survived for >200 years) (Daws, Davies, Vaes, van Gelder, & Pritchard, 2007). In many species of Fabaceae, the hilum acts as a "one-way valve" (Daws, et al., 2007). This allows loss of water from the seed, but not uptake (Hyde, 1954). Consequently, the seed MC will equilibrate to that of the lowest relative humidity experienced by the seed and remain at that MC irrespective of whether higher ambient relative humidity is subsequently experienced. This ability to remain at low MC even under high ambient relative humidity is likely to be a key factor in maximising long-term survival of these species (Daws, et al., 2007). The loss of water impermeability below 6% MC observed in seed of *C. puniceus* may therefore affect the ability of this species to survive long-

term in storage. The effect, if any, needs to be determined. There are no reports in the literature of seed of the Fabaceae losing water impermeability with drying.

The mechanism by which drying to low seed moisture alleviates hardseedness in *C. puniceus* is not known. However, for many Fabaceae treatments such as acid scarification, hot water, dry heat, and high or fluctuating temperature will alleviate hardseedness by inducing fractures in the lens, hilum, micropyle or seed coat (Hu, et al., 2008; Zeng, et al., 2005). There is no evidence that drying is involved in loss of impermeability in Fabaceae. However in some species of Geraniaceae dry storage or drying of hard seed can render the seed coat permeable. For example, after drying exhumed seeds of *Erodium* and *Geranium* species for 7 days in a desiccator over silica gel, 88-100% of the seeds germinated in one or two days compared with 0-1% in seeds that had not been dried (Van Assche & Vandelook, 2006). The current study showed a similar result for *C. puniceus* and *C. maximus* where drying for a relatively short period (10 days) in silica gel increased seed coat permeability.

The present study emphasises the importance of an understanding of the desiccation behaviour and dormancy mechanisms within seed. At the collection MCs of 19.8% 22.8% and 20.0% for *C. williamsii, C. puniceus* and *C. maximus* respectively hardseedness had not developed. Desiccation to MCs of approximately 10% induced hardseedness and resulted in low germination percentage (0-14%). Failure to recognise the decline in germination with desiccation as being a function of the imposition of dormancy can lead to the erroneous conclusion that viability loss is associated with loss of moisture.

3.2.4.2 *M. hortensium*

Dormancy in seed of *M. hortensium* is most likely a function of the seed coat (McGill, 2003). The immediate and rapid uptake of water by *M. hortensium* seed demonstrates that the seed coat of *M. hortensium* is permeable to water. Potential inhibitors of germination of viable seeds with a water permeable seed coat are the presence of one or more germination inhibiting compounds in the seed coat; the seed coat acting as a barrier to oxygen supply to the embryo; the seed coat itself acting as a barrier to germination (radical emergence) or a combination of these (Bewley & Black, 1994).

McGill (2003) proposed that the dormancy of *M. hortensium* may involve either the seed coat acting as a mechanical barrier to embryo growth, restriction of gas exchange or a combination of both. Manipulating the seed coat by peeling, cutting, chipping and pricking were effective in breaking dormancy. However the treatments used in the McGill (2003) study would both weaken the seed coat and improve gas exchange and it was not therefore possible to determine the precise dormancy mechanism or mechanisms from the work. The structure of the seed coat of *M. hortensium* also supports the suggestions that either physical constraint, restriction of gas exchange by the seed coat or both are involved in the regulation of dormancy (McGill, 2003). The seed coat surface of *M. hortensium* has a network of ridges on the surface and vascular elements below the surface allowing that coat to constrain embryo growth and there are no intercellular spaces which may restrict oxygen diffusion (McGill, 2003).

In this study, germination of de-coated seeds placed within their seed coats was high (82%) and not significantly different from de-coated seeds. This suggests that dormancy in seed of *M. hortensium* is not a function of inhibitors in the seed coat. McGill (2003) reported that germination of de-coated *M. hortensium* seeds on seed leachate or seed coats was not significantly below that of de-coated seed germinated on water. This confirms the observation in this study that germination of *M. hortensium* seeds is not prevented by water-soluble inhibitors in the seed coat.

The germination percentages of seeds that were pricked with and without applying Vaseline® to the pricked area were not significantly different. Also oxygen uptake of the intact seeds and pricked seeds with Vaseline® were not significantly different from the

pricked seeds only. This suggests that the *M. hortensium* seed coat is not preventing oxygen uptake or at least if the seed coat is restricting oxygen uptake then this is not sufficient to prevent germination. Removal of seed coat significantly increased O_2 uptake (450-570%) as well as CO_2 evolution (520-870%, Figure 3.7). The increased O_2 uptake and CO_2 release is likely to be due to increased seed respiration and the germination progress, not oxidation of phenolic compounds in the embryo of *M. hortensium*. The respiratory quotients (RQ) were almost the same in both seeds with coat intact and removed. This may suggest that oxygen is not being consumed by phenolics in the seed embryo or seed coat. In some species, the higher rate of O_2 consumption can be caused by the oxidation of phenolic substances in the seeds (Qi, Upadhyaya, Furness, & Ellis, 1993).

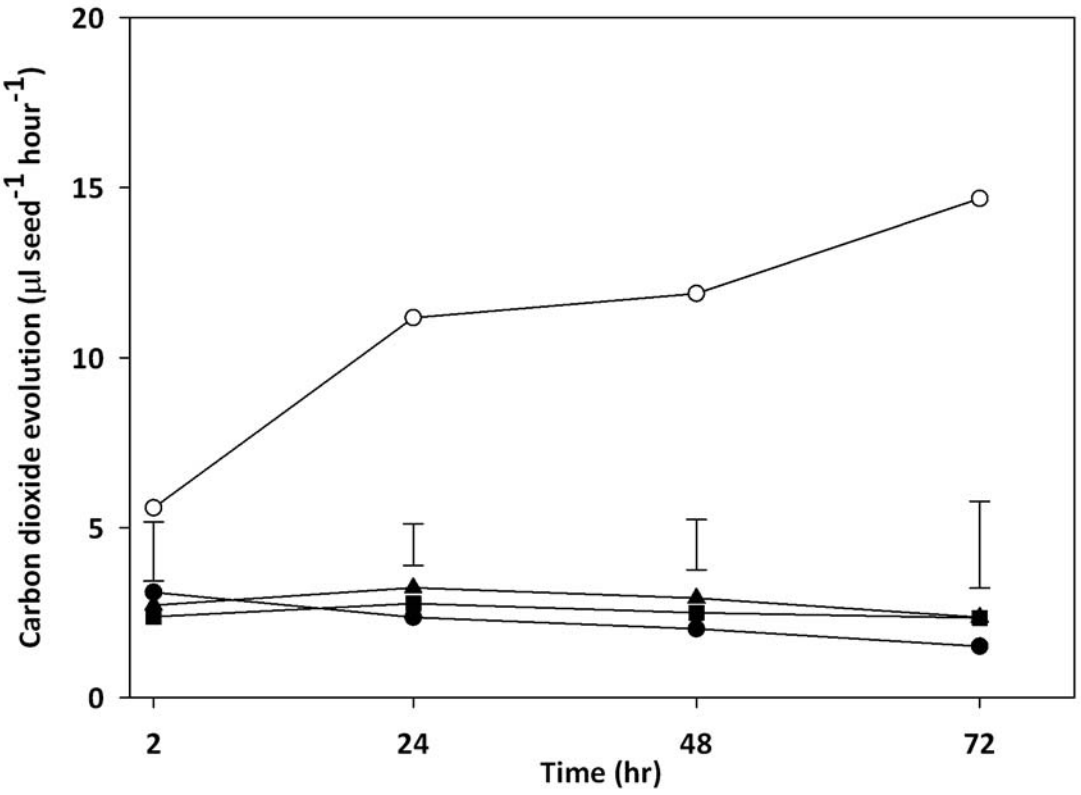

Figure 3.7 The rate of carbon dioxide evolution of intact seeds (●), decoated seeds without a seed coat (○), seeds that were pricked with a fine needle at the centre (■), and with Vaseline® applied to pricked area (▲) of *Myosotidium hortensium*. Data are the means of six separate assessments using the Gilson Respirometer with two replications per assessment. Vertical bars indicated the least significant difference between mean carbon dioxide evolutions for each time ($p \leq 0.05$).

Intact seed coats of *M. hortensium* may be permeable to oxygen, however the germination percentages for seeds pricked in the centre and seeds where Vaseline® was applied to the pricked area were significantly higher compared with intact seeds. This provides further evidence that seed coat mechanically restricts germination in *M. hortensium* seeds. This was supported by the position of seed coat rupture during germination. Whereas the intact seeds germinated with the seed coat rupturing at the pointed "radicle end" of the seed, most of pricked seed and seeds to which Vaseline® was applied germinated with the seed coat rupturing at the point of pricking during germination (Figure 3.8). If pricking allowed *M. hortensium* seed germination by relieving the mechanical constraint of embryo expansion by the seed coat, the coat might be expected to rupture primarily at the point of pricking during germination.

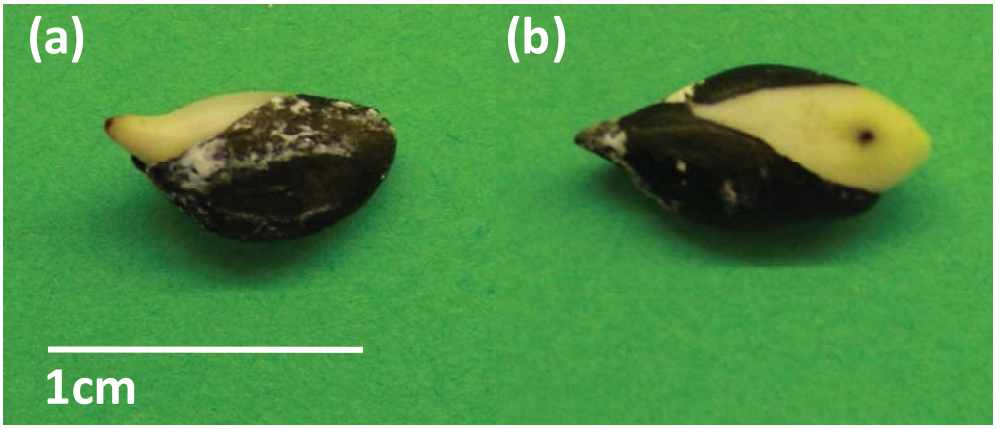

Figure 3.8 Intact seeds (a) and seeds were pricked with fine needle at the cotyledon area (b) showing the seed coat rupturing during germination in *Myosotidium hortensium*.

The significantly lower germination in the pricking treatment with or without Vaseline® than the decoating treatment is a result of an increase in percentages of dead and fresh ungerminated seeds. McGill (2003) observed the presence of bacterial slimegrowth surrounding some seeds which retained their seed coat. It may be that the seed coat harboured pathogens – decoating of the seed removed this source of infection. The fungicide was able to deal with some but not all the pathogens either because it was ineffective against them or was overwhelmed by the numbers of bacteria. Vaseline® application allowed more contamination because it was applied after fungicide treatment. Vaseline® probably created a moister seed coat, allowing bacteria to thrive

better. The higher percentage of fresh ungerminated seed with the pricking treatment suggests the treatment was insufficient to completely alleviate dormancy in all seeds in the population.

3.2.4.3 *T. speciosa*

Freshly harvested seeds of *Tecomanthe speciosa* are not dormant. This is similar to many other species of Bignoniaceae (Sautu, Baskin, Baskin, Deago, & Condit, 2007). For example, *Tebebuia impetiginosa* seeds reached over 80% germination within 5 days at 20-30°C (da Silva, Davide, Faria, de Melo, & de Abreu, 2004). Metcalf (1995) also observed that seeds of *T. speciosa* germinated (N'Nan, et al., 2008) easily in warm conditions.

3.2.4.4 *D. spectabile*

The results for *Dysoxylum spectabile* also show the seeds are not dormant and germinated quickly. Similarly, Burrows (1999b) and Court & Mitchell (1988) reported that seeds of this species are viable at dispersal and there was no evidence of dormancy. Many other species of Meliaceae also have been reported to produce non dormant seeds (Sautu, et al., 2007). This is consistent with the storage behaviour of *Dysoxylum* which will be discussed in the next chapter.

CHAPTER 4 *EX SITU* CONSERVATION APPROACH

The selection of one or more of the techniques available for the conservation of plant genetic resources depends on needs which are determined by a range of variables in terms of logical, scientific and economic criteria, possibilities of conserving the material and the target species (Maxted, Ford-Lloyd, et al., 1997). Among all the *ex situ* conservation strategies the easiest and least expensive method of preserving the world's existing plant genotypes appears to be conventional seed storage (Fay, 1992; Paunescu, 2009; Rao, 2004; Roberts, 1991). When seeds are stored as genetic resources, viability must be maintained for at least several decades, if not centuries. The longevity of seeds in storage is influenced by diverse factors such as plant species, environmental conditions during seed development, physiological status of the seed at maturity, and seed storage methods (Pritchard & Dickie, 2003). Temperature and moisture content of the seed are the main factors in determining if viability is retained in storage (Hong, Ahmad, & Murdoch, 2001; Roberts, 1973; Theilade & Petri, 2003). Seed viability varies from species to species, even if provided with identical storage conditions. For plants, *ex-situ* storage usually requires drying of seed to low moisture content (3-7% fresh weight basis), depending on species, and storage at low temperature, preferably at -18°C or cooler (FAO/IPGRI, 1994). There is an optimum moisture level at which seeds should be stored and this needs to be determined for individual species (Christina, Eric, & Steve, 1996).

Many experiments have shown that longevity may be further improved in some species if the seeds are dried to moisture contents less than that generally accepted as optimum i.e. recommended MC (3–7%) (Ellis, Hong, & Roberts, 1995; Ellis, Hong, Roberts, & Tao, 1990; Li, Feng, Chen, Yang, & An, 2007). Storage at moisture contents below the usual 3-7% is termed ultra-dry storage. This can greatly reduce the cost of constructing and maintaining the gene bank where use of ultra-dry storage allows seed to be stored at ambient temperatures for the same length of time as non- ultra-dried seed in refrigerated storage (Zheng, Jing, & Tao, 1998). However, there are limits to the beneficial effects of drying such that drying below a critical moisture content will not improve longevity and may even have detrimental effects on seed survival in storage (Ellis, Hong, Roberts, et al., 1990; Vertucci, Roos, & Crane, 1994).

Not all species are suitable for conventional seed storage methods, as some seeds are unable to withstand desiccation and are often sensitive to chilling (Engelmann & Engels, 2002; Roberts, 1973). For long-term storage of these non-orthodox seeds, cryopreservation, which is storage at ultra-low temperature, usually that of liquid nitrogen (-196°C), currently offers the only safe and cost-effective option for the long-term conservation of genetic resources of problem species. At this temperature, cellular division and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, are protected from contamination, and require very limited maintenance (Engelmann & Engels, 2002; Maxted, Ford-Lloyd, et al., 1997). However, more advanced tissue culture procedures have to be established prior to any cryopreservation and moreover there is a difficulty of regeneration of whole plants after cryopreservation (Engelmann, 2000; FAO, et al., 2004).

The objective of this study was to evaluate suitable conservation strategies including dry storage and/or cryopreservation methods for the long-term storage of *Clianthus maximus*, *Myosotidium hortensium* and *Dysoxylum spectabile*.

The results of this study will be used to aid the development of protocols for the long term preservation of these species.

4.1 Seed storage of *Clianthus maximus*

4.1.1 Introduction

This work has shown that *Clianthus maximus* will tolerate desiccation (see chapter 3.1). The impermeable seed coat of *C. maximus* will inhibit the uptake of moisture and oxygen from the surrounding atmosphere, such that it can be stored for a number of years even at room temperature.

The objective of this study was to identify optimum seed MC and storage temperatures for the long-term storage of *Clianthus maximus*. The effects of three factors, initial seed moisture content, storage temperature (+20, 0, and -20°C) and storage duration on the germination were examined.

4.1.2 Materials and methods

4.1.2.1 Seed material

Seeds of *Clianthus maximus* were sourced and handled as detailed in 3.1.2.1.

4.1.2.2 Determination of moisture content and germination

Seed moisture content and germination were determined as described in 3.1.2.2. and 3.1.2.3 using four replicates of 25 seeds and 50 seeds, respectively.

4.1.2.3 Determine storability

Seed drying

Seed lots were dried to three target moisture contents of 10%, 5% and 2.5% as described in 3.1.2.4.

Seed packaging and storage

A factorial design combining three different moisture contents (2.5-10%) and three temperatures (20°C, 5°C and -20°C) was applied to define the seed response to both desiccation and storage. For each sample at each moisture content, three samples of 300 seeds were sealed in laminated polyester/aluminium foil/polythene packets (116×118 mm) and stored in a controlled temperature room at 20°C, 5°C and -20°C. The temperature within each room was monitored and logged every 30 min using a data logger and the mean temperature during experimental storage was calculated. Samples were removed after 7-months storage and seed moisture content and germination percentage determined. Before testing these samples for germination, the seeds at <10% moisture content were humidified above water at 20°C for 24hr to avoid the possibility of imbibition injury during rehydration.

To determine hardseededness, seeds that had not germinated after 3 weeks were counted and scarified with a scalpel by cutting the seed coat in the cotyledon area. Scarified seeds were returned to 20°C to continue the germination process for another 6 days after which ungerminated seeds were classified as viable when they showed no sign of infection and were firm when pressed. Normal seedlings were assessed as described in 3.1.2.3.

4.1.2.4 Seed-surface features: Scanning electron microscopy observation

Surface features of seeds at target MC of 2.5% and 10% were examined using scanning electron microscopy (SEM); 3 seeds were used for each treatment. For all seeds examined, three regions of the seed coat could be distinctly identified: (1) a hilum (the micropyle lies on one side of the hilum and is covered by the hilum in seeds); (2) the lens, which is on the opposite end of the hilum from the micropyle; and (3) the extrahilar region, which is defined as the area not occupied by the lens and hilum. All samples were examined with FEI Quanta 200 Environmental Scanning Electron Microscope at 20 kV.

4.1.2.5 Data analysis

Data were analysed for statistical significance by analysis of variance (ANOVA). Fisher's least significant difference ($p \leq 0.05$) was used to determine whether there were significant differences between treatments. Prior to analysis, data were checked for normality using Shapiro–Wilk tests. No transformations were necessary. SAS for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

4.1.3 Results

4.1.3.1 Seed moisture content

The initial target MC's were 10%, 5% and 2.5% with actual MC's of 10.7%, 5.4% and 3.7%, respectively reached. Seed MC changed during experimental storage, i.e. between the first (beginning of storage) and final determination (after storage). Seeds lost 1.7%, 0.9% and 1.4 % MC of the original value moisture content after storage (Table 4.1).

Table 4.1 Changes in moisture content during storage at different temperatures and moisture contents of *Clianthus maximus*.

Storage treatment			MC (%)
Target MC (%)	Temperature	Storage periods (days)	
10		0	10.7 a
	20°C	210	9.0 b
	5°C	210	9.3 b
	-20°C	210	9.6 b
	Means (± S.E.)		9.6 ± 0.21
5		0	5.4 a
	20°C	210	4.6 b
	5°C	210	4.6 b
	-20°C	210	4.5 b
	Means (± S.E.)		4.7 ± 0.13
2.5		0	3.7 a
	20°C	210	2.3 b
	5°C	210	2.4 b
	-20°C	210	2.3 b
	Means (± S.E.)		2.7 ± 0.16

Numbers following the same letters are not significantly different within each target moisture content set (LSD, $p \leq 0.05$).

4.1.3.2 Storability

After 7 months' hermetic storage, no loss in seed viability was detected at all combinations of 20°C, 5°C or -20°C with 2.7–9.6% moisture contents (Table 4.2). Seeds of *C. maximus* had 99 ± 0.3% of viability after 210 days of storage treatment.

Table 4.2 Comparison of percentage normal germination after desiccation to different seed moisture content and after 210 days' hermetic storage at different temperatures and moisture contents of *Clianthus maximus*.

Storage treatment			Germination (%)
Seed MC (% ± S.E.)	Storage periods (days)	Temperature	
9.6 ± 0.21			
	0		96.5 A
	210	20°C	99.5 A
	210	5°C	98.5 A
	210	-20°C	99.5A
4.7 ± 0.13			
	0		97.5 A
	210	20°C	98.5 A
	210	5°C	99.5 A
	210	-20°C	100.0A
2.7 ± 0.16			
	0		99.0 A
	210	20°C	98.5 A
	210	5°C	98.5 A
	210	-20°C	99.5 A

Numbers following the same letters are not significantly different within each moisture content (LSD, $p \leq 0.05$).

Percentage of hardseededness of seeds stored at -20°C decreased significantly after 210 days' hermetic storage. In contrast, hardseededness percentage in storage at 5°C or 20°C remained unchanged (Table 4.3). Seeds with an impermeable seed coat required scarification to enable germination. After scarification, germination percentage increased to 97-100% (Table 4.3).

Table 4.3 Hardseededness percentages before and after 210 days' hermetic storage at different temperatures and moisture contents and changes of germination without scarification and after scarification of *Clianthus maximus*.

Seed MC (% ± S.E.)	Storage periods (days)	Temperature	Hardseededness (%)	Germination (%)	
				Without scarification	After scarification
9.6 ± 0.21					
	0		100.0 A	0.0 Bb	96.5 Aa
	210	20°C	98.5 A	1.5 Bb	99.5 Aa
	210	5°C	98.5 A	1.5 Bb	98.5 Aa
	210	-20°C	1.5 B	98.5 Aa	99.5 Aa
4.7 ± 0.13					
	0		21.0 A	76.5 Bb	97.5 Aa
	210	20°C	25.0 A	74.0 Bb	98.5 Aa
	210	5°C	21.5 A	78.5 Bb	99.0 Aa
	210	-20°C	0.5 B	99.5 Aa	100.0 Aa
2.7 ± 0.16					
	0		3.1 A	95.9 Ba	99.0 Aa
	210	20°C	4.0 A	95.5 Bb	98.5 Aa
	210	5°C	2.5 A	97.5 ABa	98.5 Aa
	210	-20°C	0.0 B	99.5 Aa	99.5 Aa

Numbers following the same uppercase letters are not significantly different within each moisture content; Numbers within rows sharing the same lowercase letters are not significantly different (LSD, $p \leq 0.05$).

4.1.3.3 Seed-surface features: Scanning electron microscopy observation

SEM results showed that all examined seeds at the target MC of 10% had no cracks in the hilum, lens and extrahilar region. However, seeds at target MC of 2.5% had cracks in the lens and extrahilar region whereas the hilum remained intact (Figure 4.1).

