Ex situ conservation of Myrtaceae. A response to Myrtle Rust in the Pacific Region

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Abstract

Many Myrtaceae species are threatened with extinction due to the arrival of myrtle rust (*Austropuccinia psidii*) in the Pacific Region. *Ex situ* conservation, in particular seed banking and cryopreservation, requires knowledge of the seed biology of the species to develop optimum long-term seed storage protocols. This information is limited for many New Zealand species. This study investigated *ex situ* conservation options for five critically endangered Myrtaceae species; *Lophomyrtus bullata* (ramarama), *Lophomyrtus obcordata* (rōhuto), *Neomyrtus pedunculata* (rōhuto), *Metrosideros bartlettii* (rātā moehau) and *Syzygium maire* (swamp maire).

Less than 14 individuals of *M. bartlettii* remain in the wild. Using six trees in cultivation, the breeding system was found to be self-incompatible, thus an unrelated pollen donor is required to produce viable seed. With many trees in cultivation closely related, hand-pollination is an essential management tool. For hand-pollination to be successful, viable pollen must be available at the precise time. This study compared viability of pollen sourced from different trees and for the same tree over two years. Pollen germination protocols, which can be used to quantify pollen viability, were developed while short- and medium- term pollen storage methods are described.

The seed morphology, seed germination requirements, seed desiccation tolerance, the combined impact of desiccation and freezing, and storability at 5°C, -18°C and -196°C of *Lophomyrtus bullata*, *L. obcordata* and *Neomyrtus pedunculata* were investigated. All three species were found to have intermediate seed storage physiology. *L. bullata* and *N. pedunculata* were sensitive to desiccation (~15% equilibrated relative humidity). *Lophomyrtus bullata* was also found to be freezing sensitive, while two of the three *L. obcordata* seed lots tested also displayed significant seed viability loss following storage at -18°C. Differential Scanning Calorimetry (DSC) analysis revealed that lipid crystallization in all three species occurred between -18°C and -52°C, and it is postulated that the lipids are in a metastable state when stored under conventional seed banking temperatures (c. -18°C). It is recommended that seeds of these three species are best stored outside of their lipid metastable temperature ranges.

Syzygium maire seed and embryos are highly recalcitrant. This study investigated the oxidative stress, thermal properties, and ultrastructure of zygotic embryo axes (EAs) desiccated to various moisture contents (MC). Using rapid desiccation, moisture content in EAs were lowered to ~0.3 g/g resulting in ~50% of the EAs surviving. Exposure to liquid nitrogen was however fatal irrespective of the moisture contents tested. The enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPOX) significantly decreased following desiccation, while there was a corresponding 7-fold increase in the production of protein carbonyls and lipid peroxides. Exogenous application of ascorbic acid (AsA) increased antioxidant activity in desiccated EAs but not by a significant amount. The decrease in antioxidant activity following desiccation as well as the presence of melt enthalpies observed on the DSC thermograms, confirmed that for cryopreservation of *S. maire* EAs to be successful, rapid desiccation needs to be combined with cryoprotection.

Plant Vitrification Solution 2 (PVS2) moderated the thermal behaviour in *S. maire* EAs with the novel droplet vacuum infiltration vitrification (DVIV) significantly improving embryo survival and plantlet development compared to droplet vitrification (DV). Neither method however resulted in embryo survival following exposure to liquid nitrogen. Longer incubation times using DVIV combined with physical desiccation should be investigated for the cryopreservation of *S. maire.*

The four fleshy New Zealand Myrtaceae species, *Lophomyrtus bullata*, *L. obcordata*, *Neomyrtus pedunculata* and *Syzygium maire* were not amenable to conventional seed banking. Seed from *Lophomyrtus* and *Neomyrtus* displayed intermediate storage physiology due to sensitivity to desiccation and storage at -18°C, while *S. maire* seeds were highly recalcitrant. Cryopreservation, including the use of cryoprotectants and optimisation of cooling/warming rates, should be investigated for *Lophomyrtus* and *Neomyrtus*. *Syzygium maire* embryos did not regenerate successfully following any of the cryopreservation methods tested in this study (rapid desiccation, encapsulation-dehydration, cryoprotection). Further optimisation of cryopreservation methods for *S. maire* should be investigated. This includes application of exogenous antioxidants, increased cooling/warming rates, longer PVS2 incubation times and combining DVIV and rapid desiccation.

Keywords: cryopreservation, desiccation, differential scanning calorimetry (DSC), endemic, freezing, liquid nitrogen, living collection, myrtle rust, pollen, PVS2, seed banking, threatened, TZ staining, vacuum infiltration, zygotic embryo.