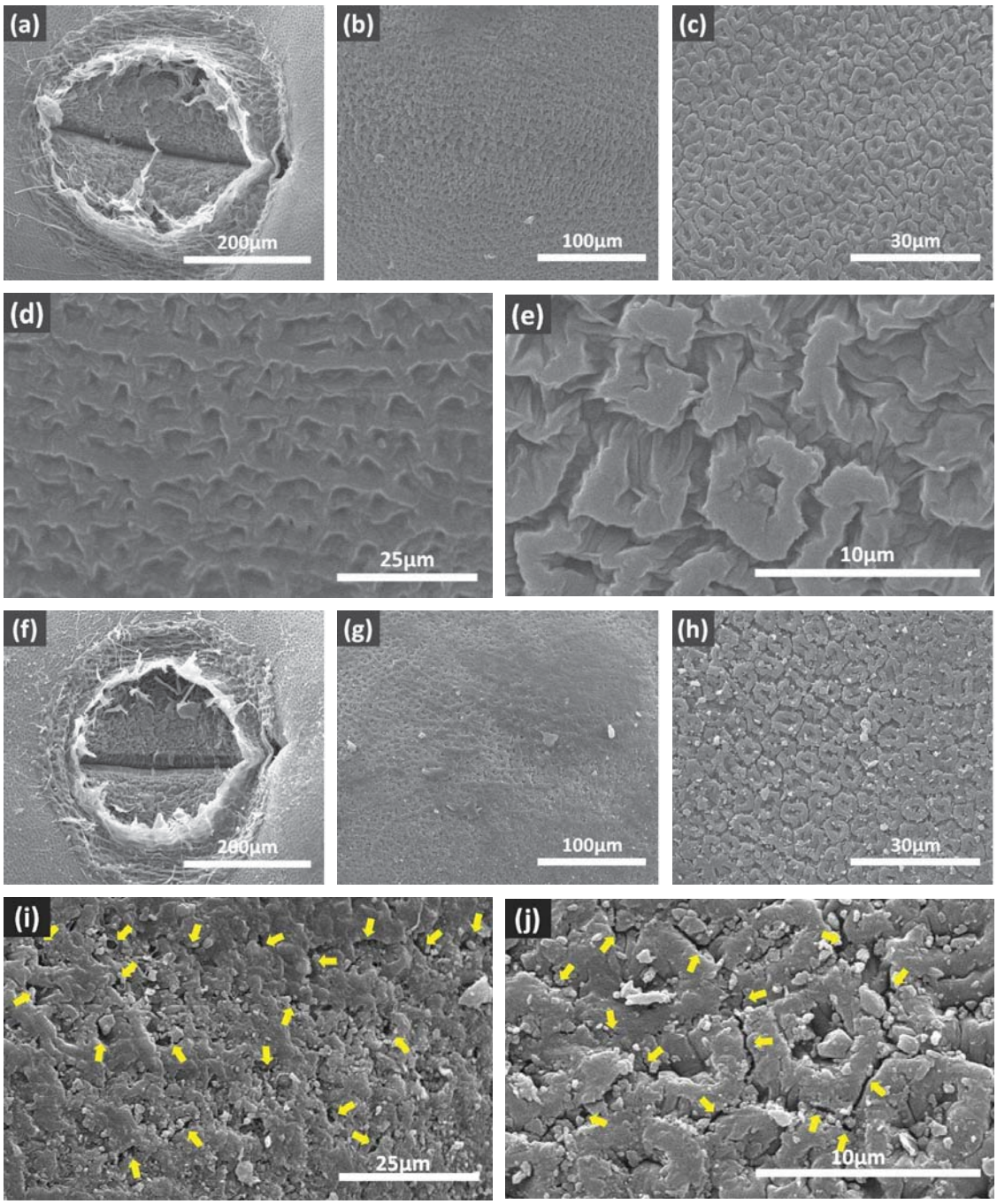

Figure 4.1. Scanning electron micrographs of seed coats of *Clianthus maximus* at 10% moisture and following desiccation to 2.5% moisture. (a-e) Seed coat of *C. maximus* seeds at 10% moisture content: (a) hilum, (b) lens, (c) extrahilar region, (d) detail of the intact lens, (e) detail of the intact extrahilar region . (f-j) Seed coat of *C. maximus* seeds at 2.5% moisture content: (f) hilum, (g) lens, (h) extrahilar region, (i) detail of the lens with cracks (arrows), (j) detail of the extrahilar region with cracks (arrows).

4.1.4 Discussion

Hong and Ellis (1996) proposed a three-step protocol to determine seed storage behaviour, *viz.* i) drying to ca. 10% MC, ii) if most seeds survived further drying to 5% MC, and iii) if most seeds survived, storage at -20°C for three months. If most seeds survived step iii, they were considered orthodox.

C. maximus seeds, showed no reduction in viability at a MC of 2.7%, and most seeds survived storage for seven months at -20°C indicating, according to the protocol presented by Hong and Ellis (1996), that seeds of *C. maximus* are orthodox.

The ability of many orthodox seeds to remain viable for tens or hundreds of years in dry storage provides a convenient mechanism for the long-term *ex situ* conservation of plant germplasm (Daws, et al., 2007; Roberts, 1973; Walters, et al., 2005). Seeds of many Fabaceae species show considerable longevity (Baskin & Baskin, 1998). For example, *Liparia villosa* is reported to have survived for >200 years (Daws, et al., 2007).

The hard seed coat of species of Fabaceae helps maintain a low level of metabolism in these dry orthodox seeds by excluding moisture and oxygen (Bonner, 2008). This ability to remain at low MC under high ambient relative humidity is likely to be a key factor in maximising long-term survival of Fabaceae (Daws, et al., 2007). As in many Fabaceae taxa (e.g. Baskin & Baskin, 1998; Eisvand, Arefi, & Tavakol-Afshari, 2006; Finch-Savage & Leubner-Metzger, 2006; Gresta, Avola, Anastasi, & Miano, 2007), *C. maximus* seeds have an impermeable seed coat. However, seeds of *C. maximus* showed a significant decline in percentage impermeability over the 7 months following low temperature storage (Table 4.3). A water impermeable seed or fruit coat is known to occur in seeds of all or most species of Fabaceae of the temperate and arctic zones, and in many of those in the tropical/subtropical zone (Baskin & Baskin, 2005; 1998). Depending on the species, the hardseedcoat can be alleviated in different ways (Baskin & Baskin, 1998). Acid scarification, hot water, dry heat and high or fluctuating temperature will make the seed coat permeable by inducing fractures in the lens, hilum, micropyle or seed coat (Hu, et al., 2008; Zeng, et al., 2005). However, there is no evidence that storing at low temperature is involved in loss of impermeability in Fabaceae. It has been suggested that seed water content and temperature are the main

factors affecting longevity during storage. The lower the seed moisture content and storage temperature the longer the seed longevity (Ellis & Roberts, 1980; Roberts, 1973). Thus, the effect, if any, of loss of water impermeability at low temperature storage in seed of *C. maximus* on the long term survival of seed in storage needs to be determined.

The cracks occurring in the lens and extrahilar region suggest that these sites are the physically weakest part of the testa, and thus preferentially cracked by desiccation (Baskin, et al., 2000; Morrison, et al., 1998; Serrato-Valenti, et al., 1995). In many species, the lens is believed to be the primary site of water entry into the seed (Baskin & Baskin, 2003; Baskin, et al., 2000; Serrato-Valenti, et al., 1995) due to its relatively thin cell walls. Seeds of *Albizia lophantha*, *Acacia kempeana*, and *Sesbania sesban* imbibed water via the lens after pre-treatment with boiling water and/or concentrated sulphuric acid treatment (Dell, 1980; Hanna, 1984; Hu, et al., 2009). However, the mechanism underlying the breaking of physical dormancy in seeds of legumes is controversial, especially with regard to site of initial water entry into the seed. Burns (1959) reported that palisade cells of the hilum in *Lupinus angustifolia* were destroyed by acid scarification and that water entered through the hilum but not through the lens. Seeds of *Vigna oblongifolia* first cracked in the hilum when pretreated with sulphuric acid (Hu, et al., 2009). In thermally scarified seeds of *Cassia leptophylla,* water entered through the micropyle (de Paula, Delgado, Paulilo, & Santos, 2012). Morrison et al. (1998) showed that dry heating caused a disruption of the seed coat at the lens of some legumes, while in other species other than the lens was the site of water entry.

In this study SEM results showed that desiccation causes disruption of the cells of the lens and extrahilar region that allows entry of water. However, further investigation into, for example, the initial site of water entry into seeds during imbibition following desiccation and low temperature storage is needed to understand dormancy mechanisms and develop an *ex situ* conservation approach for this species. To determine the point of water entry, after each desiccation treatment and/or low temperature storage, an experiment subjecting seeds to the following treatments: (1) no blockage applied (control); (2) blockage material (Vaseline®) applied to hilum area; (3) Vaseline® applied to lens area; (4) Vaseline® applied to lens and hilum area; and (5)

Vaseline® applied to extrahilar region, which is defined as the whole seed coat except the hilum and lens area could be undertaken. The numbers of imbibed and non-imbibed seeds are monitored daily. Each seed is weighed at 24-h intervals, and a seed is recorded as imbibed when the amount of water taken up exceeds 50% of its initial weight.

It should be noted that for determination of the underlying mechanism of water entry into seeds after treatments, frequent observations need to be made during the dormancy breaking process and various periods of imbibition need to be used. Taylor (2004, 2005) and (Hu, et al., 2008) suggested that at some place in the testa (such as the hilum or micropyle) other than the lens, moisture can enter seeds slowly before the lens is ruptured (and rapid imbibition occurs). This very slow imbibition could be easily missed due to the relatively short periods of time allowed for imbibition to occur. Thus, various periods of time should be used to determine if seeds imbibe, which range from 12 h to 1 month (Hanna, 1984; Hu, et al., 2009; Hu, et al., 2008; Ma, Cholewa, Mohamed, Peterson, & Gijzen, 2004; Morrison, et al., 1998; Serrato-Valenti, et al., 1995).

4.2 Seed storage of *Myosotidium hortensium*

4.2.1 Introduction

Previous studies on *Myosotidium hortensium* have shown that this species tolerated desiccation (see chapter 3.1). McGill et al. (2002) reported that *M. hortensium* seeds appear to be orthodox in their storage behaviour. However, they suggested that evaluation of the storability at lower seed moisture contents should be undertaken, in particular, because of the high lipid content of *M. hortensium* seeds (24% of the dry weight). Many oil seeds have shown increased sensitivity to variations of storage conditions of temperature, moisture and gaseous exchanges. Fast oxidation of fatty acids and proteins can reduce the viability, vigour and germination percentage in these seeds. Oil seeds are more sensitive to lipid peroxidation of plasma membrane and protein degradation (Dolatabadian & Sanavy, 2008). It has been suggested that drying increases the formation of reactive oxygen species (ROS) as a result of unbalanced metabolism and the impairment of respiratory electron transport (Hendry, et al., 1992; Pukacka & Ratajczak, 2006, 2007; Roach, et al., 2008). ROS react with cellular macromolecules, such as proteins, lipids and nucleic acid, causing oxidative damage and eventually resulting in cell death (Berjak, et al., 2007; Greggains, Finch-Savage, Atherton, & Berjak, 2001; Pukacka, Malec, & Ratajczak, 2011). Under different conditions particularly environmental stress, ROS, such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are generated (Berjak, et al., 2007; Franca, et al., 2007; Hendry, 1993). Oxidative stress, especially lipid peroxidation and de-esterification of phospholipids, leading to the loss of membrane structure, is one of the significant causes of desiccation damage and deterioration during dry storage (Berjak, et al., 2007; Buitink, Hoekstra, Leprince, & Black, 2002; Franca, et al., 2007). It has been suggested that ultra-dry storage at very low seed moisture content could be more appropriate for seeds with high lipid contents (Ellis, et al., 1989; Vertucci & Roos, 1990). For oily seed species, the values of critical moisture content which could provide maximal seed longevity at a given storage temperature are lower: 3.3% for *Glycine max*; 2.7% for *Linum usitatissimum*; 2.4% for *Guizotia abyssinica*; and 2% for *Arachis hypogaea* and *Helianthus annuus* (Ellis, 1998; Ellis, Hong, Roberts, et al., 1990; Rao, et al., 2006).

The objective of this study was to identify optimum seed MC and storage temperatures for the long-term storage of *M. hortensium*. The effects of three factors: initial seed moisture content, storage temperature (+20, +5, 0, and -20°C) and storage duration on the germination was examined.

4.2.2 Materials and methods

4.2.2.1 Seed material

Seeds of *M. hortensium* were sourced and handled as detailed in 3.1.2.1. and stored at -5°C until the experiment was conducted (March 2008).

4.2.2.2 Determination of moisture content and germination

Seed moisture content and germination were determined as described in 3.1.2.2. and 3.1.2.3. Seed moisture content and germination were determined using four replicates of 10 seeds and 40 seeds, respectively.

4.2.2.3 Determination of storability

Seed drying

Seed lots were dried to three target moisture contents of 10%, 7.5% and 5% as described in 3.1.2.4.

Seed packaging and storage

A factorial design combining different moisture contents (5 - 10%), temperatures (-20°C to +20°C), and storage periods (3–30 months, depending on treatment combination) was applied to define the seed response to both desiccation and storage. For each sample at each moisture content, 20 samples of four replicates (with 50 seeds in each replicate) were sealed in laminated polyester/aluminium foil/polythene packets (116×118 mm) and stored in controlled temperature rooms at 20°C, 5°C, 0°C and -20°C. The temperature within each room was monitored and logged every 30 min using a data logging system and the mean temperature during experimental storage was calculated. Samples were withdrawn from storage at intervals varying from 3 to 30 months; depending on temperature and moisture content (Table 4.4).

Table 4.4 Sampling schedule and treatments for the *Myosotidium hortensium* storage experiment.

Seed storage treatment			Sampling schedule (month)	No. of samples
Target MC (%)	Actual MC (%)	Temperature (°C)		
10.0	9.2 ± 0.06	20	0, 3, 6, 12, 18	5
10.0	9.2 ± 0.06	5	0, 3, 6, 12, 18	5
10.0	9.2 ± 0.06	0	0, 3, 6, 12, 18	5
10.0	9.2 ± 0.06	-20	0, 3, 6, 12, 18	5
7.5	5.9 ± 0.17	20	0, 3, 12, 24, 30	5
7.5	5.9 ± 0.17	5	0, 3, 12, 24, 30	5
7.5	5.9 ± 0.17	0	0, 3, 12, 24, 30	5
7.5	5.9 ± 0.17	-20	0, 3, 12, 24, 30	5
5.0	4.5 ± 0.24	20	0, 3, 12, 24, 30	5
5.0	4.5 ± 0.24	5	0, 3, 12, 24, 30	5
5.0	4.5 ± 0.24	0	0, 3, 12, 24, 30	5
5.0	4.5 ± 0.24	-20	0, 3, 12, 24, 30	5

All samples, together with the control (0 months storage) for each moisture content were tested for seed moisture content and germination percentage. Before testing these samples for germination, the seeds at <10% moisture content were humidified above water at 20°C for 24hr to avoid the possibility of imbibition injury during rehydration.

4.2.2.4 Ultra-dry storage experiments

To investigate the storage longevity of the ultra-dry *M. hortensium* seeds over 24 months, seeds were collected from Chatham Island in January in 2008. On arrival, seeds were handled as detailed in 3.1.2.1 and stored at -5°C until the experiment was conducted (September 2008).

For the ultra-dry storage experiments, seeds were dried at both target moisture content of 5% and 2.5% and were sealed in laminated polyester/aluminium foil/polythene packets (116×118 mm) and stored at 20°C, 0°C and -20°C, respectively. The observations on seed moisture content and germination percentage were recorded at 6, 12 and 24 months. To avoid imbibition damage prior to germination seeds were humidified at 20°C for 24hr.

4.2.2.5 Data analysis

Data were analysed for statistical significance by analysis of variance (ANOVA). Fisher's least significant difference ($p \leq 0.05$) was used to determine whether there were significant differences between treatments. Prior to analysis, data were checked for normality using Shapiro–Wilk tests. No transformations were necessary. SAS for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

4.2.3 Results

4.2.3.1 Determination of storability

Seed Moisture Content

During the storage trial, seed MC of samples initially stored at 5.9% and 4.5% fluctuated around the initial value at all storage temperature. However, seed with initial 9.2% MC stored at 20°C, 5°C and 0°C increased considerably in MC after 18 months' hermetic storage (Table 4.5). The mean MCs of seeds stored at 4.5%, 5.9% and 9.2% during the storage trial were 4.3%, 6.0% and 10.8%, respectively.

Table 4.5 Changes of moisture content during storage at different temperatures and moisture contents of *Myosotidium hortensium*. The seeds were stored at 20°C, 5°C, 0°C or -20°C with initial moisture content of 9.2%, 5.9% or 4.5%.

Target MC (%)	Temperature	MC (%)							$LSD_{0.01}$
		Storage periods (months)							
		0	3	6	12	18	24	30	
10%		9.2							
	20°C		10.9	11.7	10.8	11.5	-	-	1.15
	5°C		10.7	11.1	11.3	10.8	-	-	1.37
	0°C		10.2	11.6	10.5	11.4	-	-	1.17
	-20°C		10.3	10.5	10.7	10.8	-	-	n.s.
	Means (± S.E.)	10.8 ± 0.15							
7.5%		5.9							
	20°C		6.2	-	5.7	-	5.6	5.9	n.s.
	5°C		6.1	-	6.5	-	5.8	6.1	n.s.
	0°C		6.5	-	5.6	-	5.5	6.1	0.47
	-20°C		6.4	-	5.7	-	5.9	6.2	n.s.
	Means (± S.E.)	6.0 ± 0.08							
5%		4.5							
	20°C		4.7	-	3.7	-	3.7	4.3	0.69
	5°C		4.8	-	4.1	-	3.9	4.9	0.59
	0°C		5.0	-	3.7	-	3.8	4.3	0.83
	-20°C		4.8	-	4.1	-	3.9	4.4	0.58
	Means (± S.E.)	4.3 ± 0.11							

-: moisture content was not determined;
n.s.: not significant.

Storability

The results of the storage study (Figure 4.2) strongly indicated the orthodox storage behaviour of the seed of *Myosotidium hortensium*. The seeds tolerated desiccation to 4.3% moisture content and seeds at low moisture content were tolerant of chilling at least to -20°C. It was also observed that longevity of seeds decreased with the increase of moisture content and storage temperature.

Seeds of *M. hortensium* with MCs of 4.3% and 6.0% stored at all combination of 20°C, 5°C, 0°C or -20°C for 12 months retained their original germination percentages of 85.5% and 84.9%, respectively (Figure 4.2). However, viability declined gradually within 30 months storage periods at all storage temperatures.

Germination percentages of seeds with 4.3% MC stored at 20°C, 0°C or -20°C decreased from 85.5% to 38.7%, 37.4% or 36.5%, respectively, whereas germination percentage fell to 55.6% within 30 months storage at 5°C. But germination was not significantly different at any storage temperature (Figure 4.2).

Germinability of seeds with MC of 6.0% stored at 20°C, 5°C, 0°C or -20°C for 30 months decreased from 84.9% to 39.7%, 54.6%, 47.0% or 42.3% , respectively. There was no significant difference in the germination at any storage temperature (Figure 4.2).

Seeds at 10.8% MC showed significant reduction in viability during the 18 months of storage at all temperatures (Figure 4.2). Loss of viability was most rapid at higher temperatures. At 20°C, complete loss in viability occurred after 6 months of storage. Germination percentages of *M. hortensium* seeds stored at 5°C, 0°C or -20°C decreased gradually within 18 months, but seed viability loss was faster at 5°C than at 0°C or -20°C. Germination percentage decreased from 86.2% to 0.6 % at 5°C, whereas germination percentage fell to 11.1% and 14.5% within 18 months storage at 0°C and -20°C, respectively.

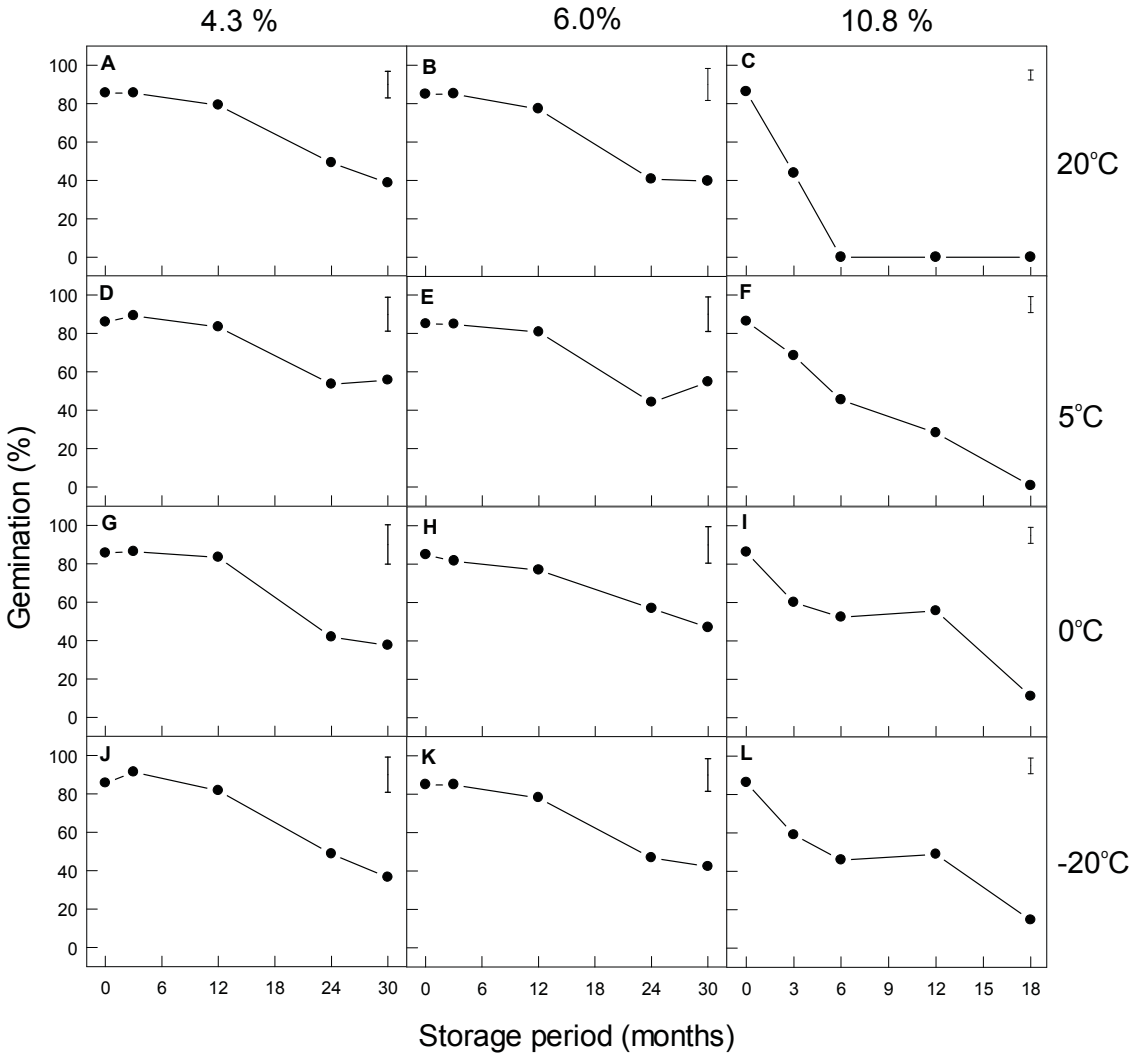

Figure 4.2 The effect of desiccation and subsequent hermetic storage on the germination (criterion, radicle emergency) of seeds of *Myosotidium hortensium*. The seeds were stored at 20°C (A-C), 5°C (D-F), 0°C (G-I) or -20°C (J-L) with 10.8 (A, D, G, J), 6.0 (B, E, H, K) or 4.3% moisture content (C, F, I, L). Vertical bars indicated the least significant difference between mean germination percentages for each treatment ($p \leq 0.05$).

4.2.3.2 Ultra-dry storage experiments

Desiccation sensitivity

The initial moisture content of *M. hortensium* was 6.9% and seeds germinated 84.4%. There was a small but insignificant decrease in the percentage of germination after desiccation, Desiccation to 4.4% and to 2.6% MC reduced germination to 80.8% and 76.6%, respectively (Figure 4.3).

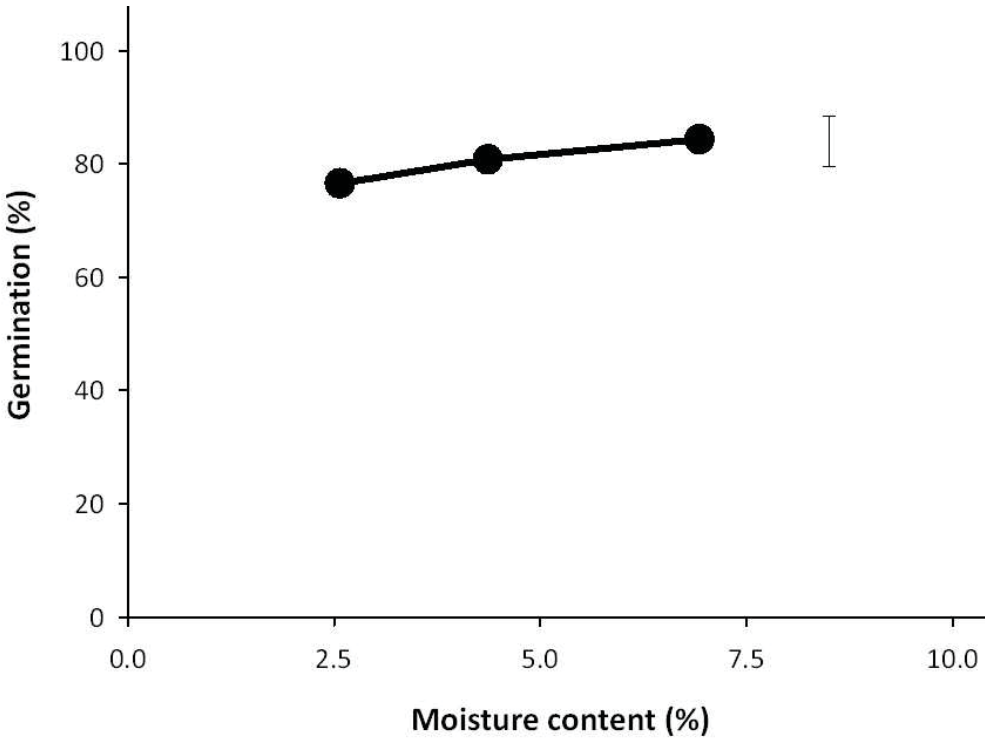

Figure 4.3 Germination capacity (%, criterion radical emergence) of seeds of *Myosotidium hortensium* during ultra-drying. Vertical bars indicated the least significant difference between mean germination percentages ($p \leq 0.05$).

Seed moisture content

During storage, the MC of seeds stored with 4.4% and 2.6% increased slightly after 24 months storage at all temperatures. The mean MC of stored seeds with 4.4% and 2.6% during storage trial was 4.5% and 2.5%, respectively (Table 4.6).

Table 4.6 Changes of moisture content during storage at different temperatures and moisture contents of *Myosotidium hortensium*. The seeds were stored at 20°C, 0°C or -20°C with initial moisture content of 4.4% or 2.6%.

Target MC (%)	MC (%)				
	Temperature	Storage periods (months)			$LSD_{0.01}$
		0	6	12	24	
5.0%		4.4				
	20°C		4.6	4.3	4.9	n.s.
	0°C		4.2	4.2	4.7	n.s.
	-20°C		4.0	4.6	4.8	0.49
	Means (± S.E.)		4.5 ± 0.09			
2.5%		2.6				
	20°C		2.2	2.3	2.8	0.22
	0°C		2.6	2.3	2.9	0.14
	-20°C		2.6	2.2	2.8	0.22
	Means (± S.E.)		2.5 ± 0.08			

n.s.: not significant.

Ultra-dry storage

Seeds of *M. hortensium* with MCs of 4.5% and 2.5% retained high germination for 12 months at all storage temperatures. However, germination declined rapidly after 12 months (Figure 4.4). Loss of viability was most rapid at high temperature with ultra-dried storage.

Germination of seeds with 4.5% MC and stored at 20°C, 0°C or -20°C decreased from 80.8% to 37.8%, 38.2% or 30.0% after 24 months storage, respectively. Germination percentages of *M. hortensium* seeds stored at 2.5% MC and 20°C, 0°C or -20°C decreased rapidly within 24 months, but seed viability loss was faster at 20°C than at 0°C or -20°C. Germination percentage decreased from 76.6% to 14.2 % at 20°C, whereas germination percentage fell to 39.7% and 29.6% within 24 months storage at 0°C or -20°C, respectively (Figure 4.4).

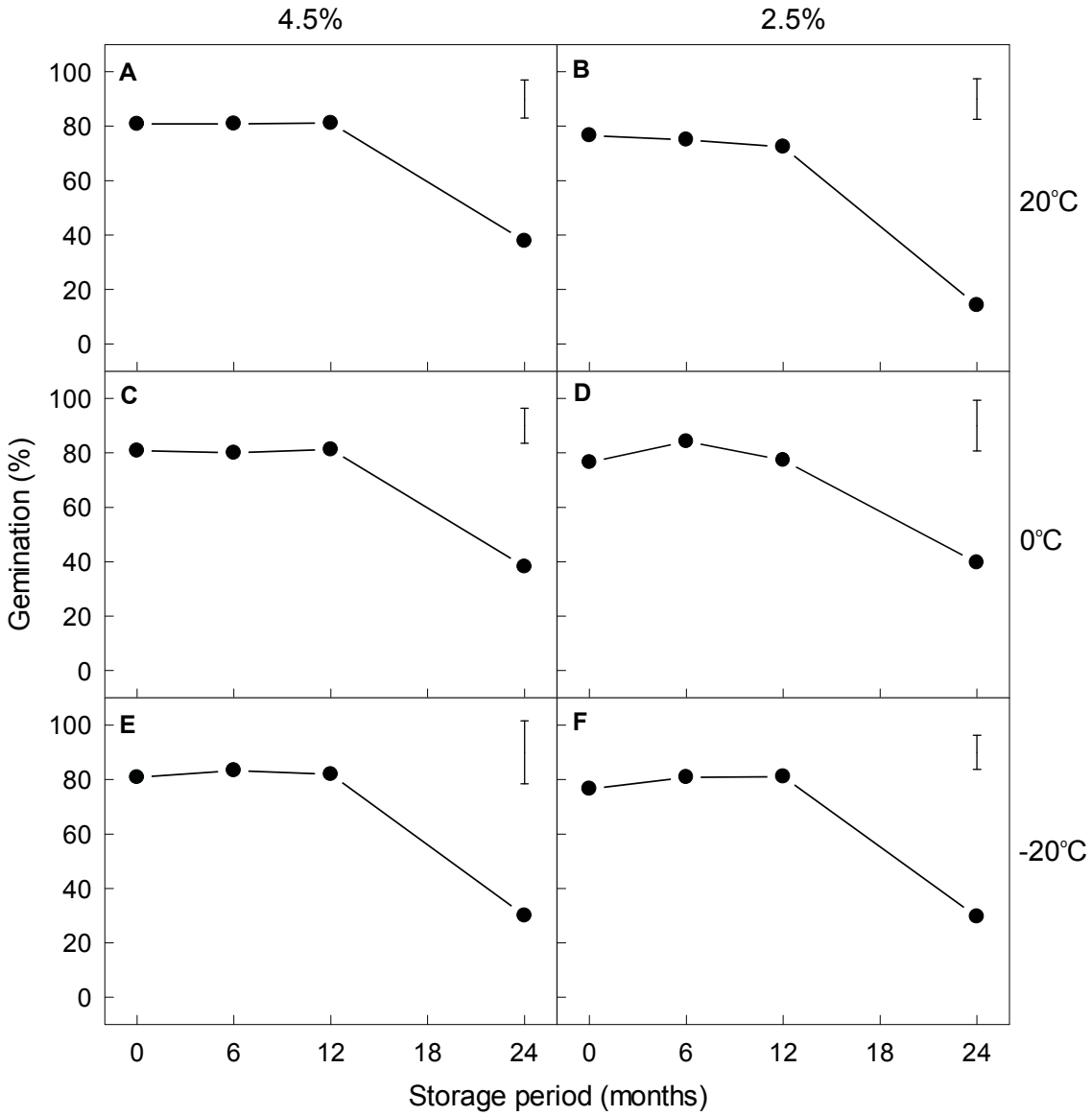

Figure 4.4 The effect of ultra-drying and subsequent hermetic storage on the germination (criterion, radicle emergency) of seeds of *Myosotidium hortensium*. The seeds were stored at 20°C (A, B), 0°C (C, D) or -20°C (E, F) with 4.5 (A, C, E) or 2.5% moisture content (B, D, F). Vertical bars indicated the least significant difference between mean germination percentages for each treatment ($p \leq 0.05$).

The results after 24 months' storage of *M. hortensium* seeds showed that ultra-dry storage compared with conventional dry storage gave neither advantage nor a disadvantage at 0°C or -20°C. The viability of seeds declined between 12 and 24 months at both storage temperatures regardless of moisture content. However, ultra-dry storage at ambient temperature seems to be disadvantageous compared with conventional dry storage (Figure 4.5). Following hermetic storage at 20°C for 12 months, normal germination percentage in ultra-dry seeds is lower than dry seeds,

whereas no difference in either normal or total germination was detected between dry and ultra-dry storage after 12 months at 0°C or -20°C (Figure 4.5).

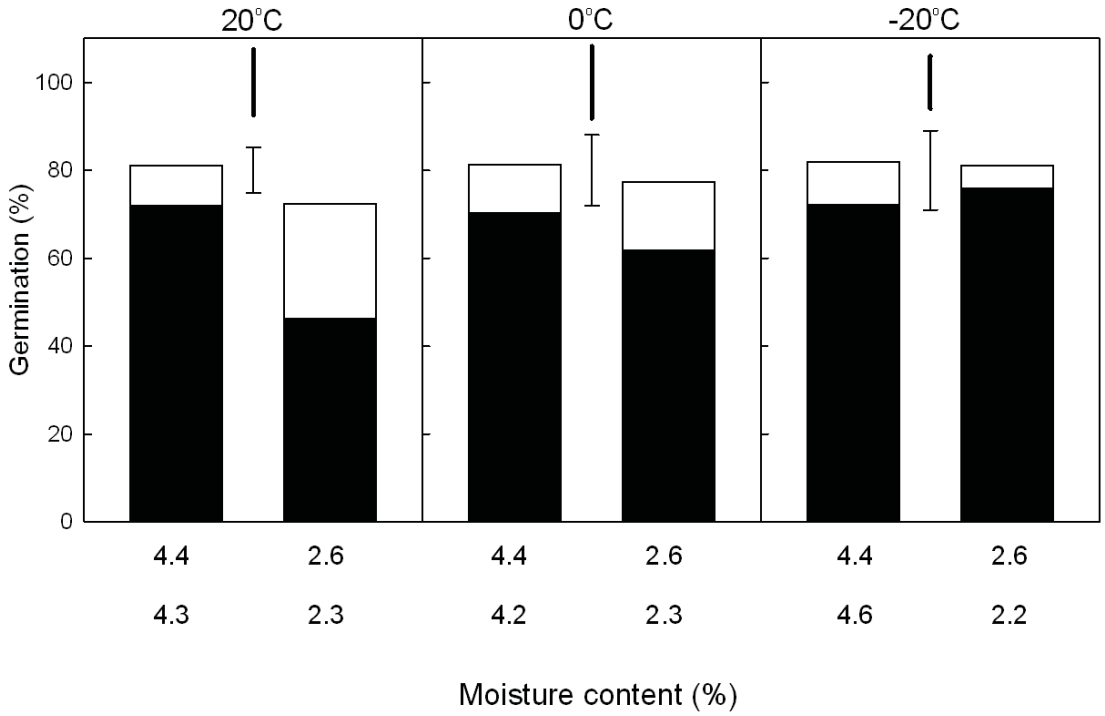

Figure 4.5 Comparison of percentage of normal germination (solid columns) and total germination (combined height of solid and open columns) of *Myosotidium hortensium* seeds stored hermetically for 12 months at 20°C , 0°C and -20°C at two moisture contents (upper values determined when storage began in 2008, lower values after storage). Vertical bars indicated the least significant difference for comparison of normal germination (lower bar) and total germination (upper bar) between moisture contents ($p \leq 0.05$).

4.2.4 Discussion

Roberts (1973) defined orthodox seeds as those that survive drying to low moisture contents and whose longevity is increased (within broad limits) with decreases in moisture content and temperature. *M. hortensium* seeds tolerate drying to low moisture, and therefore their longevity would be expected to be increased by reducing moisture content and temperature, i.e. the seeds could be described as orthodox. However, this work suggests that viability will decline significantly within 12-24 months, even under standard seed-banking conditions, indicating that *M. hortensium* are likely to be short lived in storage. Thus seeds of *M. hortensium* can be placed in the sub-orthodox category of seeds of Bonner (1990), who subdivided orthodox seeds into (a) true orthodox which "can be stored for long periods at seed moisture contents of 5–10% and subfreezing temperatures" and (b) sub-orthodox which "can be stored under the same conditions, but for shorter periods due to high lipid content or thin seed coats".

A number of studies have reported potential correlations of seed longevity in dry storage with seed mass, oil content, carbohydrate composition, taxonomy and climate (e.g. Horbowicz & Obendorf, 1994; Pritchard & Dickie, 2003; Walters, et al., 2005). However, the suggested link between high oil content and short storage life-span has not been supported by recent analyses (Pritchard & Dickie, 2003; Probert, et al., 2009; Walters, et al., 2005). Probert et al. (2009) surveyed species with both longevity and lipid composition data and reported a significant negative correlation between seed lipid content and seed viability, but the relationship did not hold when families with widely varying longevities and compositions, such as the Asteraceae or Fabaceae were considered separately. Furthermore, species within the Poaceae showed diverse longevities, despite fairly similar chemical compositions.

Nonetheless, it is possible that lipid composition is important in determining longevity since the degree of fatty acid saturation will impact on the potential for lipid peroxidation which has been considered to be a primary reaction in ageing contributing to free-radical production and subsequent attack on other macro-molecules (Benson, 1990; Probert, et al., 2009). Polyunsaturated fatty acids are more susceptible to peroxidation than are monounsaturated fatty acids, while saturated fatty acids are the most resistant. Thus preferential loss of polyunsaturated fatty acids in seeds during

storage may serve as an indication of lipid peroxidation. For example, in *Pisum sativum* seeds, both linoleic (18:2) and linolenic (18:3) fatty acids decrease during storage; saturated and monoenic acids show no changes (Harman & Mattick, 1976). Moreover, the rapid decrease in the quantity of linoleic and linolenic fatty acids is strongly correlated with ageing and viability loss. Similar findings have been reported for *Phaseolus vulgaris* (Lin & Pearce, 1990), and *Acer platanoides* seeds (Pukacka, 1991).

Peroxidation of storage lipids may contribute to a progressive decline in seed viability, not only because of the depletion of nutrient reserves, but also because of the generation of toxic products of peroxidation - aldehydes, ketones, organic acids, and hydrocarbons. Oil rancidity was linked to the loss of viability of *Pinus palustris* seeds (Kaloyereas, 1958). It is probable that the composition or ratios of saturated and unsaturated acids need to be considered rather than the total oil content.

M. hortensium seeds have predominantly the unsaturated oleic (40.7%), linoleic (15.2%), and γ-linolenic acids (9.0%) (McGill, et al., 2002). Thus these polyunsaturated fatty acids may contribute to short storage life of this species, but further research is required. This is the most puzzling aspect of the data where there appears to be no difference in the rate of viability loss at 5, 0 or - 20°C or with declining moisture content as normally occurs with orthodox seed.

Several reports have indicated that ultra-dry storage is a feasible option for germplasm preservation. Seeds may survive longer if they are stored at water contents of less than 5%, even if they are stored at ambient temperature (i.e. 20°C) (Demir & Ozcoban, 2007; Ellis, et al., 1988, 1989; Hong, et al., 2005; López, Dávila-Aranda, Flores, & Pritchard, 2003; Perez-Garcia, et al., 2007; Vertucci & Roos, 1990; Walters & Engels, 1998). More recently Hong et al. (2005) indicated that ultra-dry stored carrot, groundnut, lettuce, oil seed rape and onion seeds maintained viability and vigour after 10 years at 20°C.

The question of whether hermetic storage of *M. hortensium* at ultra-dry moisture contents (2.5%) would provide better survival compared with storage of seeds at 5±1% MC (dry storage) was addressed in a second storage trial. Species specific responses to

ultra dry storage indicate that the chemical components of seed may affect storage longevity. Some earlier studies have shown that those species that contain higher oil content are amenable to ultra dry storage, for example, pepper, onion and lettuce seeds (Demir & Ozcoban, 2007; Roberts & Ellis, 1989). Pepper and onion seeds stored at higher moisture content had lower viability than those stored at ultra-dry moisture at ambient temperature (20°C) (Demir & Ozcoban, 2007). However, poor correlations between longevity and oil content have also been observed (Lima, et al., 2005; Medeiros, Probert, Sader, & Smith, 1998). Usually, storage at -20°C rather than 20°C is beneficial to seed survival (Demir & Ozcoban, 2007; Hong, et al., 2005).

Generally orthodox seed longevity is assumed to increase as seed MC is lowered and storage temperatures decrease (Ellis & Roberts, 1980). Research examining the thermodynamic properties of water in *Pisum sativum* seeds (Vertucci & Roos, 1990; Vertucci, et al., 1994) and molecular mobility of water in *Impatiens walleriana*, *Pisum sativum* and *Typha latifolia* seeds (Buitink, Leprince, Hemminga, & Hoekstra, 2000) suggests that there is an optimum MC for each storage temperature. This MC is proposed to correspond to one that minimizes cellular fluidity, but with water present in sufficient quantities for maintaining protective effects e.g., hydrophobic and hydrophilic interactions of membrane lipids and proteins are not altered ((Buitink, et al., 2000; Vertucci & Roos, 1990). Interestingly, increases in tree seed longevity are observed when seed is stored at higher MC. For example, 20% MC for *Ulmus carpinifolia* seed (Tompsett, 1986) and 12% MC for *Pinus taeda* (Bonner, 1994). These higher MC may permit the repair of cellular damage, which occurs as seed deteriorates during storage (Beardmore, Wang, Penner, & Scheer, 2008).

Metabolic rates can also be minimized with low temperatures, both for orthodox and for recalcitrant seeds. The storage moisture content determines just how low temperatures can be set for seed storage. From freezing to –15 °C, 20% is the approximate upper moisture limit. Below –15 °C, the limit is about 15% (Bonner, 2008). Many desiccation-tolerant seeds can be safely stored at low temperature. Thus, storage conditions for long-term seed conservation usually follow the international standard, i. e. stored seed with 3-7% seed moisture content (fresh weight basis, depending on the species) at -18°C or cooler (FAO/IPGRI, 1994). These conditions are considered optimal for the

majority of desiccation-tolerant seeds (Terry, Probert, & Linington, 2003) and have been used successfully to store collections of wild species from around the world for many years. For example, analysis of 20-year re-test data from collections stored at the Millennium Seed Bank of the Royal Botanic Gardens, Kew, has shown that close to 86% of the 2388 collections re-tested had maintained viability (Probert, 2003). While it is not known if sub-orthodox seeds have the same tolerance of low temperatures as the true orthodox seeds, it is known that they can be stored for a few years at temperatures as low as –20 °C (Bonner, 2008).

The longevity of seeds held in dry storage is mainly determined by seed moisture content and storage temperature, with life-span increasing predictably with decreasing temperature and moisture content (Ellis & Roberts, 1980; Probert, et al., 2009). With regard to the effect of temperature, the quantitative effect of temperature on seed longevity does not differ greatly, or at all, among orthodox species (Dickie, Ellis, Kraak, Ryder, & Tompsett, 1990; Ellis, et al., 1988; Ellis, Osei-Bonsu, & Roberts, 1982; Tompsett, 1986). Jayasuriya et al. (2008) suggested that longevity is increased by a factor of almost 3 if storage temperature is reduced from 20ºC to 10ºC; by 2.4 from 10ºC to 0ºC; by 1.9 from 0ºC to –10ºC; but by only 1.5 from –10ºC to –20ºC.

The response of *M. hortensium* seed longevity to hermetic storage environment was described for a range of moisture contents and temperatures (Figure 4.2). The results indicated that there was a similar effect of temperature on longevity within the temperature range investigated. Accordingly, it can be concluded that the relative effect of temperature on seed longevity in *M. hortensium* is different from that in other orthodox species.

Consequently, for operational storage of *M. hortensium* it is recommended to reduce moisture content to 4-6% and store in hermetically sealed containers for up to 1-2 years. For longer time periods of seeds storage, it is recommended that further research on storage at higher MC is required. According to McGill et al. (2002), drying to 7.5% MC significantly increased the storability of M. *hortensium* up to 21 months.

4.3 Cryopreservation of *Dysoxylum spectabile*

4.3.1 Introduction

Cryopreservation has been considered the most promising method for long-term storage of recalcitrant seeds (Engelmann, 2000, 2004). Several temperate recalcitrant or intermediate species have been successfully cryo-stored for nearly 10 years with 80-100% survival rate of the embryo axes (Pence, 2003). There have been also reported that some of tropical recalcitrant species were successfully cryopreserved (Abdelnour-Esquivel & Engelmann, 2002; Mroginski, et al., 2008; Sant, Taylor, & Tyagi, 2006).

Different techniques are used for the cryopreservation of plant germplasm. The method used for recalcitrant seeds usually involves desiccation of zygotic embryonic axes prior to immersing the axes into liquid nitrogen (Berjak, et al., 1998). Because of the inherent inability of intact recalcitrant seeds to tolerate desiccation to water levels that would permit non-injurious exposure to liquid nitrogen temperatures, development of cryopreservation protocols must include desiccation-sensitivity studies. Rapid drying has generally been found to be superior to slower dehydration for recalcitrant material, as rapid drying reduces the duration the tissues spend at water concentrations during which injurious metabolism occurs (Pammenter & Berjak, 1999; Pammenter, et al., 1998; Walters, Pammenter, Berjak, & Crane, 2001; Yap, et al., 1998). The rate of cooling, warming and conditions of rehydration are other factors that markedly influence the success of a cryopreservation protocol. Sufficiently high cooling rates in relation to tissue water concentration have been found to be critical for the successful cryopreservation of embryonic axes of *Aesculus hippocastanum* (Wesley-Smith, Walters, Pammenter, & Berjak, 2001), *Camellia sinensis* (Wesley-Smith, Vertucci, Berjak, Pammenter, & Crane, 1992), *Poncirus trifoliata* (Wesley-Smith, Walters, Berjak, & Pammenter, 2004) and *Quercus robur* (Berjak, et al., 1998); while rapid rehydration has been shown to be better than slow rehydration for the embryonic axes of *Ekebergia capensis* (Peran, Pammenter, Naicker, & Berjak, 2004), and possibly for all desiccation-sensitive tissues.

Another cryopreservation technique, encapsulation-dehydration, was introduced in the early 1990's and is based on technology developed for the production of synthetic seeds

(synseeds) (Yap, et al., 1998). This technique has been utilised for cryopreservation on the premise that alginate encapsulation should allow tissues to withstand manipulation of water without damaging the tissue (Yap, et al., 1998). Although encapsulation has been applied mainly to apices excised from *in vitro* plantlets and somatic embryos of several species, the technique has also been tested on zygotic embryos or embryonic axes of some species, including *Citrus madurensis* (Cho, et al., 2002a) and *Hevea brasiliensis* (Yap, et al., 1998). Compared with other preservation techniques, encapsulation–dehydration has several advantages: manipulation of explants is easy (Martínez, et al., 1999; Takagi, 2000), large quantities of delicate tissues can be stored (Paul, Daigny, & Sangwan-Norreel, 2000), non-toxic cryoprotectants (sucrose, glycerol) are applied (Martínez, et al., 1999; Niino & Sakai, 1992; Takagi, 2000) and explants are efficiently protected during dehydration (Verleysen, Van Bockstaele, & Debergh, 2005).

Vitrification-based methods involve pretreatment of samples with highly viscous cryoprotectant solution. On rapid freezing the highly concentrated solute inside the tissue will form a meta-stable 'glass' allowing the avoidance of lethal ice crystallization. A vitrification protocol involves the following successive steps: pregrowth or pre-conditioning of mother plants; preculture of explants; treatment (loading) of samples with cryoprotective substances; dehydration with highly concentrated vitrification solutions; rapid cooling and rewarming; removal of cryoprotectants (unloading); and recovery (Reed, 2008).

The vitrification solutions most commonly employed for freezing plant tissues and organs include the Plant Vitrification Solutions (PVS). The PVS solutions have the following composition (% expressed in w/v): 22 % glycerol + 15 % ethylene glycol (EG) + 15 % propylene glycol + 7 % dimethylsulfoxide (DMSO) + 0.5 M sorbitol (PVS1, Uragami, et al., 1989); 30 % glycerol + 15 % EG + 15 % DMSO + 0.4 M sucrose (PVS2, Sakai, et al., 1990); 50 % glycerol + 50 % sucrose (PVS3, Nishizawa, et al., 1993); and 35 % glycerol + 20 % EG in medium containing 0.6 M sucrose (PVS4, Sakai, 2000). A solution developed by Steponkus' group consists of 7 M EG + 0.88 M sorbitol + 6 % bovine serum albumin (Langis, Schnabel, Earle, & Steponkus, 1989).

Seeds of *D. spectabile* (see chapter 3.1) exhibit recalcitrant storage behaviour with viability being lost on drying to comparatively high water contents (in the region of 25% fresh weight basis). As recalcitrant seeds start to lose viability whilst 'free' or 'bulk' water is present, whole seeds are unsuitable for storage at sub-zero temperatures that could lead to ice crystal formation and subsequently cell damage and death. Consequently, options for storing these seeds are limited to either a short term storage of whole seeds at temperatures that minimise germinative growth (Pritchard, 2004) or cryopreservation of embryonic axes (Pence, 2003; Wesley-Smith, et al., 2004).

Desiccation at the proper developmental stage can also be critical for maximizing survival of tissues through cryopreservation (Pence, 1995). The development and sensitivity to desiccation of *D. spectabile* seeds have not been reported previously. In this study, the relationship between seed development and desiccation tolerance, as well as the influence of drying rate on seed desiccation tolerance is assessed in order to provide information for the development of cryopreservation protocols for this species. Seed tolerance to desiccation (e.g. for storage purposes) is often dependent on the developmental status of a seed and is acquired at completion of morphological development, i.e. around mass maturity (completion of reserve accumulation) (Golovina, Hoekstra, & Van Aelst, 2001; Hay & Smith, 2003; Kermode, Finch-Savage, & Black, 2002; Pritchard, 2004). Moreover, seeds capable of a high level of germination prior to maturity may not have attained their maximal tolerance to desiccation and storage (Pritchard, 2004).

The moisture content (MC) of seeds at the time of immersion in liquid nitrogen must be regarded as the most critical factor in cryopreservation. Indeed, seed moisture should be reduced to such an extent that the formation of intra-cellular ice crystals during ultra-rapid freezing (produced by the direct immersion of seeds in liquid nitrogen) is made impossible, thus preserving the integrity of seed tissues (Lambardi, et al., 2004). Differential scanning calorimetry (DSC) has been used to determine the optimal moisture content for cryopreservation (Dussert, et al., 2001; Hor, et al., 2005; Vertucci, 1989). DSC provides a means of calculating the quantities of osmotically active (OA) and osmotically inactive (OI) water in a sample during cooling and rewarming, which

are equivalent to the frozen and unfrozen water fractions under the conditions of the experiment (Zachariassen, Hammel, & Schmidek, 1979).

Optimal results from cryopreservation require a combination of adequate conditioning of plant materials, properly performed protocols, and good regrowth conditions. Cryopreservation protocols that are effective for diverse species or cultivars are needed for conservation programs and genebanking, and sometimes available techniques can be directly applied to new species (Reed, Okut, D'Achino, Narver, & DeNoma, 2003).

The overall aim of this study was to develop an optimum cryopreservation protocol for the seeds of *D. spectabile* and to investigate its potential application for *ex situ* conservation of this species. To achieve this aim, the following aspects were studied:

(1) The effect of seed maturity on desiccation tolerance and cryopreservation in *D. spectabile*

(2) Determination of optimum desiccation method for seed and embryo

(3) Determination of optimum dehydration windows for cryopreservation of embryos using DSC thermal analysis

(4) The effect of desiccation and cryopreservation on germination of the embryonic axes

(5) The efficacy of different cryopreservation protocols for long-term storage of *D. spectabile*

4.3.2 Materials and methods

4.3.2.1 Seed materials

To determine optimal developmental stage, seeds were collected at three-weekly intervals between 21 March 2009 and 26 June 2009. Fruits were considered completely matured as they began to be shed from the tree at this last date.

For cryopreservation experiments, mature fruits were collected from the 28 May and 26 June 2009. The collected fruits were couriered to Millennium Seed Bank (UK) in plastic bags within 10 days of collection.

Seeds were handled as detailed in 3.1.2.1.

4.3.2.2 Determination of moisture content

Seed moisture content (MC) determination was as described in 3.1.2.2. Water content of embryonic axes was determined using three replicates of 10 axes. The seeds were cut in half vertically then embryonic axes were dissected from the cotyledons by hand with gentle pressure. Excised embryos were weighed with a digital balance accurate to 0.001 g before and after drying for 17 hours in a 103°C ± 2°C oven. MC was calculated as the percentage of water on a fresh weight basis.

4.3.2.3 Determination of viability

Seeds

Seed germination was determined using four replicates of 50 seeds. Seeds were placed between moist folded 38 lb regular weight seed germination paper (Anchor Paper Company, St. Paul, Minnesota) held in closed plastic boxes and incubated at 20°C ± 2°C, under a 16 hr light/8 hr dark photoperiod. Normal seedlings were assessed as described in 3.1.2.3.

Embryonic axes

The seed coat was removed and embryonic axes were dissected from the cotyledons. Excised embryonic axes were surface sterilized in 70% (v/v) ethanol for 10 sec, followed by 10% (v/v) sodium hypochlorite solution (Domestos, Lever Bros.) for 2 min. Axes were then rinsed three times in sterile distilled water. Surface sterilized embryonic axes were cultured on standard medium: ½ strength Murashige & Skoog (1962) (MS) medium supplemented with 30 g/l sucrose and 8 g/l Plant Agar. The cultures were incubated at 20 ± 2°C, under a 16 hr light/8 hr dark photoperiod. Survival was assessed as the percentage of embryonic axes that exhibited any kind of growth, including normal development and development of the root pole only after 14 days culture. Three replicates of 10 axes were used for the viability test.

4.3.2.4 Determination of the effect of seed maturity on desiccation tolerance

To determine the effect of desiccation on seed survival at different development stages, sets of immature seeds (harvested on 2 May) and mature seeds (harvested on 26 June) were dried to a number of target moisture contents of 50%, 40%, 30%, 20%, 10% and 5% chosen according to the IPGRI screening protocol (IPGRI-DFSC, 2004) as described in 3.1.2.4.

Once the target weight was reached seed moisture content was determined as described in 3.1.2.2. Seeds was germinated in plastic boxes (220×120×40mm) and incubated at 20°C ± 2°C, under a 16 hr light/8 hr dark photoperiod. Seeds were placed upright in moist, heat-sterilized sand, which was previously sterilized at 103°C for 24 hr. Seeds were pressed into the surface of the sand until half covered. The remaining exposed seed was then covered with a layer of sterilized sand. Sand was remoistened when necessary to maintain moist conditions during germination. Normal seedlings were assessed as described in 3.1.2.3. Seed moisture content and germination were determined using four replicates of 10 seeds and 50 seeds, respectively.

4.3.2.5 Determination of optimum desiccation method

Different drying methods were used to determined desiccation sensitivity of seed and embryonic axes as described below.

Slow drying methods

Seeds were equilibrated to 5, 15, 30, 50, 65 & 80% relative humidity (RH). For 30, 50, 65, 80 and 100% RH, seeds were placed in air-tight containers which were calibrated at appropriate RH environment using lithium chloride salt solutions and/or distilled water. For 5% RH, seeds were placed in an air tight container with silica gel and for 15% RH, samples of seeds were dried at, by placing in a dry-room maintained at 15% RH.

Equilibrium relative humidity (eRH) of seeds was determined using with a ROTRONIC hygrometer. Once the seeds had equilibrated to the target RH, moisture content and viability of seeds and embryonic axes were determined as described in 4.3.2.2 and 4.3.2.3.

Rapid drying methods

To determine seed desiccation sensitivity, seed lots were dried to target moisture contents of 40%, 35%, 30%, 25%, 20% and 15% chosen according to the IPGRI screening protocol (IPGRI-DFSC, 2004) as described in 3.1.2.4. Once the target weight was reached, moisture content and viability of seeds were determined as described in 3.1.2.2 and 4.3.2.3.

For embryonic axes, the sterilized embryonic axes were placed on sterilized filter paper in 9 cm Petri dishes for desiccation. The axes were desiccated for between 0 and 6h under a laminar air-flow air current and, at the end of the prescribed period of desiccation, embryo moisture content and viability was determined described in 4.3.2.2 and 4.3.2.3.

Encapsulated-dehydration methods

The sterilized embryonic axes were placed in 3% (w/v) sodium-alginate prepared in ½ strength MS medium without calcium at pH 5.7. Beads were formed by suspending embryonic axes in sodium-alginate and dripping them into a calcium chloride solution (½ strength MS medium with 1% $CaCl_2$). Beads were allowed to polymerize for 20 min. Beads were blotted dry on sterile filter paper, transferred to an open Petri dish and dehydrated between 0 and 6 hrs in a laminar air-flow cabinet. At the end of the prescribed dehydration period, the moisture content and viability of the beads containing the embryos was assessed as described in 4.3.2.2 and 4.3.2.3.

4.3.2.6 Thermal analysis

Differential scanning calorimetry incorporating both cooling and warming cycles of *D. spectabile* cotyledons, embryonic axes and beads of each treatment was undertaken using a DSC model Perkin Elmer DSC with Pyris 7 software. The instrument was calibrated with zinc and indium and pure water as a standard for cryogenic operations (Benson, Reed, Brennan, Clacher, & Ross, 1996). Individual samples were placed in aluminium pans, sealed with the aid of a Perkin Elmer crimper. Scans were performed from + 25ºC to –100ºC with a cooling/warming rate of + 10ºC min^{-1}.

After thermal analysis, sample pan lids were pierced and dried in an oven at 103°C ± 2°C for 17 hr to determine dry weight. The total water content of the sample and the proportion of osmotically active (OA) and inactive (OI) water were determined. Calculations of the proportion of OA water in the samples were based on the enthalpy of water (334.5 $J.g^{-1}$) using the melt endotherm together with the total water content of the sample (Block, 2003). The quantity of OI water was calculated as the difference between total water and OA water contents. Three replicates were examined by DSC for each treatment.

4.3.2.7 Cryopreservation of embryonic axes

Rapid desiccation protocol

For each of five desiccation times (0, 3.5, 3.75, 4 and 4.25 hr; duration determined based on DSC thermal analysis), 120 embryonic axes were desiccated in open Petri dishes on a laminar flow bench. Following desiccation, half of the embryos were used for cryopreservation and the other half were used as controls. For cryopreservation, embryos were transferred into a 2 ml cryotube and immersed directly in liquid nitrogen for 24 hr. Thawing was performed by immersing the cryotubes in a water bath at 40 ± 2ºC for 5 min. Control embryos were directly cultured on standard recovery medium after desiccation. Water content and viability of embryonic axes before (non-cryopreserved controls) and after cryopreservation were determined as described in 4.3.2.2 and 4.3.2.3.

Encapsulation-dehydration protocol

Embryonic axes were encapsulated as described previously. Sucrose was included in the alginate bead, so sucrose pre-treatment was omitted to avoid over-drying by osmotic effects. 120 beads then blotted dry on sterile filter paper and desiccated in Petri dishes for five desiccation times (0, 4.5, 4.75, 5 and 5.25 hr; duration determined based on DSC thermal analysis). Following desiccation, half of the beads were used for cryopreservation and the other half were used as controls. Cryopreservation was performed as previously described.

Vitrification protocol

Preculture

The embryonic axes were precultured for one, two and three days on solidified preculture medium: 0.75M sucrose + ½ strength MS medium and incubating them at 20 ± 2ºC under a 16 hr light/8 hr dark photoperiod.

Loading treatment

Following preculture, embryos were placed in a 2.0ml cryovial and treated with loading solution comprised of the following compounds (% expressed in w/v) in ½ strength liquid MS medium: 0.4 M sucrose + 2 M glycerol for 20 min at room temperature (~25 °C).

Treatment with vitrification solution

Two different vitrification solutions were used to optimize the conditions of treatment. These were prepared in ½ strength MS liquid medium, as: 30 % glycerol + 15 % ethylene glycol (EG) + 15 % DMSO + 0.4 M sucrose [PVS2 (Sakai, et al., 1990)]; 50 % glycerol + 50 % sucrose [PVS3 (Nishizawa, et al., 1993)].

The effect of the duration of application of the vitrification solution was then evaluated. For this purpose, embryonic axes were treated with vitrification solution for 0, 30, 60 and 90 min at 0°C.

Cooling, storage and rewarming

At the end of the vitrification treatment, the vitrification solution was renewed and the final volume in the cryotubes adjusted to 1.5 ml. The embryonic axes were either unloaded and transferred to standard recovery medium (non-cryopreserved controls) or cryopreserved by rapid immersion of the cryotubes in liquid nitrogen (LN). After 24 hr

of storage at - 196 °C, the embryonic axes were rewarmed by placing the cryotubes in a water-bath at 40 ± 2ºC for 5 min.

Unloading treatment and growth recovery

The vitrification solution was removed and replaced by 1.5 ml ½ strength liquid MS medium added with 1.2M sucrose for 20 min (unloading treatment). The embryonic axes were retrieved from the liquid medium, blotted dry on sterile filter paper and placed on standard medium. After 24 hr, they were transferred on to standard medium for growth recovery and survival was assessed as described 4.3.2.3.

4.3.2.8 Data analysis

Data were analysed for statistical significance by analysis of variance (ANOVA). Fisher's least significant difference ($p \leq 0.05$) was used to determine whether there were significant differences between treatments. Prior to analysis, data were checked for normality using Shapiro–Wilk tests. No transformations were necessary. SAS for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

4.3.3 Results

4.3.3.1 Optimal seed development stage for cryopreservation

Physical characteristics of seeds during development

Changes in fresh and dry weight of seeds during development are shown in Figure 4.6 and 4.7. Seed and embryo dry weight increased from c. 85 mg and 0.4 mg respectively at 22 March to maxima of 221 mg and 1.1 mg respectively at 26 June. Seed fresh weight reached a maximum at 23 May and that of the embryo at 2 May. The fresh weight per seed and per embryo did not change significantly after 23 May. The moisture content of seeds and embryos decreased throughout the whole development period investigated and reached 48% MC and 70% respectively by 26 June.

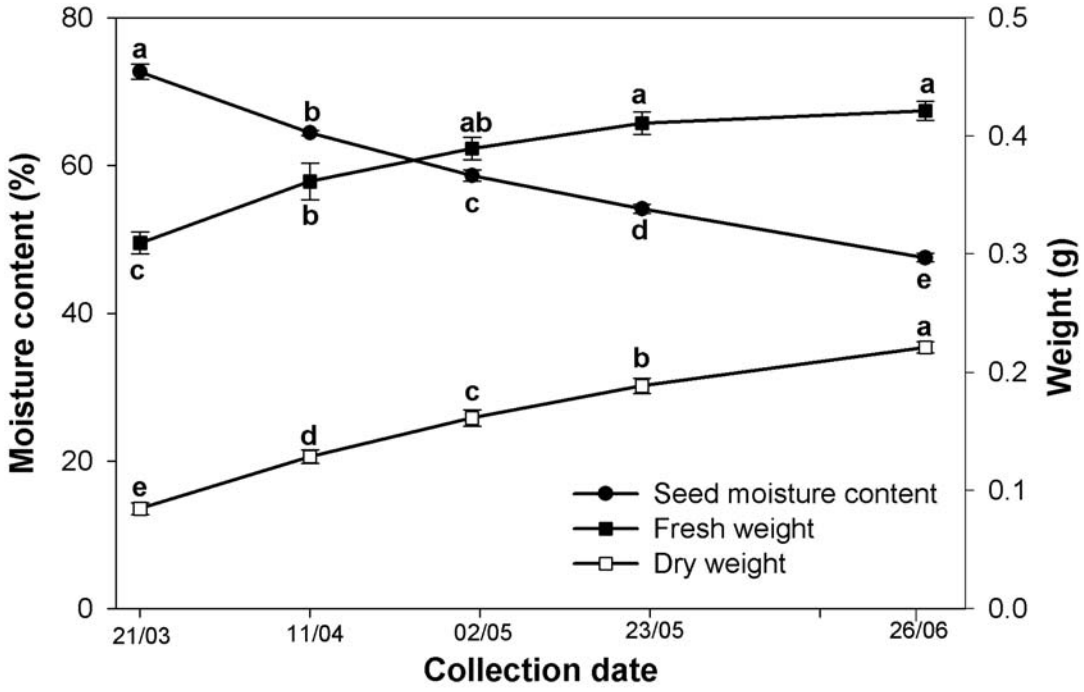

Figure 4.6 Changes in moisture content (●), fresh weight (■) and dry of weight (□) of developing seeds of *Dysoxylum spectabile*. Bars correspond to SE of means of four replications. Values with different letters are significantly different (LSD, $p \leq 0.05$).

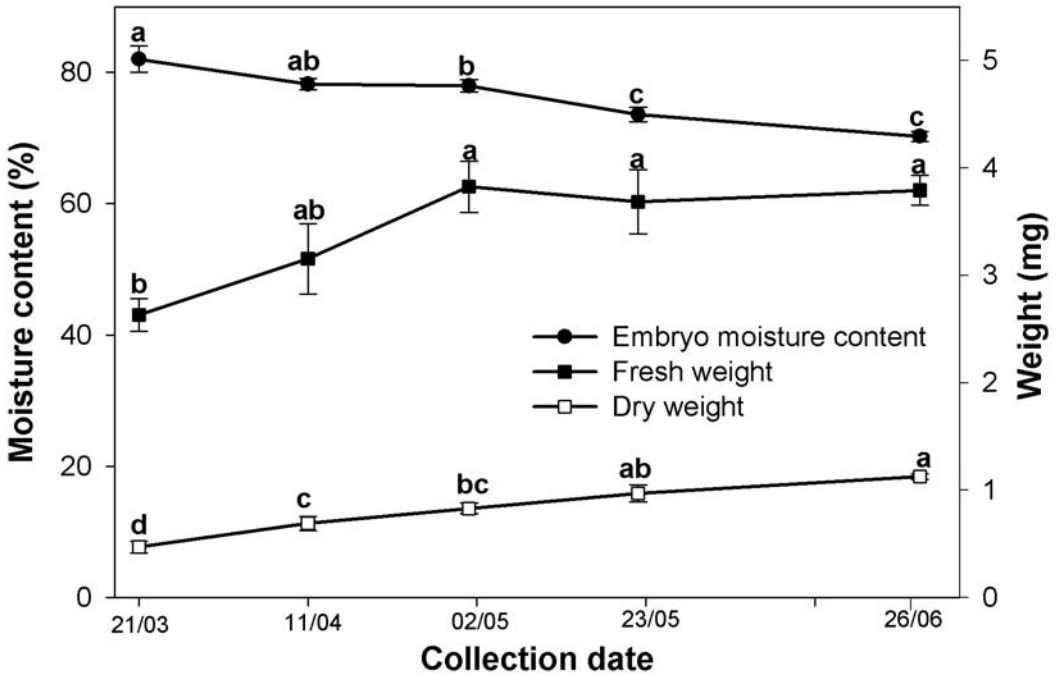

Figure 4.7 Changes in moisture content (●), fresh weight (■) and dry of weight (□) of developing embryonic axes of *Dysoxylum spectabile*. Bars correspond to SE of means of four replications. Values with different letters are significantly different (LSD, $p \leq 0.05$).

Germination percentage was not significantly different throughout the whole development period investigated but time taken for 50% germination decreased from 20 days at 23 May to 2.3 days at 26 June (Table 4.7).

Table 4.7 Germination (%) and time taken for 50% germination (T_{50}) of developing seeds of *Dysoxylum spectabile.*

Collection date	Germination (%)	T_{50} (days)
21/03	98.9 ab	20.1 a
11/04	97.5 a	10.3 b
02/05	100.0 a	4.9 c
23/05	100.0 a	2.3 d
26/06	93.3 b	2.3 d

Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Effects of desiccation on seeds survival at different development stage

For the immature seeds harvested on 2 May (58% seed MC), an initial reduction in seed moisture content resulted in significant increase in the percentage of germination when compared to the initial germination (0hr), the percentage of germination increased from 30.0% (initial) to 56.0% (max germination) within 5h (Figure 4.8). However, further reduction in the moisture content resulted in a substantial reduction in germination. Germination percentage of immature seeds decreased to 12% after drying to 22.0% MC; no seed germinated below this moisture level.

For mature seeds harvested on 26 June (50% seed MC), the germination percentage decreased from 93% to 58% after drying to 29% MC. The percentage of germination was dramatically reduced to 3% when MC was 21% and no seed germinated below this moisture level (Figure 4.8).

The desiccation tolerance differed depending on the stage of seed development. When the MC fell to ca. 30%, the germination percentage of immature and mature seeds were 21% and 58%, respectively.

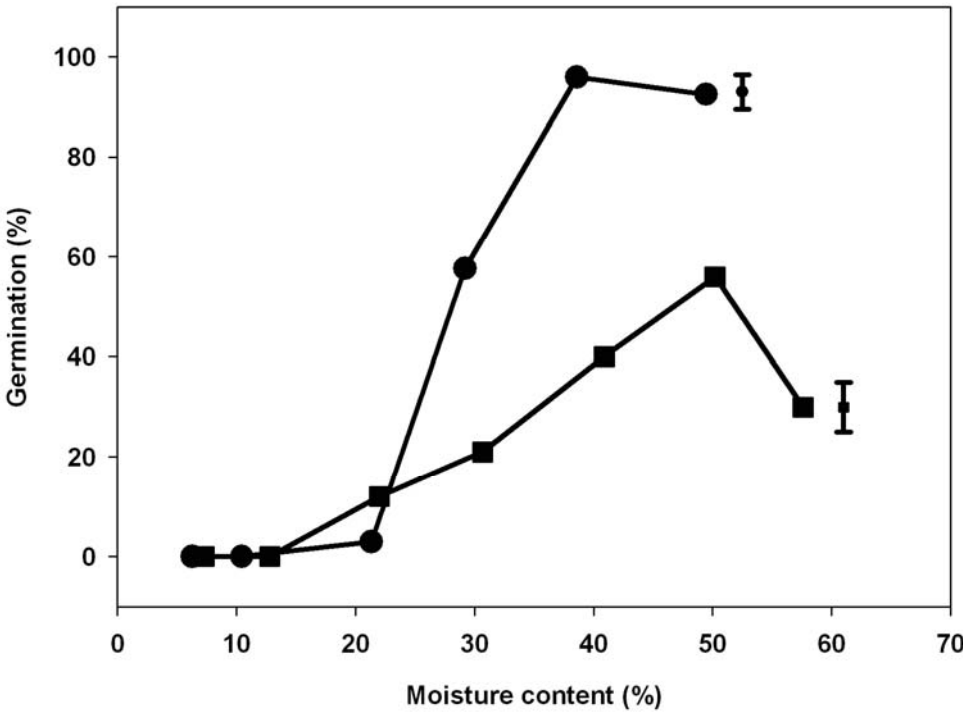

Figure 4.8 Effect of desiccation on subsequent ability of *Dysoxylum spectabile* seeds to germinate (%, criterion normal germination) at different maturity stage seed lots: mature seed (●), immature seed (■).Vertical bars indicated the least significant difference between mean germination percentages for each maturity stage ($p \leq 0.05$).

4.3.3.2 Desiccation sensitivity

Slow drying methods

The equilibrium relative humidity (eRH) of fresh seed was 99.1% at 20.8°C. The MC of the seeds was allowed to reach equilibrium at 30, 50, 65, 80 and 100% RH with appropriate lithium chloride solutions and/or distilled water after 4-20 days. The mean equilibrium seed RH in each RH chamber were 98.6%, 88.7%, 74.3%, 57.5%, 46.8% and 31.9%, respectively, and the respective seed MCs of 54.1%, 20.9%, 10.9%, 7.8%, 6.5% and 5.1% were obtained (Table 4.8).

Table 4.8 Changes in seed eRH, seed (SMC) and embryo moisture content and germination of seeds and excised embryos of *Dysoxylum spectabile* at various RH environments.

	Seed eRH (%)	SMC (%)	MC of embryonic axes (%)	Viability (%)	
				Seed	Embryonic axes
Initial	97.0 ± 0.29	47.5 b	74.3 a	93.3 a	93.3 a
100	98.6 ± 0.83	54.1 a	71.9 a	43.3 b	66.7 b
80	88.7 ± 0.41	20.9 c	33.7 b	0 c	0 c
65	74.3 ± 0.32	10.9 d	15.2 c	0 c	0 c
50	57.5 ± 0.03	7.8 e	10.2 d	0 c	0 c
30	46.8 ± 0.25	6.5 ef	8.1 de	0 c	0 c
15	31.9 ± 1.38	5.1 f	5.9 e	0 c	0 c

Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

The germination percentage decreased dramatically, with no seed or embryonic axes survival following desiccation below 80% RH (34% embryonic axes MC and 21% seed MC). When seeds and embryonic axes were maintained at 100% RH for 4 days, there was a significant deleterious effect where viability of seeds and embryonic axes decreased from 93% to 43% and 67%, respectively (Table 4.8).

Rapid drying methods

Fresh seeds were desiccated to various target moisture contents using silica gel. Viability decreased from 100% germination for seeds at 48% MC to 12% germination for seeds at 25% MC. This reduction of viability to 12% occurred at 37% MC for embryonic axes. No seeds germinated below this moisture level (Table 4.9). There was a decline in the rate of germination, as indicated by an increase T_{50}, during desiccation.

Table 4.9 Changes in moisture content, percentage of normal seedlings and time to 50% germination (T_{50}) of *Dysoxylum spectabile* during desiccation.

Drying time (hr)	Target MC (%)	MC of seeds (%)	MC of embryonic axes (%)	Normal seedlings (%)	T_{50} (days)
0	Initial	47.8 a	68.3 a	100 a	3.1 d
9	40	40.6 b	63.6 b	100 a	5.4 c
15	35	33.8 c	54.3 c	88 b	7.2 b
22	30	29.0 d	48.9 d	38 c	8.4 a
30	25	25.1 e	36.6 e	12 d	-
40	20	20.6 f	27.0 f	0 e	-
58	15	12.5 g	6.4 g	0 e	-

Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Moisture content of excised embryonic axes dropped from 74% to 8% after 6h desiccation. The survival of *D. spectabile* embryonic axes decreased from 69% to 40% after 3hr desiccation. Only 10% of the embryonic axes survived after 6h desiccation (Figure 4.9).

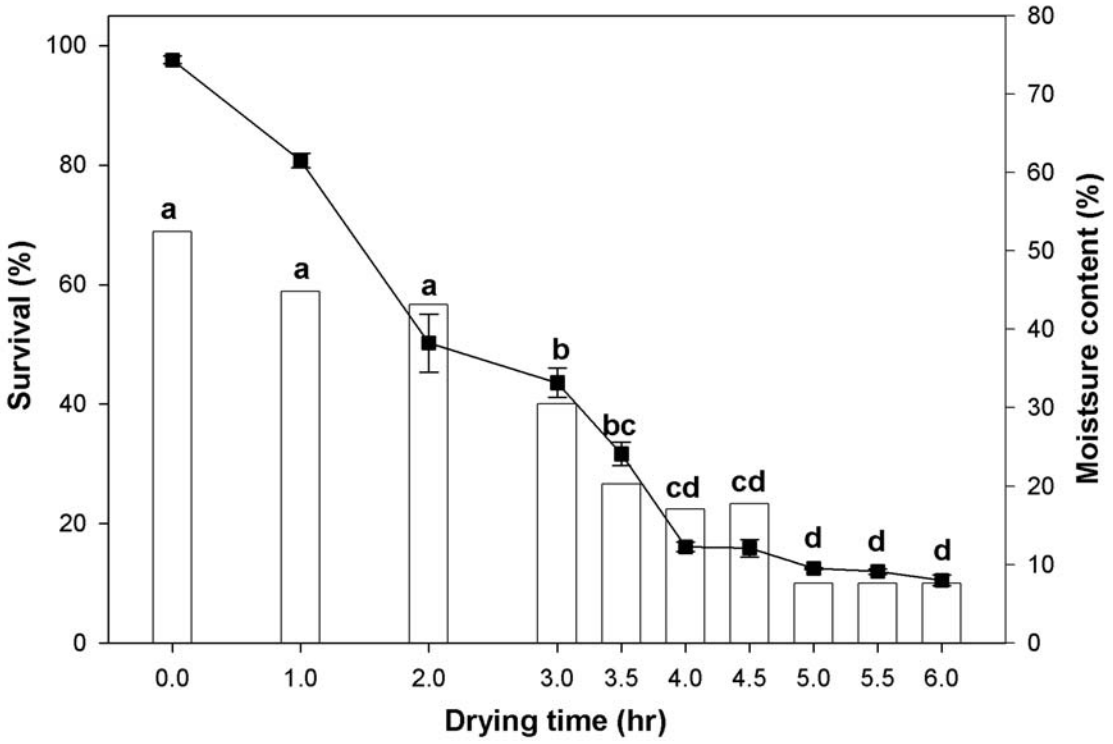

Figure 4.9 Changes in moisture content (line graph) and percentage survival (bar graph) of embryos of *Dysoxylum spectabile* after various periods of desiccation. Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Encapsulation-dehydration methods

The initial water content of beads with embryonic axis before desiccation was 93% on a fresh weight basis. This rapidly decreased to 34% within the first 3h and then gradually dropped to 17% after 6h. Survival decreased from 59% to 40% after 4h dehydration and then dropped to 17% after 6 hr dehydration (Figure 4.10).

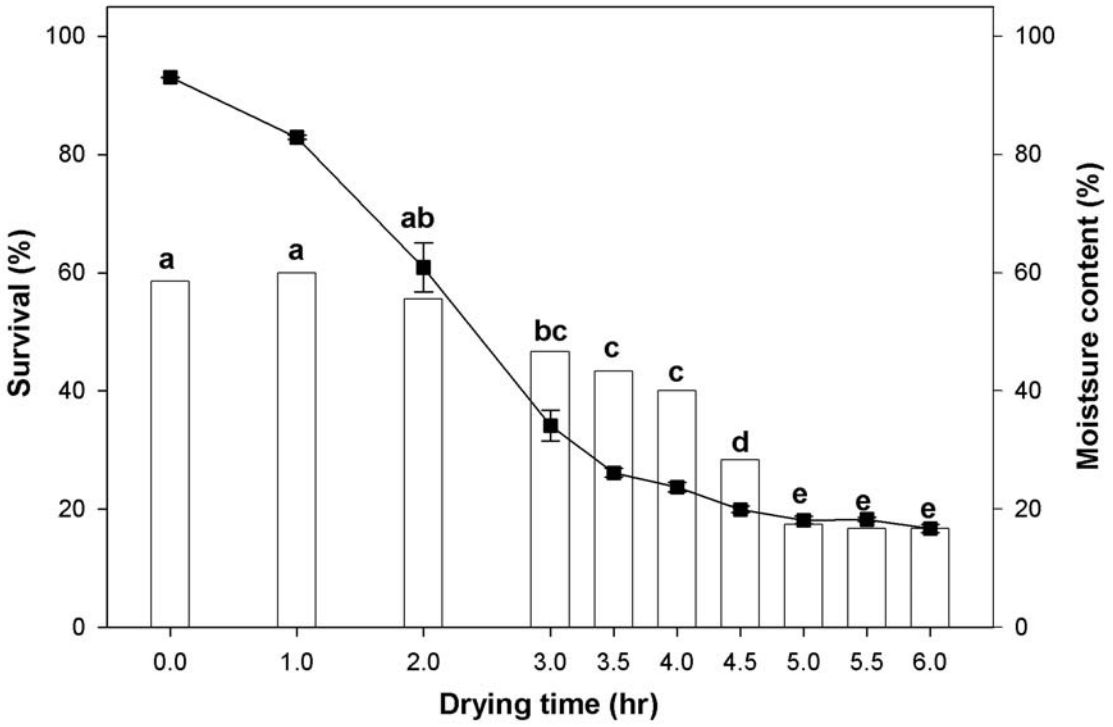

Figure 4.10 Changes in moisture content (line graph) and percentage survival (bar graph) of alginate encapsulated embryos of *Dysoxylum spectabile* after various periods of desiccation. Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

4.3.3.3 Thermal analysis

The DSC thermograms provided information on critical parameters such as melt onset temperature, the enthalpy of melting, and the amount of OI water across the range of treatments for excised embryonic axes, cotyledon and bead containing embryonic axes.

Representative thermograms for control embryonic axes (Figure 4.11) showed an ice nucleation peak with a corresponding exothermic enthalpy variation of 177.52 ± 7.07 $J.g^{-1}$ during cooling and on melting during subsequent rewarming an endothermic event with enthalpy variation of 203.3 ± 0.32 $J.g^{-1}$, respectively.

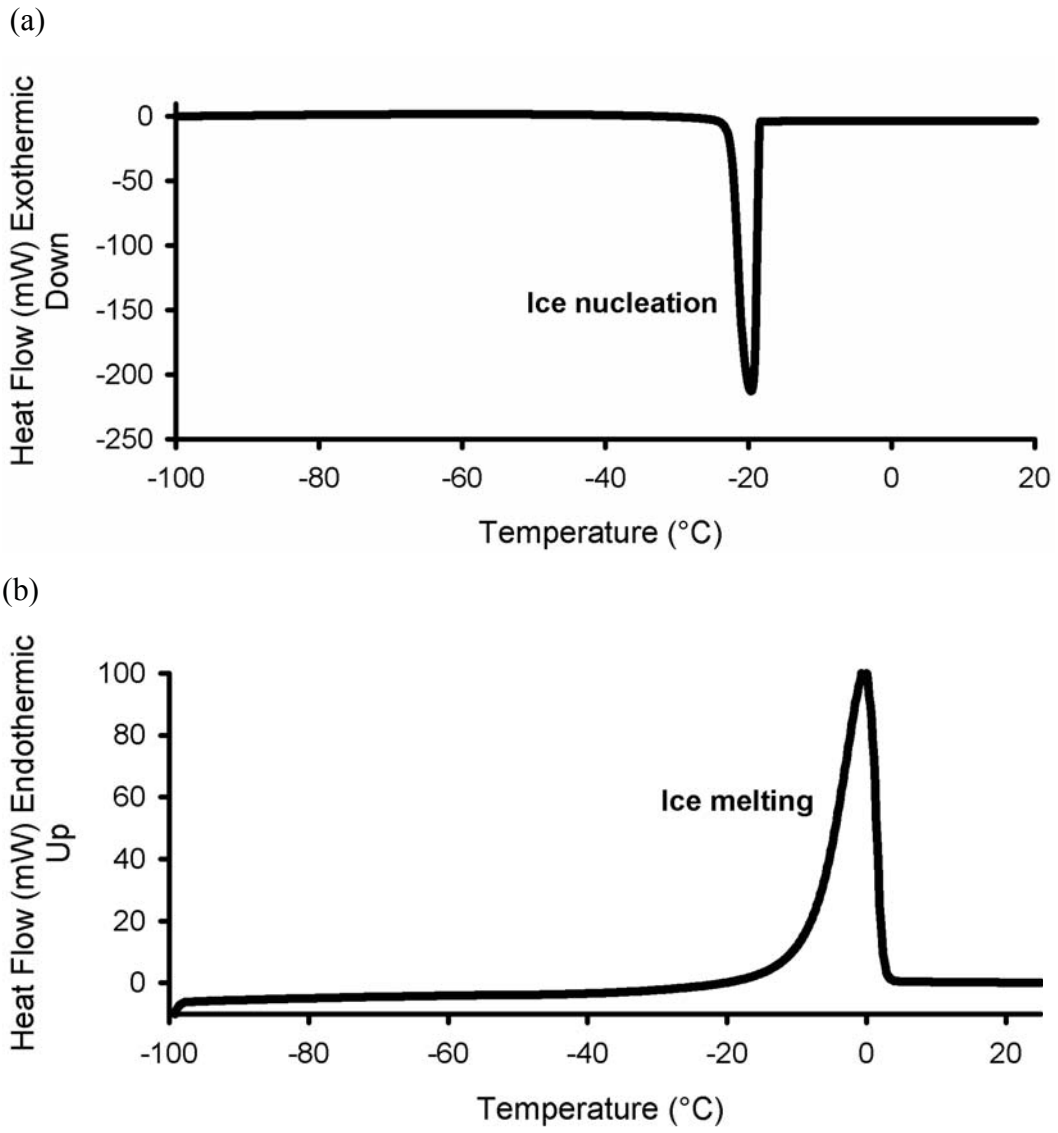

Figure 4.11 DSC cooling (a) and warming (b) thermograms for control *Dysoxylum spectabile* embryonic axes. Samples were held at 25ºC for 1 min cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

Thermograms for control samples of cotyledons are given in Figure 4.12, two main exotherms (peaks A and B) were detected during cooling as an ice nucleation peak with a corresponding exothermic enthalpy variation of 74.7 ± 5.56 $J.g^{-1}$ and 8.9 ± 0.31 $J.g^{-1}$, respectively. On melting an endothermic event (peaks C and D) with enthalpy variation of 96.8 ± 4.82 $J.g^{-1}$ and 14.3 ± 0.72 $J.g^{-1}$, respectively (Figure 4.12) were observed. Peak A (cooling) and peak C (warming) represent water melts while peak B (cooling) and peak D (warming) represent lipid phase transitions.

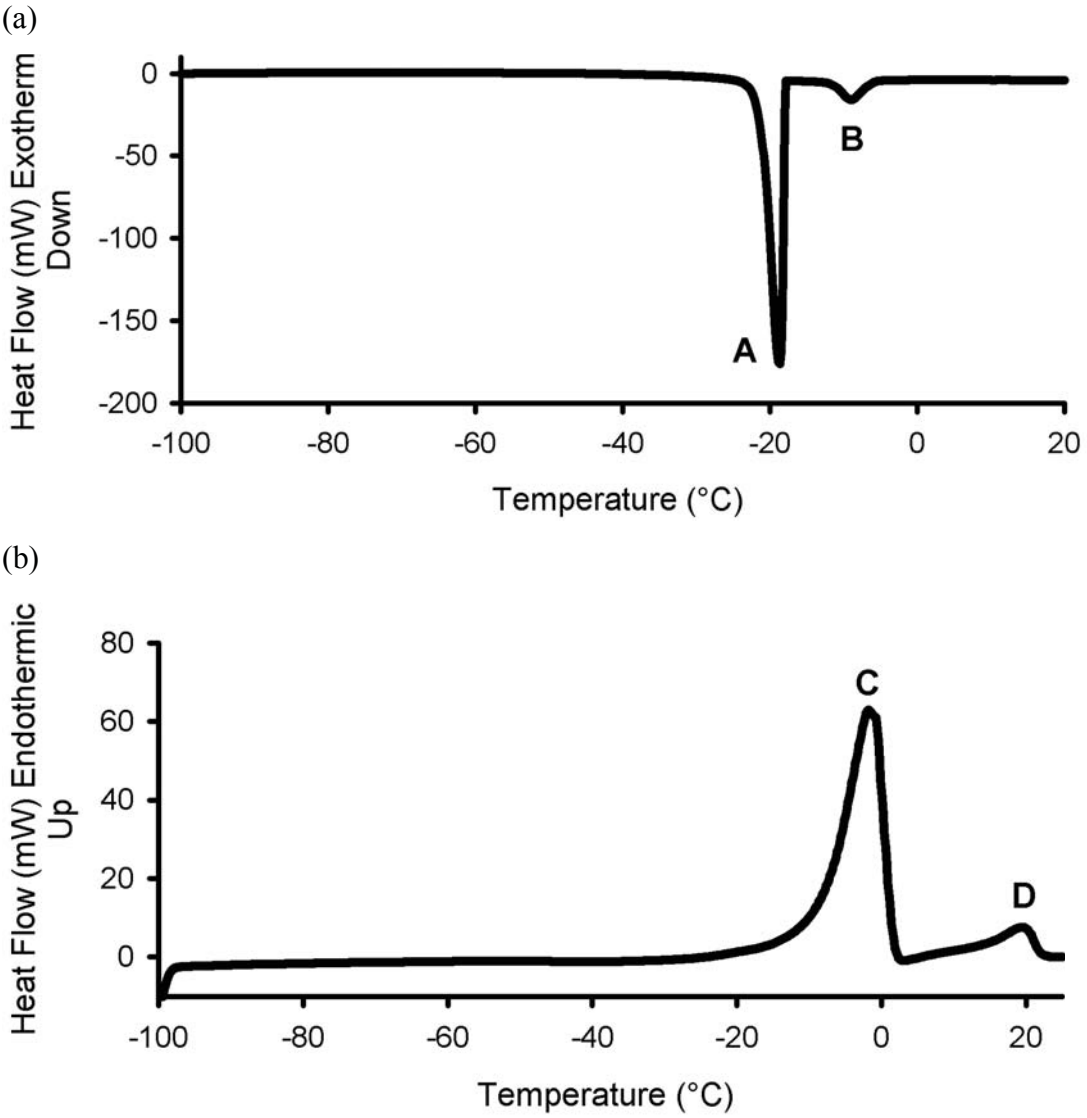

Figure 4.12 DSC cooling (a) and warming (b) thermograms for control *Dysoxylum spectabile* cotyledon. Samples were held at 25ºC for 1 min cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

Slow drying methods

Analyses of warming cycles revealed melting events in embryonic axes between c. -26°C and 4°C. The main melting peak was centred around -0.4 to -13°C when seed had been equilibrated in an atmosphere above 80% RH. However at lower humidity embryonic axes showed thermodynamically stable profiles (Table 4.10, Figure 4.13).

Table 4.10 Rewarming thermodynamic properties of embryos of *Dysoxylum spectabile* equilibrated at different RH.

Relative humidity	MC of embryonic axes (%)	Thermal Event	Onset (°C)	Mid-point (°C)	Endpoint (°C)	Enthalpy ($J.g^{-1}$)
Control	74.4 ± 0.51	Ice Melt	-8.1 ± 0.29	-0.4 ± 0.06	3.8 ± 0.94	203.3 ± 0.32
100	71.9 ± 0.44	Ice Melt	-8.1 ± 1.21	-1.1 ± 0.14	3.3 ± 0.55	193.6 ± 2.13
80	33.7 ± 3.57	Ice Melt	-25.7 ± 2.37	-13.4 ± 2.44	-8.2 ± 2.18	30.5 ± 8.04
65	15.2 ± 0.27	θ	NA	NA	NA	NA
50	10.2 ± 0.14	θ	NA	NA	NA	NA
30	8.1 ± 0.05	θ	NA	NA	NA	NA
15	5.9 ± 0.05	θ	NA	NA	NA	NA

Data points are means of three replicates (± standard error).

* θ = stable profile, no thermodynamic event detected in all replicates.

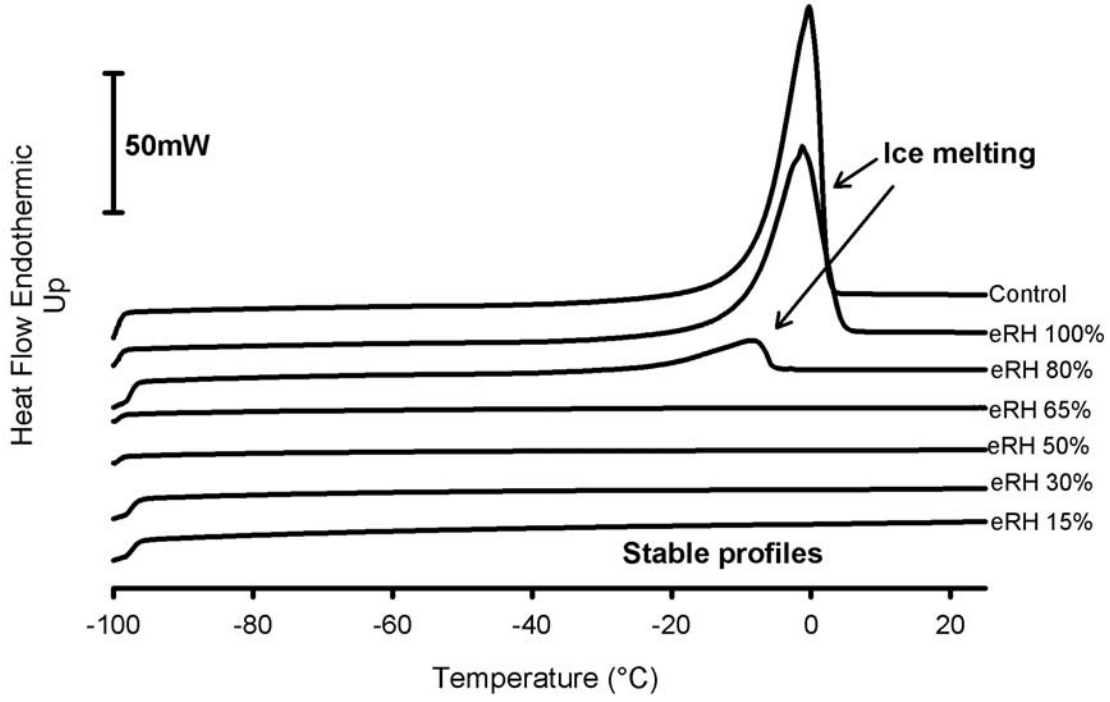

Figure 4.13 Rewarming thermodynamic properties of embryos of *Dysoxylum spectabile* equilibrate at different RH. Samples were held at 25ºC for 1 min cooled to –150ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

Warming thermograms of *D. spectabile* samples of seed cotyledons at different water contents are given in Figure 4.14. At low water contents (<0.18 g H_2O g^{-1} DW, below 80% RH) two main endotherms (peaks C' and D) were observed. For peak D, there was no apparent increase in either size or a change in their onset and mid temperatures as seed water contents varied, hence, it can be concluded that peaks D are the lipid melts. Peak C' in dry seeds is included some lipid re-crystallizations events (L). At higher water contents, the size and the onset temperature of peak C increased (Figure 4.14). This peak belongs to water melts as it disappeared at low water contents.

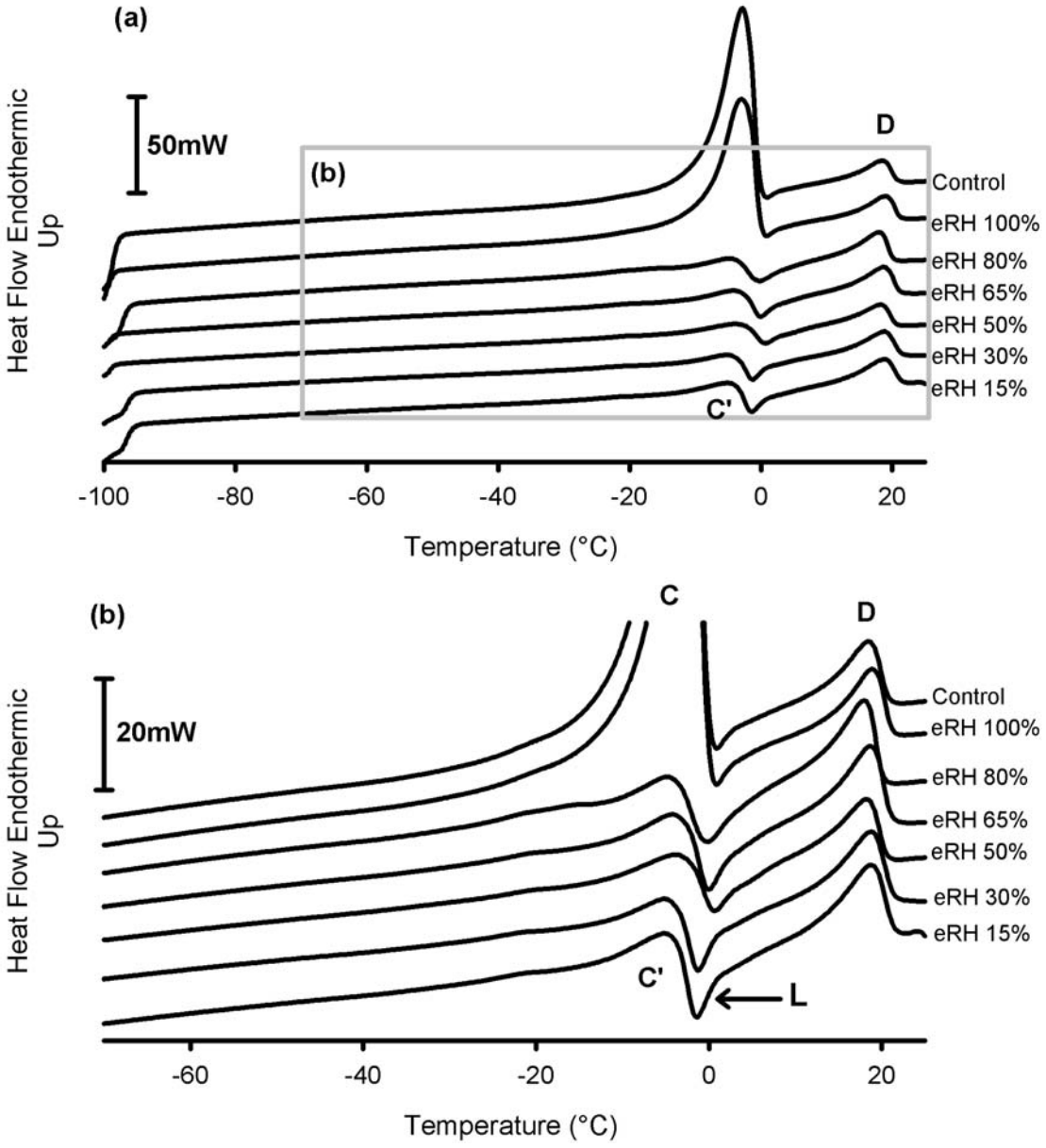

Figure 4.14 DSC rewarming thermograms of cotyledon samples of *Dysoxylum spectabile* equilibrated at different RH showing water and lipid melting traces (a) with a enlarged from boxed area (b). Samples were held at 25ºC for 1 min cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

Similar observation was made during the cooling phase. Peak A belongs to ice crystallization and only occurred for control and 100% eRH (Figure 4.15). Peak B belongs to lipid crystallization and occurred in every sample and there was no significant difference in peak size and enthalpy for these samples. (Figure 4.15).

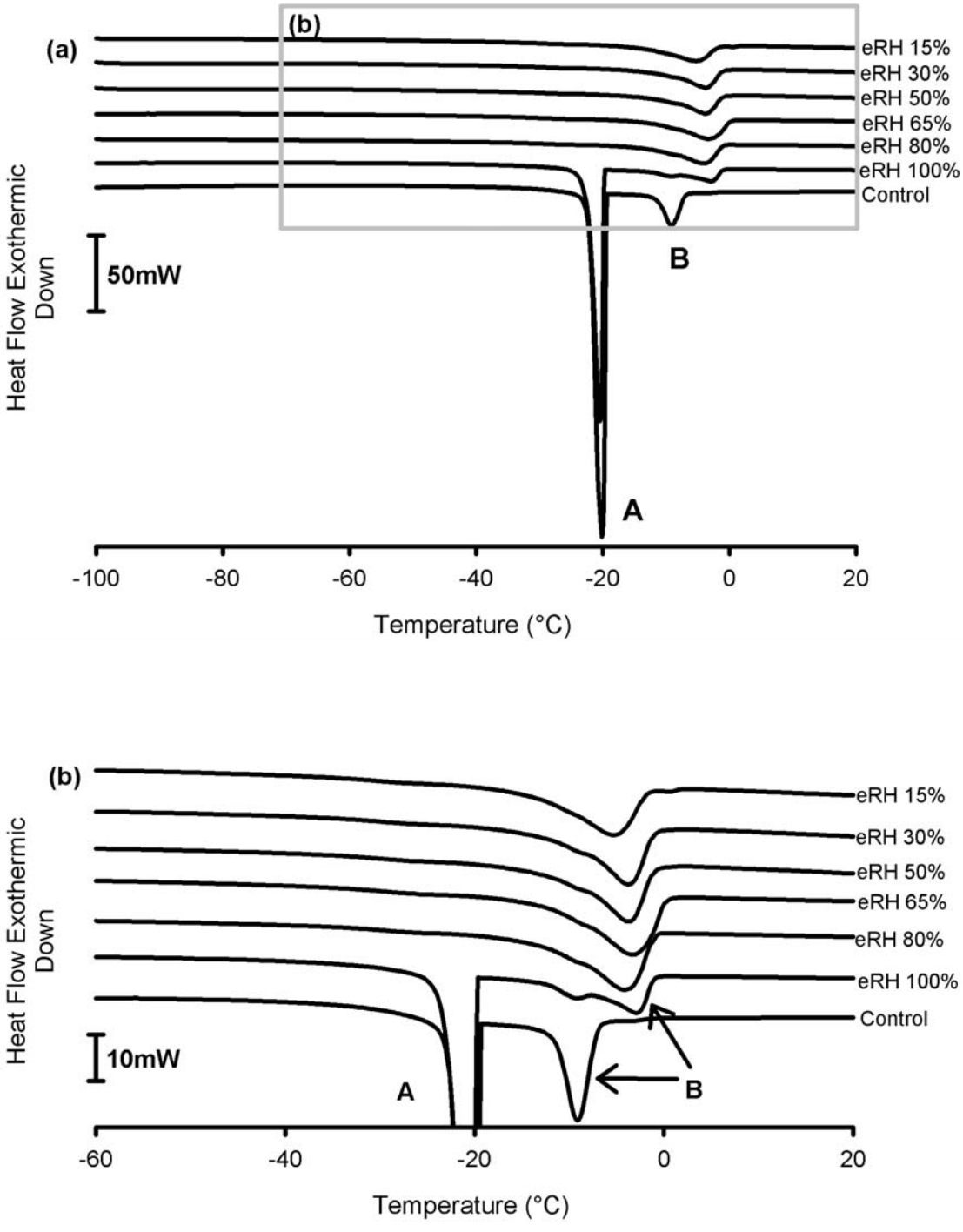

Figure 4.15 DSC cooling thermograms of seed samples of *Dysoxylum spectabile* equilibrated at different RH showing water and lipid phase transitions (a) with a enlarged from boxed area (b). Samples were held at 25ºC for 1 min cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

Rapid drying methods

During the warming phases, endothermal events were observed in embryonic axes dehydrated for up to 3.5 hr, indicating the occurrence of ice melting. The melt onset and peak temperatures decreased with a concomitant reduction in melt enthalpies as desiccation duration increased (Table 4.11 and Figure 4.16, a). After 4h desiccation a Tg with a heat capacity of 0.4 ± 0.14 J. $g^{*}°C^{-1}$ (melting enthalpy (Table 4.11 and Figure 4.16, b) was observed. No endotherms were detected after 5 hr desiccation (Figure 4.16, b).

Table 4.11 Rewarming thermodynamic properties of embryos of *Dysoxylum spectabile* after different drying periods.

Drying time (hr)	Thermal Event	Onset (°C)	Mid-point (°C)	Endpoint (°C)	Enthalpy ($J.g^{-1}$)	Heat Capacity (J. $g^{*}°C^{-1}$)
Control	Ice Melt	-8.1 ± 0.29	-0.4 ± 0.06	3.8 ± 0.94	203.3 ± 0.32	
1	Ice Melt	-10.7 ± 0.39	-2.2 ± 0.15	1.1 ± 0.54	149.5 ± 2.47	
2	Ice Melt	-13.2 ± 0.72	-5.7 ± 1.26	-1.3 ± 1.49	62.8 ± 12.86	
3	Ice Melt	-15.7 ± 1.08	-6.0 ± 0.36	-1.4 ± 0.94	62.1 ± 7.62	
3.5	Ice Melt	-17.6 ± 0.90	-7.2 ± 0.35	-2.5 ± 0.38	22.6 ± 2.83	
4	Tg	-47.1 ± 0.42	-36.4 ± 2.10	-23.8 ± 5.08		0.4 ± 0.14
4.5	Ice Melt	-19.4 ± 1.84	-10.9 ± 2.01	-8.2 ± 1.76	3.7 ± 1.82	
	Tg*	-47.33	-36.86	-32.34		0.52
5	θ	NA	NA	NA	NA	NA
5.5	θ	NA	NA	NA	NA	NA
6	θ	NA	NA	NA	NA	NA

Data points are means of three replicates (± standard error).

* = event occurs in one replicate out of three; θ = stable profile, no thermal event detected in all replicates.

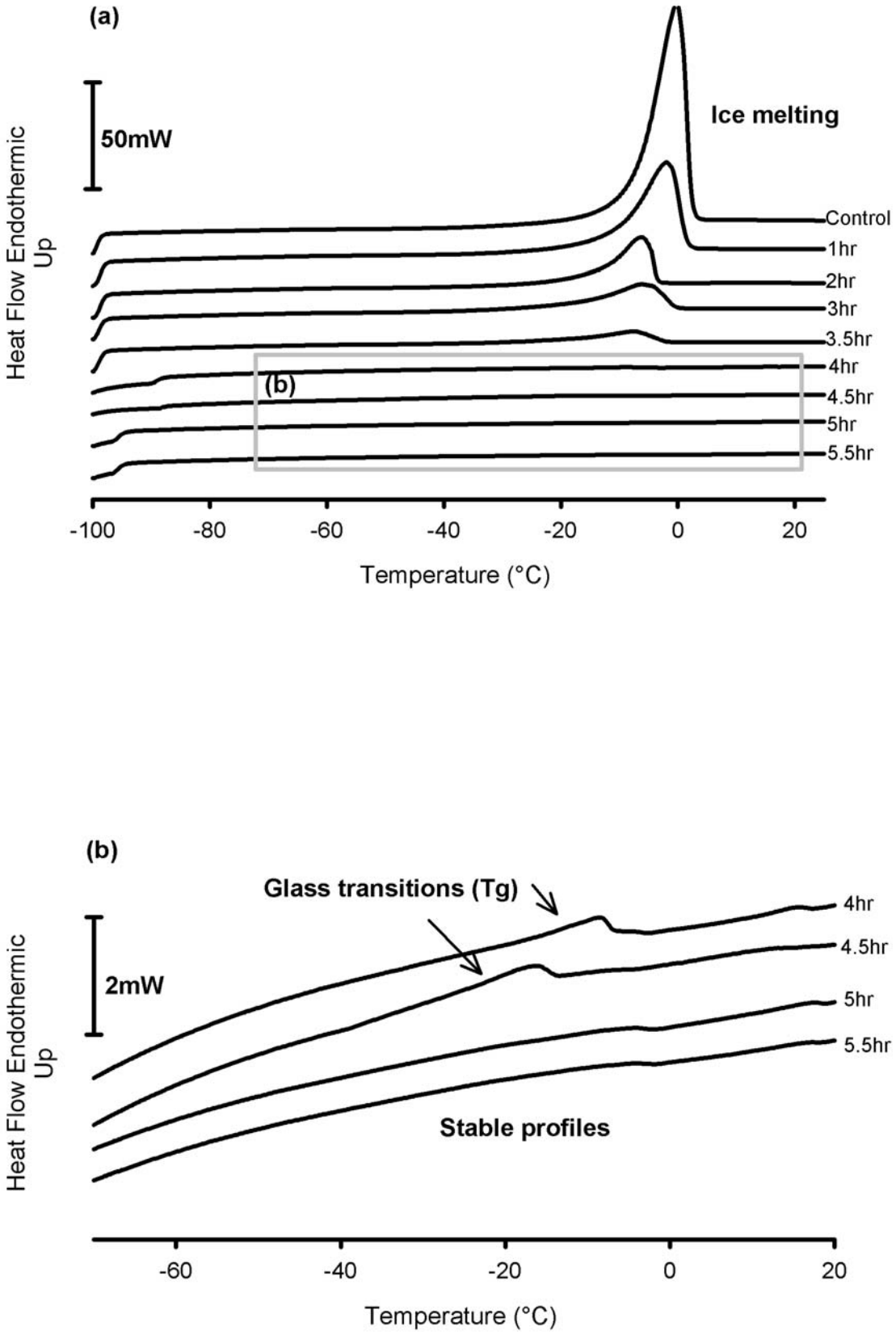

Figure 4.16 DSC warming thermograms for embryonic axes of *Dysoxylum spectabile* after different desiccation periods showing ice meting peaks for higher moisture contents (a) and glass transitions for 4 and 4.5 hr drying flowed by stable thermal profiles at lower MCs (b). Samples were held at 25ºC for 1 min, cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

The proportion of osmotically inactive water increased considerably as the drying periods increased (Figure 4.17). Before drying embryonic axes the osmotically inactive water fraction comprised ca. 18% of the total water content, whereas following dehydration for 3.5 hr it had increased to ca. 60% and after 4 hr desiccation it had reached 96%. Table 4.12 shows the water composition in embryonic axes resulting from the desiccation treatments.

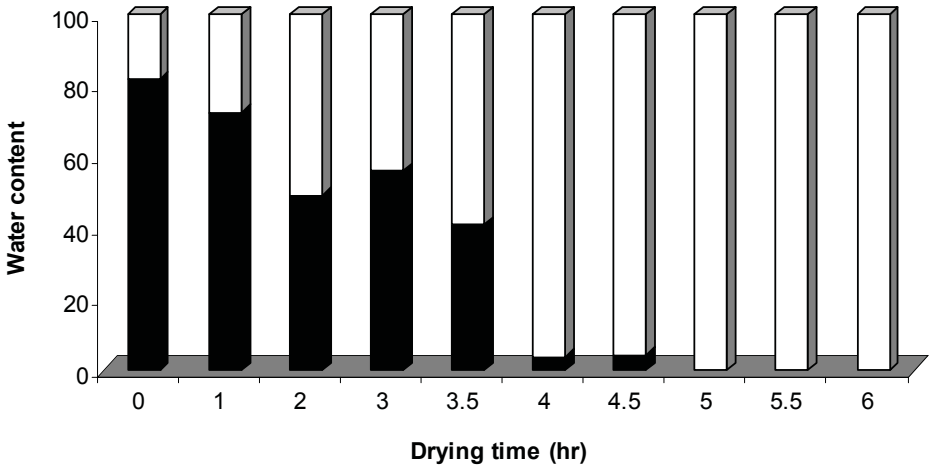

Figure 4.17 Summary profiles of osmotically active (■) and inactive water (□) contents calculated as percentage of total water content of embryonic axes of *Dysoxylum spectabile* following dehydration treatment.

Table 4.12 Total, osmotically active and osmotically inactive water contents of embryos of *Dysoxylum spectabile* following various dehydration treatments.

Treatment	% Water content (FW)	Total water content (g H_20 [g DW]$^{-1}$)	Osmotically active water content[1] (g H_20 [g DW]$^{-1}$)	Osmotically inactive water content [2] (g H_20 [g DW]$^{-1}$)
Control	74.4 ± 0.51	2.90 ± 0.078	2.37 ± 0.046	0.53 ± 0.033
1	61.5 ± 0.94	1.60 ± 0.065	1.16 ± 0.049	0.44 ± 0.017
2	38.3 ± 3.69	0.6. ± 0.942	0.31 ± 0.079	0.32 ± 0.016
3	33.2 ± 1.86	0.50 ± 0.042	0.28 ± 0.039	0.22 ± 0.031
3.5	24.1 ± 1.47	0.32 ± 0.026	0.09 ± 0.013	0.13 ± 0.006
4	12.3 ± 0.63	0.14 ± 0.008	0.005 ± 0.0026	0.13 ± 0.006
4.5	12.1 ± 1.11	0.14 ± 0.014	0.005 ± 0.0027	0.11 ± 0.001
5	9.5 ± 0.12	0.11 ± 0.001		0.11 ± 0.001
5.5	9.1 ± 0.34	0.10 ± 0.004		0.10 ± 0.004
6	8.0 ± 0.69	0.09 ± 0.008		0.09 ± 0.008

Data points are means of three replicates (± standard error).

DW= Dry weight.

Encapsulation dehydration methods

Melt onset and mid-temperatures and enthalpy of beads were markedly decreased after desiccation (Table 4.13). The melt enthalpy for control beads was 258 ± 1.59 $J.g^{-1}$. As the bead water content decreased with 2h dehydration, the melt enthalpy declined to 121 ± 16.0 $J.g^{-1}$ and thereafter to 13.2 ± 1.86 Jg^{-1} following 4.5h drying. After 5h desiccation a Tg with a heat capacity of 0.3-0.4 J. $g^{*}°C^{-1}$ was observed during rewarming of beads containing embryonic axes, although a small melting phenomena were also observed (Table 4.13 and Figure 4.18).

Table 4.13 Rewarming thermodynamic properties of alginate-encapsulated embryos of *Dysoxylum spectabile* after different drying periods.

Drying time (hr)	Thermal event	Onset (°C)	Mid-point (°C)	Endpoint (°C)	Enthalpy ($J.g^{-1}$)	Heat Capacity (J. $g^{*}°C^{-1}$)
Beads	Ice Melt	-5.6 ± 0.16	2.2 ± 0.25	6.9 ± 0.37	261.4 ± 1.89	-
Control	Ice Melt	-6.0 ± 0.26	1.3 ± 0.09	6.0 ± 0.02	258.7 ± 1.59	-
1	Ice Melt	-7.1 ± 0.83	0.4 ± 0.91	5.5 ± 1.26	220.2 ± 3.80	-
2	Ice Melt	-14.7 ± 1.57	-1.1 ± 0.58	5.4 ± 0.09	120.9 ± 16.03	-
3	Ice Melt	-14.6 ± 0.53	-3.6 ± 0.25	1.1 ± 0.58	22.2 ± 3.05	-
3.5	Ice Melt	-17.1 ± 4.73	-4.3 ± 2.57	-0.6 ± 2.22	13.5 ± 2.20	-
4	Ice Melt	-16.6 ± 0.40	-5.8 ± 1.01	0.9 ± 0.48	21.1 ± 1.25	-
4.5	Ice Melt	-18.2 ± 2.39	-7.0 ± 1.70	-3.0 ± 2.16	13.2 ± 1.86	-
5	Ice Melt*	-24.72	-11.97	-7.66	1.92	
	Tg	-45.8 ± 1.91	-36.5 ± 0.99	-23.7 ± 1.36		0.3 ± 0.03
5.5	Ice Melt	-24.5 ± 3.39	-12.9 ± 2.08	-9.2 ± 1.57	0.9 ± 0.29	
	Tg	-46.4 ± 0.19	-34.8 ± 1.22	-24.0 ± 1.62		0.4 ± 0.08
6	Ice Melt	-21.6 ± 0.09	-11.3 ± 1.21	-8.7 ± 1.22	0.9 ± 0.08	
	Tg	-46.2 ± 0.97	-34.4 ± 0.68	-23.8 ± 1.92		0.4 ± 0.01

Data points are means of three replicates (± standard error).

* = event occurred in one replicate out of three.

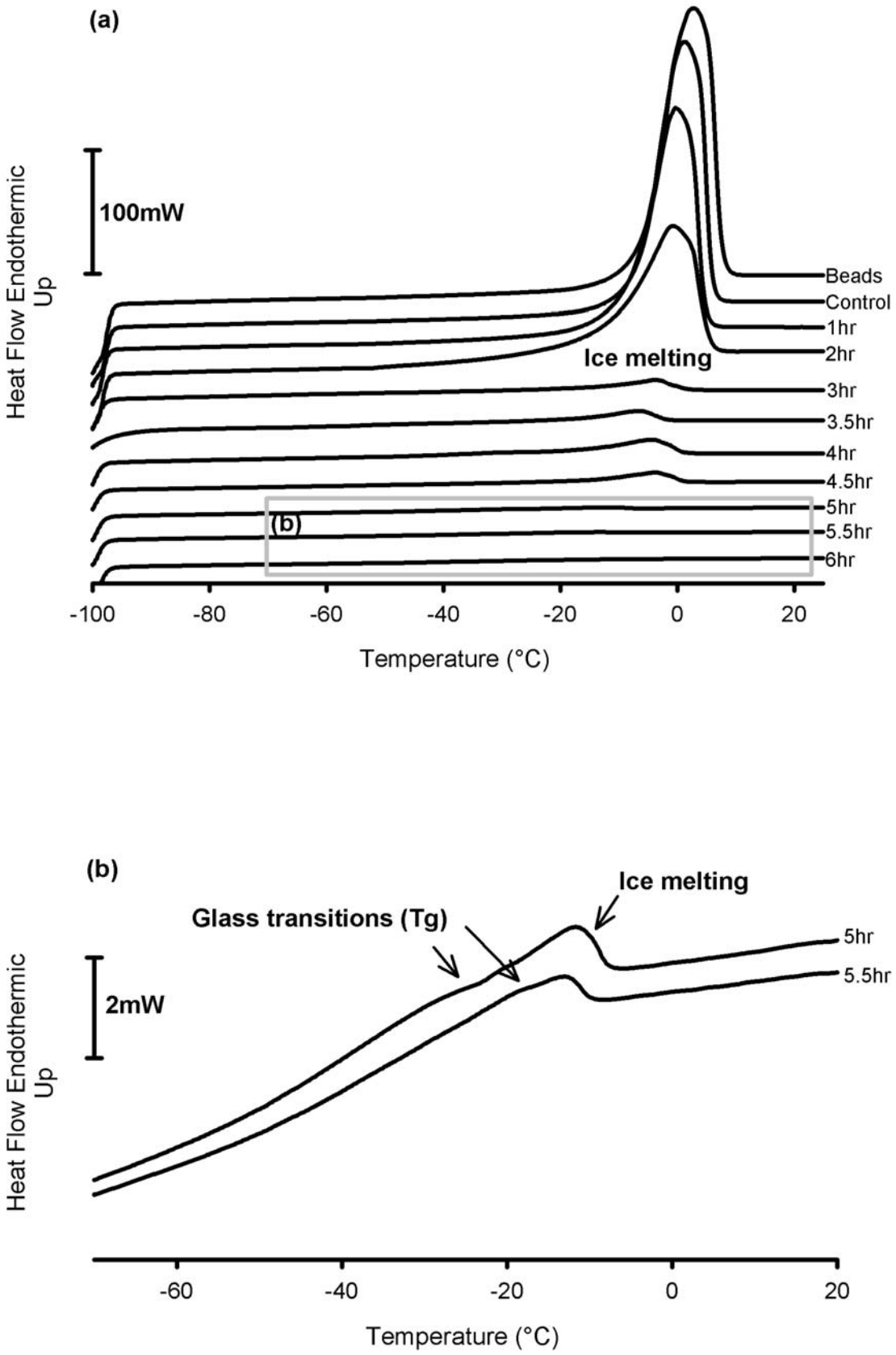

Figure 4.18 DSC warming thermograms for alginate-encapsulated embryos of *D. spectabile* after different desiccation periods showing ice melting peaks for higher moisture contents (a) and glass transitions with ice melting peaks for 5 and 5.5 hr drying (b). Samples were held at 25 ºC for 1 min, cooled to –100ºC, held for 1 min and rewarmed to 25ºC at a rate of ±10ºC per min.

The proportion of osmotically inactive water as compared to the total water content was ca. 17% for control beads. This increased to 40% and 97% after 2h and 5h desiccation, respectively (Figure 4.19 and Table 4.14). Table 4.14 shows the water composition in embryonic axes resulting from the desiccation treatments.

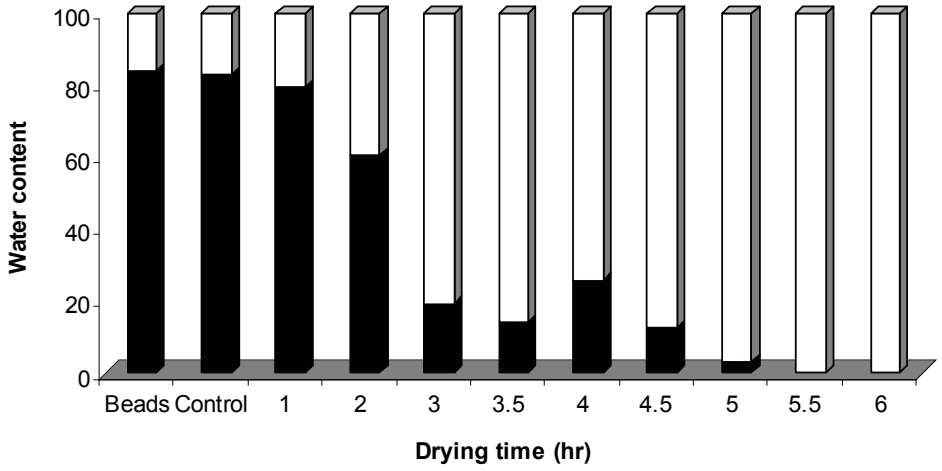

Figure 4.19 Summary profiles of osmotically active (■) and inactive water (□) contents calculated as percentage of total water content of alginate-encapsulated embryos of *Dysoxylum spectabile* after different dehydration treatments.

Table 4.14 Total, osmotically active and osmotically inactive water contents of encapsulated embryos of *Dysoxylum spectabile* following various dehydration treatments.

Treatment	% Water content (FW)	Total water content ($g\ H_20\ [g\ DW]^{-1}$)	Osmotically active water content[1] ($g\ H_20\ [g\ DW]^{-1}$)	Osmotically inactive water content [2] ($g\ H_20\ [g\ DW]^{-1}$)
Control	92.6 ±1.55	13.60 ± 2.538	11.43 ± 2.046	2.16 ± 0.492
0	93.0 ± 0.06	13.38 ± 0.118	11.12 ± 0.123	2.26 ± 0.070
1	82.9 ± 0.35	4.85 ± 0.116	3.85 ± 0.092	1.00 ± 0.084
2	60.6 ± 4.41	1.60 ± 0.286	0.97 ± 0.222	0.63 ± 0.067
3	34.1 ± 2.56	0.52 ± 0.060	0.10 ± 0.017	0.42 ± 0.045
3.5	26.2 ± 0.79	0.35 ± 0.015	0.05 ± 0.010	0.31 ± 0.005
4	23.7 ± 0.92	0.31 ± 0.158	0.08 ± 0.010	0.23 ± 0.011
4.5	19.9 ± 0.55	0.25 ± 0.009	0.03 ± 0.016	0.21 ± 0.008
5	18.1 ± 0.64	0.22 ± 0.010	0.006	0.22 ± 0.007
5.5	18.3 ± 0.32	0.22 ± 0.005	-	0.22 ± 0.005
6	16.7 ± 0.73	0.20 ± 0.010	-	0.20 ± 0.010

Data points are meansof three replicates (± standard error).

DW = Dry weight.

4.3.3.4 Cryopreservation of embryonic axes

Rapid desiccation protocol

Survival of non-desiccated embryos was over 90%. However, survival decreased to 10-13% after dehydration for 3.5hr, 3.75hr, 4hr and 4.25hr (Table 4.15). After dehydration and cryopreservation, germination of embryonic axes was only 0–10 %. None of the desiccated and cryopreserved embryos developed further into normal seedlings: the root and shoots had elongated, and shoot area had showed green colouring, but, no further seedling development was observed.

Table 4.15 Changes in moisture content, survival of desiccated embryonic axes of *Dysoxylum spectabile* before and after 24hr cryo-storage.

Drying time (hr)	Moisture content (%)	Survival (%)	
		Before cryo	After cryo
0	70.3a	93a	0a
3.5	20.8b	13b	10a
3.75	17.9c	13b	7a
4	16.4d	13b	7a
4.25	15.2d	10b	0a

Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Encapsulation-dehydration protocol

Approximately 50% of embryonic axes survived dehydration up to 20% water content i.e. 4.75h desiccation (Table 4.16). However, survival decreased to 23% after dehydration for 5.25hr (MC=17.3%). Following dehydration and cryopreservation, only 13–20% embryos survived. None of the desiccated and cryopreserved embryos developed further into normal seedlings: the root and shoots had elongated, and shoot area had showed green colouring, but, no further seedling development was observed.

Table 4.16 Changes in moisture content, survival of desiccated alginate encapsulated embryos of *Dysoxylum spectabile* before and after 24hr cryo-storage.

Drying time (hr)	Moisture content (%)	Survival (%)	
		Before cryo	After cryo
0	93.0a	90a	0b
4.5	22.6b	50b	20a
4.75	19.7c	47b	20a
5	18.0d	33c	20a
5.25	17.3d	23c	13a

Numbers followed by the same letter are not significantly different (LSD, $p \leq 0.05$).

Vitrification protocol

Sucrose pre-culture

The effect of sucrose pre-culture was tested on the germinability of the embryos before cryopreservation. There was a significant difference in the pre-cryopreservation germination of the embryos which were pre-cultured with sucrose compared to those which were not (Figure 4.20). Germination percentage decreased from 90% for 0 day pre-culture to about 50% for 1-3 day pre-culture. However, there was no significant difference in germination for treatment with different preculture time (1, 2 and 3 days) before cryopreservation (Figure 4.20).

For cryopreserved embryonic axes, no survival was achieved in the control axes. However, a significant effect of the sucrose pre-treatment was observed with an increase of post-cryopreservation survival percentage to about 20 % following 1, 2 and 3 days sucrose pre-culture treatments.

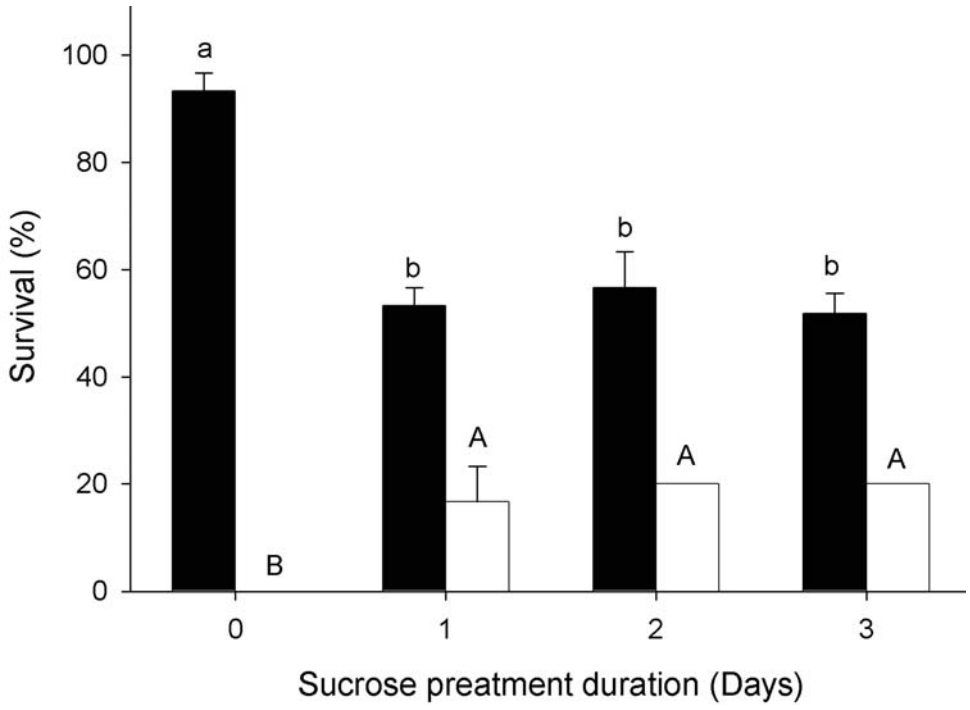

Figure 4.20 of sucrose preculture on survival rate (%) of *Dysoxylum spectabile* before (■) and after 24hr cryo-storage (□). Vertical bars represent the SE and columns with same shade marked with the same letter are not significantly different (LSD, $p \leq 0.05$).

PVS vitrification

The survival percentage decreased significantly as exposure time to PVS2 increased from 0 to 90 min except for the 1 day preculture duration [Figure 4.21, (a), (b), (c)]. No significant difference was observed among PVS3 treatments for survival for 1 and 2 days sucrose pre-culture treatment [Figure 4.21, (d) and (e)]. However, after 3 days of pre-culture with 0.75 M sucrose, the survival of embryonic axes was reduced from 52% to 33% [Figure 4.21, (f)].

There was a significant difference in the survival of embryos before and after cryopreservation. The survival of control (non-frozen) embryos ranged from 33 to 63%, while, after freezing, survival of embryos ranged from 7 to20% (Figure 4.21).

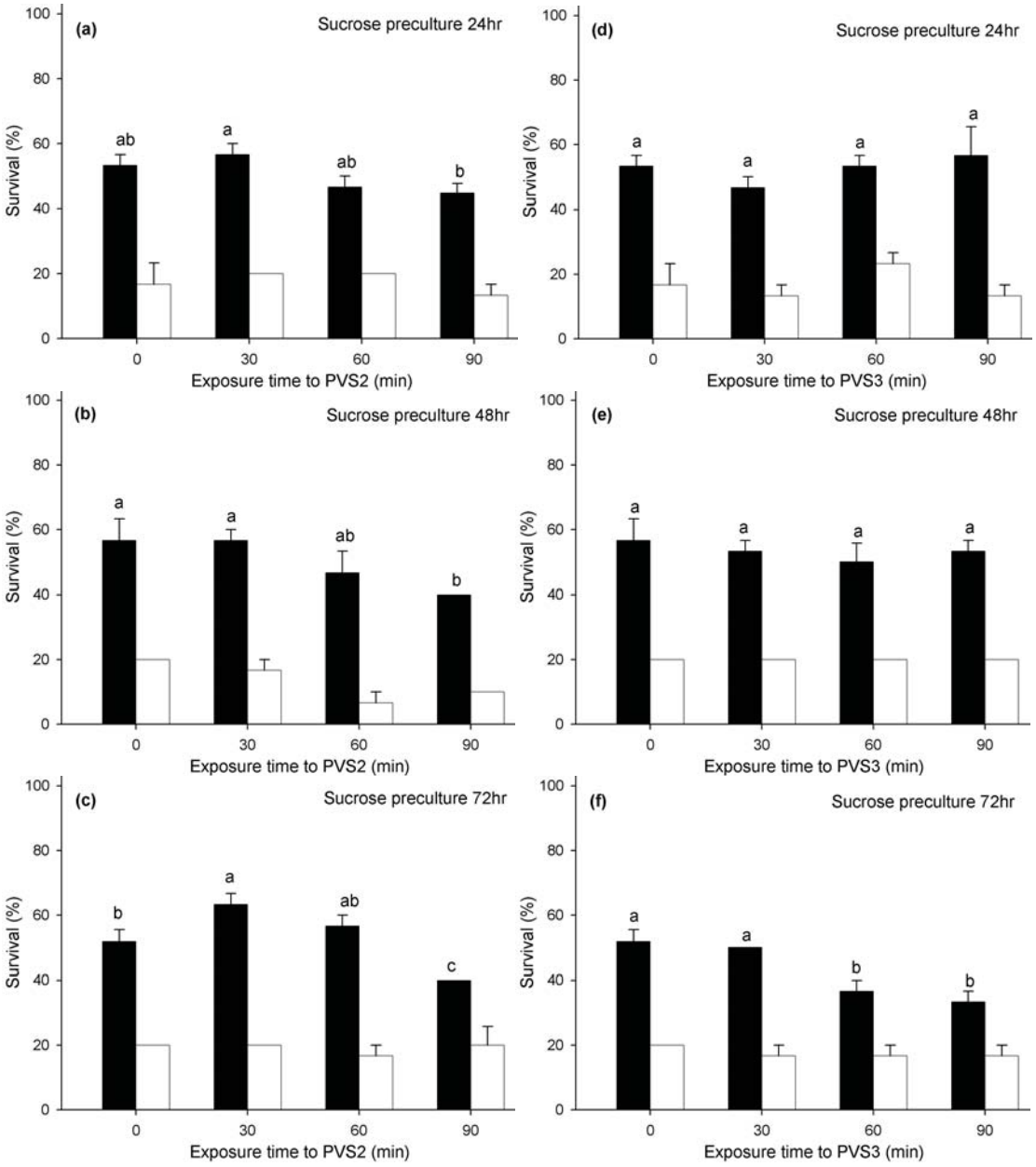

Figure 4.21 Effects of sucrose preculture and exposure time to PVS2 and PVS3 on survival rate (%) of *Dysoxylum spectabile* before (■) and after 24hr cryo-storage (□). Vertical bars represent the SEM and values for each preculture duration marked by the same letter are not significantly different (LSD, $p \leq 0.05$) dependent on the PVS2 and PVS3 exposure time.

4.3.4 Discussion

4.3.4.1 Seed developmental stage

Developmental stage is a very important factor in developing a successful cryopreservation protocol as the seed or embryo axes must be capable of acquiring desiccation and freezing tolerance and be able to regenerate plants after cryogenic storage (Engelmann, 2000, 2004). Thus, linking desiccation tolerance with the various seed developmental stages may be an effective approach for cryopreservation (Fu, Jin, Peng, & Xia, 1994). It has been suggested that recalcitrant seeds at different developmental stages differ in desiccation sensitivity (Chandel, Chaudhury, Radhamani, & Malik, 1995). The decrease in water content towards the end of development is accompanied by an increase in desiccation tolerance in most recalcitrant seeds (Pammenter & Berjak, 1999). Walters (2000) suggested that dry matter accumulation is an important feature in conferring tolerance and most of the tolerance in developing embryos appears to be acquired during dry matter accumulation.

An effect of developmental status on seed desiccation tolerance is well known for many recalcitrant species. For example, desiccation tolerance has been shown to increase with development, albeit not to the same extent as orthodox species, for seeds of *Hopea hainanensis* (Lan, et al., 2007), *Acer pseudoplatanus* (Hong & Ellis, 1990), *Aesculus hippocastanum* (Farrant & Walters, 1998; Tompsett & Pritchard, 1993), *Camellia sinensis* (Berjak, et al., 1993) and *Landolphia kirkii* (Pammenter, et al., 1991). In this study, *D. spectabile* seeds and embryonic axes reached physiological maturity at 23 May (around 50% seed moisture content) by which time the seed attains its maximum fresh weight and full viability. Though the dry weight is higher on 26 June than on 23 May (Figure 4.6), the decline in viability from 100% to 93% between 23 May and 26 June supports 23 May as the date at which physiological maturity is reached. Desiccation tolerance of seeds was maximum at this developmental stage. Similarly, the acquisition of desiccation tolerance by oil palm immature embryos was associated with an accumulation of dry matter (Aberlenc-Bertossi, Chabrillange, Corbineau, & Duval, 2003). However, it appears that not all recalcitrant seeds behave in the same manner. Once they have become capable of germination, the desiccation tolerance of seeds of *Avicennia marina* did not change during further development (Farrant, Berjak, & Pammenter, 1993). In other species for instance, *Clausena lansium, Litchi chinensis*

seeds (Fu, et al., 1994) and *Archontophoenix alexandrae* (Shao, et al., 2009), survival decreased as development continued after physiological maturation. This was also observed in *D. spectabile*.

In the present study, a slight decrease in seed moisture content increased the germination of immature seeds. This increase in germination was observed at relatively high moisture content (c. 50%) and may be due to the continuation of the embryo development as the seed dried from 60% to 50% moisture rather than maturation drying which is an essential part of seed development in orthodox seed (Bewley & Black, 1994). This phenomenon has also been observed in other recalcitrant species., e.g. *Acer pseudoplatanus* (Hong & Ellis, 1990), *Aesculus hippocastanum* (Tompsett & Pritchard, 1998) and *Laurus nobilis* (Konstantinidou, Takos, & Merou, 2008) and as well as in orthodox seeds (Hong & Ellis, 1990). The lower germination percentage (30%) of immature seeds shown in this study (Figure 4.8) was most likely a function of fungal infection during germination in the sand, which was a high moisture environment.

4.3.4.2 Desiccation sensitivity

Drying rate is also an important factor in determining the dehydration tolerance of seeds (Bonner, 1996; Liang & Sun, 2000; Pammenter & Berjak, 1999; Song, Patricia, Pammenter, Ntuli, & Fu, 2003). Generally fast drying has been found to permit recalcitrant seeds or excised axes to survive to lower water contents, and to improve the survival after cryopreservation (Potts & Lumpkin, 1997; Pritchard & Manger, 1998; Shao, et al., 2009; Song, et al., 2003).

Dehydration at different rates significantly influenced the desiccation response of *D. spectabile* seeds and embryonic axes; the more rapidly the seeds or embryonic axes were dehydrated, the lower was the water content to which the seeds or embryonic axes could be dried before viability was lost. When seed dried slowly, no germination was retained in desiccated seeds and embryonic axes even at relatively high moisture contents. These results are in agreement with those found in seeds of *Landolphia kirkii* (Berjak, Farrant, Mycock, & Pammenter, 1990) and excised axes of *Trichilia dregeana*,

Castanospermum australe, *Camellia sinensis* (Pammenter, Berjak, & Walters, 2000) and *Clausena lansium* (Huang, Song, & Wu, 2009), *Artocarpus heterophyllus* (Wesley-Smith, Pammenter, et al., 2001), *Hopea hainanensis* (Lan, et al., 2007), *Mangifera persiciformis* (Tang, et al., 2008). However, there are contradictory reports on the effect of drying rate on survival of axes excised from other recalcitrant seeds e.g. *Shorea chinensis* (Yan, et al., 2007), *Aquilaria agallocha* (Kundu & Kachari, 2000) and *Theobroma cacao* (Liang & Sun, 2000). Liang and Sun (2002), identified an optimal drying rate for cocoa (*Theobroma cacao*) and ginkgo (*Ginkgo biloba*) embryonic axes, at which maximum desiccation tolerance could be achieved.

In this study, the rate of germination was slowing before any loss of germination was observed (Table 4.9). This suggests that even a slight reduction from 68.3% to 63.6% in embryo axis MC resulted in a deterioration of the seed (loss of vigour) as evidenced by slower rate of germination. By an axis MC of 48.9% even though 38% of the seed still produced normal seedlings they had also deteriorated. This assumes that the 8.4 days includes only normal seedlings - T_{50} is based on radicle emergence that went on to develop normal seedlings.

The decrease in survival observed in this study was not only a direct response to dehydration. In the slow dehydration experiment, the water contents of excised embryonic axes did not change when equilibrated from their initial moisture content to 100% RH for 4 days, but viability decreased from 93% to 67%. Whereas, in the seeds the water content increased significantly during the 4 days exposure to 100% RH, and decreased viability to 43%. Similar results have been reported for *Clausena lansium* (Huang, et al., 2009). From this perspective, desiccation damage is a time dependent process – an ageing phenomenon (Walters, Farrant, Pammenter, Berjak, & Black, 2002). Therefore, dehydration time cannot be ignored in the dehydration experiments (Huang, et al., 2009; Pammenter & Berjak, 1999).

In this study, the MC of excised embryonic axes could be reduced to lower MC more rapidly than that of whole seeds. This response is likely to be due to smaller size and more uniform shape of the embryos compared to the rest of the seed. When drying the intact seed, even when the seed water content has been reduced to a low level, that of

the embryo's remained high (Table 4.9). This differential in water content across tissues means that in seeds dried to a relatively low MC, lethal ice crystals will still form in the embryo (high water content) when the whole seed is frozen in liquid nitrogen. The measurement of whole seeds to determine the moisture content in the embryo axis (which is critical to seed survival when the seed is placed in liquid nitrogen) is misleading because of the high ratio of non-embryo axis tissues relative to the embryo axis. A similar difference in water content between whole seed and embryonic axes has been reported in *Quercus faginea* (Gonzalez-Benito & Perez-Ruiz, 1992) and *Archontophoenix alexandrae* (Shao, et al., 2009). These species also failed to survive cryopreservation despite low seed water content for similar reasons. This and the enhanced survival of excised embryos to lower MC with rapid dehydration indicates that use of embryonic tissue rather than whole seeds is appropriate for long-term cryopreservation of *D. spectabile* germplasm.

4.3.4.3 Thermal analysis

Block (2003) suggests that the success of cryopreservation of plant germplasm mainly depends on lowering the frozen water content of the sample to as low a level as is possible, without losing viability. Dehydration is considered a critical step for maintaining cell viability and integrity, and for preventing the formation of ice crystals (Engelmann, 2004; Farrant, Pammenter, & Berjak, 1986; Yamuna, et al., 2007). The high moisture freezing limit (HMFL) where freezing injury occurs varies depending on the species, e.g. 9.6% for sesame to 28.5% for bean (Stanwood, 1983). There is therefore a need to assess the optimum water content to provide optimal conditions for vitrification and reduce the possibility of glass destabilisation during rewarming and to ensure maximum viability of cryopreserved plant germplasm (Block, 2003; Shao, et al., 2009).

Corroborating survival data with thermal stability studies of glass transitions has important implications for cryopreservation improvement and especially the manipulation of water status with respect to germplasm that has low desiccation tolerance (Sherlock, Block, & Benson, 2005). Thermal analyses of plant materials using

differential scanning calorimetry (DSC) demonstrates a strong relationship between the presence of freezable water or melting transitions and where damage at subfreezing temperatures is likely to occur (Benson, et al., 1996; Dereuddre & Kaminski, 1992; Dumet, Block, Worland, Reed, & Benson, 2000; Kim, Yoon, Kim, Engelmann, & Cho, 2005; Martínez, Ángeles Revilla, Espina, & García, 2000; Sherlock, et al., 2005; Vertucci, 1989). Evidence of water transitions (i.e., freezing and melting) are observed in seeds, naked shoot tips and shoot tips encapsulated in calcium alginate beads that contain more than 0.25–0.4 g H_2Og dm^{-1} (dm; dry mass). Plant organs that survive liquid nitrogen temperatures usually contain this amount of water or less (Volk & Walters, 2006).

DSC thermal analysis in this study showed that excised embryonic axes and alginate beads dried to 12% and 18% (fresh weight basis; 0.14 and 0.22 g H_2O. g DW^{-1}), respectively form stable glasses that do not form ice crystals on cooling and melting upon rewarming, although this level of MC was accompanied by loss of viability. Too extreme desiccation of naked embryonic axes (moisture content to less than 10%) resulted in very low recovery (10%). The desiccation time that will maximise survival is 4 hr for excised naked embryonic axes and 5 hr for alginate encapsulated embryos. Glass transitions were noted at these desiccation times suggesting that these moisture contents (give values) were sufficient to remove freezable water from the excised embryonic axes.

The optimum MC level of excised embryonic axes found in this study is very close to those previously reported for optimum desiccation levels before cryopreservation. Chandel, et al. (1995) reported that in *Carmellia sinensis* and *Artocarpus heterophyllus* the axes with 10% and 14% water content, respectively, could be cryopreserved. Reduction in sample viability and growth due to dehydration was also observed in *Cocos nucifera* (Assy-Bah & Engelmann, 1992), *Veitchia merrillii* and *Howea fosteriana* (Chin, Krishnapillay, & Alang, 1988). These data suggest the importance of appropriate dehydration and rehydration protocols for embryos during the cryopreservation process (Steinmacher, et al., 2007).

Several cryopreservation studies (Engelmann, 2000; Jekkel, et al., 1998; Martínez, et al., 1999; Steinmacher, et al., 2007) using the encapsulation-dehydration technique have indicated that the optimum water content of the encapsulated samples ranges from 15% to 25% (fresh weight basis) for optimum survival. Again this data is consistent with the observations in this study. The survival of alginate encapsulated embryos remained 20% while that of naked embryonic axes was 10% at this moisture level. This means that the water content of alginate encapsulated embryos can be reduced to a much lower level than that of the excised embryonic axes while maintaining similar or slightly higher survival. The use of alginate encapsulated embryos/embryonic axes is also an advantage as alginate protects tissues from injury during dehydration and freeze–thaw treatments, and alleviates excessively rapid dehydration of the samples (Benson, 1999; Engelmann, 1997b; Fabre & Dereuddre, 1990; Grospietsch, Stodulková, & Zámecnık, 1999). The success of the method is largely dependent upon the plant's desiccation tolerance and the ability to circumvent ice nucleation during cooling and warming (Gupta & Reed, 2006).

DSC analysis of *D. spectabile* cotyledons also showed lipid crystallization (Figure 4.12, peak B) and lipid melting peaks (Figure 4.14, peak D) during cooling and warming phases, respectively, without any significant difference in enthalpy and onset temperature at seed moisture levels below 15% (RH ≤ 80%). The lipid crystallization and melting peaks were obvious only at MC 15% and below. Above this MC, it is likely that the water phase transitions overlap with lipid phase transitions.

For treatments that recorded post-cryopreservation survival there was no freezable water detected by DSC analysis. This present result offers additional evidence that lipid-rich seeds do not withstand the presence of freezable water in their tissues during the cooling/thawing process. Vertucci (1989) suggested that, in oily seeds, the interaction between storage lipids and water somehow promoted formation of ice crystals large enough to cause lethal damage. *D. spectabile* seeds contain 29.2% oil, which consists Palmitic (52.0%), linoleic (40.6%), Stearic (3.2%) and Linolenic (2.8%) and therefore can be considered an oilseed (Brooker, 1960).

4.3.4.4 Cryopreservation of embryonic axes

This study was designed to develop cryopreservation protocols for embryonic axes of *D. spectabile* using commonly used protocols; rapid desiccation, encapsulation dehydration and PVS2 and PVS3 vitrification. Post-cryopreservation survival of embryonic axes was low (20%). Although post-cryopreservation survival of desiccation encapsulated embryonic axes was higher than those of naked axes, no embryonic axes developed into normal seedlings. While radicle elongation occurred, shoot development did not. This result suggests that the shoot meristems of *D. spectabile* are more sensitive to desiccation and freezing than root meristems.

Farrant, et al. (1986), Goveia, et al. (2004), Kioko et al. (1998) and Pence (1992) have also reported a lack of/or poor capacity for shoot formation by desiccated axes before and after cryopreservation. In many cases, axes surviving after cryostorage produced roots or callus, but often did not form plantlets (Gonzalez-Benito, Prieto, Herradon, & Martin, 2002). Peran et al. (2006) pointed that this could be for one or both of the two reasons; i) the apical meristem of the embryonic axes of many species lacks a protective covering layer, and during the partial drying treatment may dry to a lower water concentration than other parts of the axis and consequently suffer more desiccation damage. This has been shown to be the case for axes of *Trichilia emetica* (Kioko, Berjak, & Pammenter, 2006), *Araucaria hunsteinii* (Pritchard & Prendergast, 1986), and *Quercus robur* (Poulsen, 1992) in which shoot meristems were more sensitive to desiccation than root meristems. However, the response is not consistent, e.g. in *Aesculus hippocastanum* roots are more sensitive to desiccation (Pence, 1992); ii) the process of excision may inflict mechanical damage on the meristem. This physical damage has been reported for *Trichilia dregeana* (Goveia, et al., 2004). In contrast to excised embryos, encapsulated embryos are protected during dehydration (Verleysen, et al., 2005), but in this study, no shoot growth was observed even in encapsulated embryos. There are reports in the literature that encapsulated - explants showed lower rate of regrowth, later recovery of growth after cryopreservation and a longer dehydration procedure than vitrification (Sakai, et al., 2000).

Over the past decade, the vitrification method has improved survival of cryopreserved cells or other tissues after cryopreservation (Turner, Senaratna, Bunn, et al., 2001;

Turner, Senaratna, Touchell, et al., 2001). In order to successfully cryopreserve tissues using vitrification the dehydration/ cryoprotective procedures need to be carefully controlled so that the highly concentrated vitrification solution (PVS solution) do not cause injury by chemical toxicity or excess osmotic stresses (Nishizawa, et al., 1993; Rall, 1987). Many different pretreatment techniques have been developed to increase dry mass, stabilize proteins and membranes, decrease freezing point, and enhance the glass forming tendency of the remaining solution during storage in liquid nitrogen (Grospietsch, et al., 1999; Luo & Reed, 1997). This includes loading with different cryoprotectant materials (sucrose, polyols, glycerol, proline, and DMSO). Using these methods, combined with preculturing or preconditioning of donor plants, embryonic axes of several non-orthodox plants have been successfully cryopreserved by vitrification (Cho, Hor, Kim, Rao, & Engelmann, 2001; Gagliardi, et al., 2002; Malik & Chaudhury, 2006; Thammasiri, 1999).

Sucrose preculture is known to enhance the survival rate of tissues in cryopreservation treatments followed vitrification (Halmagyi & Pinker, 2006). Although the precise impact of such pretreatments on the physiological state of the plant material still remains unclear (Lu, et al., 2009), the beneficial effect of sucrose in cryopreservation could be due to two effects. First, sucrose acts as an osmoticum, thereby decreasing intracellular water content (Reinhoud, Schrijnemakers, Vaniren, & Kijne, 1995; Santos & Stushnoff, 2003; Tanaka, Niino, Isuzugawa, Hikage, & Uemura, 2004). Moreover sucrose is able to enter the cells (Dumet, Engelmann, Chabrillange, Duval, & Dereuddre, 1993), inducing a range of physiological responses in plant tissues including an increase in soluble sugar content (Bachiri, Bajon, Sauvanet, Gazeau, & Morisset, 2000; Hitmi, Coudret, Barthomeuf, & Sallanon, 1999; Wang, et al., 2004) and total fatty acids (Ramon, Geuns, Swennen, & Panis, 2002; Zhu, Geuns, Dussert, Swennen, & Panis, 2006), along with significant changes in protein composition (Carpentier, et al., 2007; Wang, et al., 2004; Zhu, et al., 2006) and cell ultrastructure (Bachiri, et al., 2000). The accumulation of sucrose within the tissue contributes to the retention of viability when the freezable water is removed to the point of reaching the glassy state during vitrification during storage in liquid nitrogen (Helliot, et al., 2003; Jitsuyama, Suzuki, Harada, & Fujikawa, 2002; Pinker, Halmagyi, & Olbricht, 2009).

In the present study, sucrose preculture was shown to improve the survival of cryopreserved *D. spectabile* embryonic axes from 0 to 20%. However, the embryonic axes also appeared to be sensitive to the high concentration (0.75M of sucrose used in this study) of sucrose pretreatment, as shown by a reduction in survival of embryonic axes during preculture. Similar results were observed in *Cocos nucifera* (Hornung, Domas, & Lynch, 2001), *Syzygium francissi* (Shatnawi, Johnson, & Torpy, 2004), *Humulus lupulus* (Martínez, et al., 1999), *Mangifera indica* L. (Wu, et al., 2003), *Citrus* spp. (Gonzalez-Arnao, et al., 2003) and *Musa* spp. (Panis, Totte, VanNimmen, Withers, & Swennen, 1996). The use of a high sucrose concentration, generally above 1.0 M, may cause excessive cellular dehydration resulting in osmotic stress and a toxic effect on the cell. The damage is related to alterations of the cell membrane integrity, loss of cell turgidity and irreversible cytoplasm contraction, thus affecting the functional and structural proprieties of the cells (Steinmacher, et al., 2007). In contrast, other studies suggested that a progressive increase in sucrose concentration (above 1.0 M) could increase cell tolerance level to dehydration (Dumet, et al., 1993; Martínez, et al., 1999). For plant materials that are sensitive to desiccation and high sugar content, a step-wise increase in sucrose concentration in the preculture medium has been suggested to be more effective (Wang, et al., 2004; Wilkinson, Wetten, Prychid, & Fay, 2003).

Vitrification solutions in high concentrations prevent intracellular ice formation, but are also potentially harmful due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Thinh, et al., 1999; Towill & Jarret, 1992). Therefore, for successful cryopreservation of plant tissues by vitrification, it is essential to carefully control the loading and dehydration procedures so as to provide enough dehydration while at the same time preventing injury caused by chemical toxicity and sudden osmotic stress (Touchell, et al., 2002).

In this study, prolonged exposure to vitrification solutions resulted in injury, most likely, from chemical toxicity or excessive desiccation. Similar results has been reported in *Castanea sativa* (Vidal, Sanchez, Jorquera, Ballester, & Vieitez, 2005), *Camellia sinensis* (Kuranuki & Sakai, 1995), and *Rauvolfia serpentine* (Ray & Bhattacharya, 2008). The optimum exposure time to the vitrification solutions and the concentration of vitrification solutions is weight dependent and species-specific (Niino, Sakai, Yakuwa,

& Nojiri, 1992). For example, maximum shoot formation rates have been reported to be obtained after 20 minutes exposure of PVS2 in shoot tip meristems of *Ribes nigrum* (Benson, et al., 1996) and 150 min for excised meristems of *Bletilla striata* (Ishikawa, et al., 1997). In this study the exposure to the vitrification solutions ranged from 30 to 90 minutes. Further research is necessary to determine if shorter or longer exposure times and the concentration of PVS2 would improve the survival rate of *D. spectabile* embryonic axes.

The selection of vitrification solutions is one of the most important factors for successful cryopreservation. It is generally considered that PVS3 vitrification solution is less toxic than PVS2 solution and has been successfully used for freezing shoot tips of species which showed high sensitivity to PVS2, including *Asparagus officinalis* (Nishizawa, et al., 1993) and *Allium sativum* (Baek, Kim, Cho, Chae, & Engelmann, 2003; Kim, et al., 2004; Kim, Kim, et al., 2005). Effective cryopreservation with PVS3 often requires a longer exposure period than PVS2, perhaps indicating a longer time needed to accumulate intracellular concentrations of glycerol sufficiently high for cryoprotection when ethylene glycol and DMSO are not present (Volk, Harris, & Rotindo, 2006).

Ethylene glycol and DMSO exhibit surfactant effects on membrane permeability, increasing the diffusion of glycerol into cells (Baudot & Odagescu, 2004). Glycerol is considered as a significant component in preculture media or cryoprotectants which have high post-LN survival for vitrification. It is thought that the hydroxyl groups arranged along one side interact more efficiently with the membrane bilayer, consequently providing better protection and subsequent survival during cryopreservation (Fuller, 2004; Turner, Senaratna, Bunn, et al., 2001; Turner, Senaratna, Touchell, et al., 2001). However, while glycerol has low toxicity to living cells if exposure is short term (Fuller, 2004), an increase in intracellular glycerol concentration may be damaging (Turner, Senaratna, Bunn, et al., 2001; Turner, Senaratna, Touchell, et al., 2001). Volk et al. (2006) reported that glycerol was a major component causing damage in mint shoot tips, confirming it is essential to carefully control the exposure duration and concentration of the vitrification solution for successful cryopreservation.

This study found that embryonic axis survival did not decrease as exposure time to PVS3 increased but that it did for PVS2. This suggests that PVS3 is less toxic than PVS2. However there was an interaction between preculture time and exposure time to the PVS solutions. PVS3 toxicity was observed in embryonic axes precultured for three days on the high sucrose medium after 60 and 90 minutes exposure to PVS3. In contrast there was no difference in survival of embryonic axes precultured for 1 day after 0 and 90 minutes exposure to PVS2. It may be that excessive cellular dehydration, as a result of osmotic stress in the pre-treatment conditions, may enhance the toxicity of the PVS solutions.

Highest post-cryopreservation survival (20%) for *D. spectabile* embryos were achieved following encapsulation-dehydration or chemical dehydration with PVS techniques. However, post-cryopreservation germinated embryonic axes failed to develop into normal seedlings. This is the main limitation to cryopreservation of embryonic axes of *D. spectabile*. Berjak et al. (2000) reported that embryonic axes of some recalcitrant species are not appropriate for cryopreservation as no survival was recorded even when all parameters involved in the cryopreservation protocol were optimised. This may be because of the histological composition of the tissues and the cellular structure of the embryonic axis. In these situations, other types of explants for cryopreservation, such as apices, buds or somatic embryos could be used as the conservation material (Engelmann, 2000; Pence, 1995). This could be the case for *D. spectabile* but, before this can be definitively concluded further studies, in particular determination of the causes of the shoot meristem failing to elongate, are required.

CHAPTER 5 GENERAL DISCUSSION

5.1 Overview of main findings/outcome

This research study makes a significant contribution to our understanding of the seed biology of New Zealand plant species. These findings facilitate the development of *ex situ* conservation strategies, including options for conventional seed storage and cryopreservation of threatened genetic diversity. The six threatened New Zealand species as well as *D. spectabile* are of main concern for the investigation of *ex situ* conservation strategies because of their conservation priority, potential extinction, and probable non-orthodox seed storage behaviour and lack of the corresponding techniques for their long term *ex situ* conservation.

As seed of endangered species are difficult to obtain in sufficient quantities for this research, and *D. spectabile* might share the same characteristics as those of endangered species with respect to intolerance of dehydration and conventional long term storage, this species was selected as a representative species. It is hoped the protocols developed with *D. spectabile* will be applicable to other New Zealand's endangered species.

The key findings of this study were:

- Seed of *Carmichaelia williamsii*, *Clianthus puniceus*, *C. maximus*, *Hibiscus diversifolius*, *Myosotidium hortensium*, *Tecomanthe speciosa* tolerated desiccation to low moisture contents. Overall, the decreasing order of species tolerance to rapid seed desiccation, without a significant reduction in percentage of germination, was *Carmichaelia williamsii* (<4%MC), *Clianthus puniceus* (<4%MC), *C. maximus* (<3%MC), *Hibiscus diversifolius* (<3%MC), *Myosotidium hortensium* (<3%MC) *Tecomanthe speciosa* (>5%MC). Thus, conventional seed storage is recommended in these species, due to their tolerance to desiccation to low moisture contents.

- The tolerance to both desiccation and storage of seeds of *C. maximus* and *M. hortensium* indicates that these species are 'essentially' orthodox in seed storage behaviour. However, *M. hortensium* seeds showed very short storage life.

- The intolerance to desiccation of seeds of *Dysoxylum spectabile* indicates that this species is 'essentially' recalcitrant in seed storage behaviour. A noticeable decrease in germination percentage was observed when the MC of the seeds was reduced to 25%. The developmental status and drying rate influenced the desiccation response of this species. The desiccation tolerance of seeds maximised at physiological maturity and fast drying permit seeds to survive to lower moisture contents.

- Notwithstanding the above finding, there was a differential level of response in seeds and embryonic axes of *D. spectabile* following desiccation. The MC of excised embryonic axes can be reduced to after rapid dehydration much lower than that of whole seeds. Thus, for long-term storage the germplasm of this species cryopreservation using embryonic axes is recommended. It is the most promising method but cannot be recommended until a method is found to achieve not only survival but also normal plants following cryopreservation.

- Non-scarified seeds of *C. williamsii*, *C. puniceus*, *C. maximus* and *H. diversifolius* did not imbibe water, whereas mechanically scarified seeds took up water rapidly. Further, mechanically scarified seeds germinated to 96–100% indicating that the embryo is non-dormant. Thus, these species have a coat-imposed dormancy, and the hard seed coats act in preventing water absorption.

- *C. clianthus* and *C. maximus* were found to differ in seed coat characteristics from other species of the Fabaceae. These species lost water impermeability by desiccation and low temperature storage, and this may affect their storage life.

- The poor germination observed in *M. hortensium* seeds may be attributed to a function of the seed coat. A study of the water imbibition and respiration rate indicated that the *M. hortensium* seed coat is permeable to water and respiratory

gases. And no significant differences were observed between the germination capabilities of de-coated seeds and de-coated seeds in contact with loose seed coats. Thus it was concluded that radicle emergence is inhibited mechanically by seed coat in *M. hortensium* seeds.

- Both high germination levels and rapid rates of seedling development in *T. speciosa* and *D. spectabile* suggested that seeds of these species are not dormant.

- Thermal analysis of water phase transitions across the range of drying for excised embryonic axes and cotyledons provided information on critical parameters to aid the development of cryopreservation procedures for *D. spectabile*. Clear evidence of ice nucleation during cooling and ice melting on warming were noted for control embryonic axes and cotyledons. These thermograms confirmed the existence of non-vitrified water which formed ice during cooling and melted on rewarming and, as expected this ice was lethal to the excised embryonic axes and cotyledons.

- This study has demonstrated how DSC can be used to elucidate cryogenic behaviour in combined cryoprotection strategies applied to recalcitrant plant species. Importantly increased survival has been systematically associated with a decrease in the enthalpies of ice crystallization/ ice melting events and recovery is possible using rapid desiccation and encapsulation dehydration protocols leading to the production of stable glasses

- Cryopreservation of embryonic axes of *D. spectabile* using commonly used protocols, rapid desiccation, encapsulation dehydration and PVS2 and PVS3 vitrification, were demonstrated.

- With *D. spectabile*, encapsulation-dehydration or chemical dehydration with PVS solutions before freezing was the most successful technique for cryopreservation, as shown by the higher percentage survival (20%) achieved.

5.2 Future research studies

These research findings highlighted some key areas for future studies. These include:

- The unique characteristics of the seed coat of *Clianthus* species requires further study to improve our understanding of the dormancy breaking process and to develop an *ex situ* conservation strategy. Exploration of the anatomical mechanism of seed physical dormancy breakage by drying or low temperature storage and determination of the underlying mechanisms of water entry into the seed during imbibitions after treatments may help

- In *M. hortensium* a noticeable decrease in viability was observed after 24 months storage. It has been suggested that oxidative stress, especially lipid peroxidation and de-esterification of phospholipids, leading to the loss of membrane structure, is a significant cause of deterioration during dry storage (Buitink, et al., 2002; Franca, et al., 2007). Further biochemical analyses of *M. hortensium* seeds during storage should prove useful in understanding the storage behaviour of this species.

- Further investigations on cryopreservation of seeds of *M. hortensium* would be valuable as a potential strategy for long-term storage of this species.

- The relatively low survival (20%) of *D. spectabile* excised embryonic axes post cryopreservation means optimisation is required to improve the efficiency of the vitrification-based protocol and encapsulation-dehydration protocol demonstrated in this study. However, it may be necessary to develop other cryopreservation protocols such as encapsulation vitrification methods and to research other tissues for cryopreservation such as apices, buds or somatic embryos.

- Further research on achieving not only better survival but also normal seedling development for excised embryonic axes post cryopreservation of *D. spectabile*.

5.3 Conservation options in New Zealand threatened species

Many New Zealand native species have been historically vulnerable to genetic erosion from insufficient or degraded habitat, human activities and alien species introductions. The urgent need for *in situ* and *ex situ* conservation of existing wild biodiversity is widely recognised. The research findings of the present study on seed biology and storage behaviour of New Zealand native species contributes significantly to future efforts to conserve this germplasm by providing options such as conventional seed storage and cryopreservation.

It increases acceptance of *ex situ* efforts as an integral component of biodiversity conservation. Among the various *ex situ* conservation methods, seed storage seems to be one of the most convenient for long-term conservation. *Ex situ* samples have become integrated with protection of plants in their wild habitat, through their use in population reinforcement as a source of seeds for growing plants to plant where existing populations are reduced, reintroduction and recovery plans (Godefroid, et al., 2010; Maunder, et al., 2004; Paunescu, 2009). To this end, seed banks are a good way of conserving biodiversity, providing that seeds are of high quality and at maximum viability and remain so during storage (Godefroid, et al., 2010).

As seeds are living material, they require proper storage conditions and continuous monitoring to ensure that viability is maintained in storage (Walsh, et al., 2003). Despite the number of established *ex situ* facilities, there is relatively little information on seed viability of wild species in seed banks. Relative longevity data from experiments where seed is rapidly aged in an attempt to mimic long-term storage and comparisons between species have been considered, however, assessing the response of seed to storage conditions over the long term is required for each species. The current research project was therefore aimed at a better understanding of the seed storage behaviour of New Zealand native species. This is a prerequisite for their long-term preservation.

Viability testing through germination is essential for the maintenance of a seed collection and can be a rapid way of identifying problems with the seed storage conditions (Walsh, et al., 2003). Also, seeds are to be used in restoration programs. It is

therefore essential to know the right protocol to maximise the chance of germination, allowing seed banks to increase their efficiency and enhance their role in biodiversity conservation. Seed supplies of New Zealand threatened native species can be limited and erratic. This means that understanding of optimum conditions for germination and methods to overcome dormancy are needed as complementary conservation strategies. Considerable research effort has been directed towards understanding seed germination and dormancy traits in the New Zealand flora (Burrows, 1995a, 1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d, 1996e, 1999a, 1999b; Conner & Conner, 1988; Court & Mitchell, 1988; Fountain & Outred, 1991; Gruner & Heenan, 2001; Mackay, McGill, Fountain, & Southward, 2002; McGill, et al., 2002; McGill, et al., 2000; Metcalf, 1995; Metcalf, 1987; Moore, Bannister, & Jameson, 1994; Norton, Ladley, & Sparrow, 2002; Philipson, 1989; Rowarth, Hampton, & Hill, 2007; Simpson & Webb, 1980; Williams, Norton, & Nicholas, 1996). Despite considerable progress, there remain many prominent plant families and species in New Zealand with largely unresolved seed-germination requirements.

Physical barriers to germination may lead or may have led to confusing reports on the storage behaviour of many other species where the failure to germinate may be interpreted as a loss of viability rather than dormancy. This includes three species assessed in this study, *C. williamsii*, *C. puniceus* and *C. maximus*. Thus, appropriate dormancy-breaking techniques must be selected based on the requirements of each species.

Cryopreservation has been demonstrated as a potential strategy for long-term storage of seeds of many desiccation intolerant species and is recommended in this study for the New Zealand native species, *D. spectable*. A number of studies found cryopreservation as the safest option, even in the desiccation tolerant species (Kholina & Voronkova, 2008). Walters et al. (2004) predict a half-life of 500 to 3400 years for lettuce seeds stored by cryopreservation. Thus, cryopreservation also has the clear advantage of extending the longevity of stored seeds.

Cryopreservation was shown to be an *ex situ* conservation option for many rare and endangered species and seeds with limited longevity (Engelmann, 2004). This study

reports for the first time cryopreservation results for *D. Spectabile.* Though further research is needed to optimise the cryopreservation protocol to maximise survival, it has led to a putative way forward for conservation of desiccation sensitive New Zealand species.

New Zealand threatened native species are best conserved by utilizing strategies that include a combination of both *in situ* and *ex situ* approaches and these could include:

- Protection and management of *in situ* wild populations
- Cultivation as commercial horticultural crops
- Field germplasm collections and reference species in botanic gardens
- *Ex situ* storage of seeds
- *Ex situ* storage of plant material long term by cryopreservation:
    - Seeds
    - Embryonic axes (further optimisation required)
- *In vitro* storage through micropropagation (also facilitating multiplication of germplasm for restoration programs)

In conclusion, cryopreservation is should be regarded as a complementary conservation strategy for New Zealand threatened species, allowing the development for the first time of a comprehensive and effective *ex situ* conservation strategy for New Zealand native species. Long-term conservation of germplasm of threatened species is important to ensure future access to genetic material for regeneration and restoration programs. However, there are still limitations to the implementation of effective *ex situ* conservation strategy including conventional seed bank storage and alternative storage

techniques. These include (1) the need for sustainable funding and infrastructure for the *ex situ* storage of endangered species especially for non-orthodox seeded species, such as *D. spectabile*, by non conventional storage techniques (i.e. cryopreservation), (2) the lack of knowledge of seed biology of these species to inform effective maintenance of *ex situ* storage and (3) the acquisition of more fundamental knowledge of the mechanisms for the desiccation tolerance in seeds to improve seed survival after drying and subsequent dry storage. In order to address these limitations, further investment and study is needed for New Zealand threatened native species.

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