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List of abbreviations

ABA	Abscisic acid
ANOVA	Analysis of Variance
BGCI	Botanic Gardens Conservation International
Ca	Calcium
CPA	Cryoprotective agent
CV	Conventional vitrification
CR	Critically Endangered
DSC	Digital Scanning Calorimeter
DMSO	Dimethyl sulfoxide
EA	Embryonic Axes
EN	Endangered
FR	Free Radicles
Gly	Glycerol
GSPC	Global Strategy for Plant Conservation
GTA	Global Tree Assessment
IUCN	International Union for the Conservation of Nature
MS	Murashige and Skoog Basal media
MSB	Kew's Millennium Seed Bank
MUFA	Monounsaturated fatty acids
NaDCC	Sodium dichloroisocyanurate
NE	Nationally Endangered
NC	Nationally Critical
NV	Nationally Vulnerable
LC	Least Concern
LN	Liquid Nitrogen
MC	Moisture content
n	Number of samples
PUFA	Polyunsaturated fatty acids
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
SD	Standard deviation

SEM	Scanning Electron Microscope
Spp.	Several species
Suc	Sucrose
TAG	Triacylglycerols
TEM	Transmission electron microscope
v/v	Volume per volume
VU	Vulnerable
w/v	Weight per volume
WC	Water content

Chapter 1.

General introduction and study rationale

1.1 Myrtaceae biodiversity loss

Myrtaceae is one of the largest plant families globally and consists of 140 genera and more than 5,800 species (Christenhusz *et al*., 2017). There is however concern about the conservation status of Myrtaceae with more than 40% of the evaluated species under threat (IUCN, 2022). As with many tree taxa worldwide, Myrtaceae species are threatened by forest clearing for agriculture, development, logging, and grazing while climate change and severe weather are also having adverse impacts on many species (GTA, 2021, Guerrant *et al.* 2004, Potter *et al.*, 2017, IUCN, 2022, Sharrock, 2020). More recently the fungal pathogen, myrtle rust (*Austropuccinia psidii*), has become a significant threat to native as well as economically important trees in several genera of Myrtaceae globally (Beenken, 2017). Myrtle rust, which is native to South America, has spread globally causing extensive damage to nurseries, *Eucalyptus* plantations, commercial guava production and the allspice (*Pimenta officinalis*) industry (see Toome-Heller *et al.*, 2020 and references within). Myrtle rust has already impacted Myrtaceae dominated ecosystems, for example in Australia the rust has killed mature trees in forest ecosystems in less than four years (Carnegie *et al.*, 2015). Similar ecosystem impacts are predicted for Mexico (Esperón-Rodríguez *et al.*, 2018), New Caledonia (Soewarto *et al.*, 2018) and South Africa (Roux *et al.*, 2015). On a species level, myrtle rust has contributed to the ongoing decline of *Eugenia koolauensis*, a critically endangered Hawaiian species (Chock, 2018; Keir, 2018) and caused local extinctions in formerly widespread Australian species such as *Rhodamnia rubescens* and *Rhodomyrtus psidiodes* as well as *Lophomyrtus* species in New Zealand (Fensham *et al.*, 2020; Sommerville *et al.*, 2020; Sutherland *et al.*, 2020). Within New Zealand the Myrtaceae comprises 28 species in six genera (NZPCN, 2021), all of which are of conservation concern based on the predicted impact from myrtle rust (de Lange, 2018). Susceptibility of New Zealand Myrtaceae species to myrtle rust is based on infection survey data (Toome-Heller *et al.*, 2020) and resistance testing (Smith *et al.*, 2020) (Table 1.1).

Table 1.1: Myrtaceae species native to New Zealand, their susceptibility to myrtle rust, conservation status and distribution

Scientific Name	Common Name	Confirmed myrtle rust host) (Genus/species)	Conservation Status	Distribution
Kunzea amathicola	Rawiritoa, kānuka	Yes (genus)	NV	Widespread but fragmented in relic habitats
Kunzea ericoides	Kānuka	Yes (genus)	NV	Northern South Island
Kunzea linearis	Rawiri mānuka, kānuka	Yes (species)	NV	North Island, Te Paki to northern Waikato and Wairarapa
Kunzea robusta	Rawirinui, kānuka	Yes (species)	NV	Widespread and common
Kunzea salterae	Moutohora kānuka	Yes (genus)	NV	Local endemic to Moutohora (Whale Island)
Kunzea serotina	Makahikatoa, kānuka	Yes (genus)	NV	Widespread in North and South Island
Kunzea sinclairii	Great Barrier Island kānuka	Yes (genus)	NC	Great Barrier Island
Kunzea tenuicaulis	Geothermal kānuka	Yes (genus)	NE	North Island on geothermal fields
Kunzea toelkenii	Bay of Plenty kānuka	Yes (genus)	NC	Restricted to Bay of Plenty in North Island
Kunzea triregensis	Three Kings kānuka	Yes (genus)	NC	Endemic to Three Kings Islands
Leptospermum scoparium var. *incanum*	Mānuka	Yes (species)	NV	Confined to upper Northland Peninsula
Leptospermum scoparium var. *scoparium**	Mānuka	Yes (species)	DEC	Widespread in New

Scientific Name	Common Name	Confirmed myrtle rust host) (Genus/species)	Conservation Status	Distribution
				Zealand and Australia
Lophomyrtus bullata	Ramarama	Yes (species)	NC	North and South Islands, but scarce in South Island
Lophomyrtus obcordata	Rōhutu	Yes (species)	NC	Patchy distribution on North and South Islands
Metrosideros albiflora	White flowering rātā, akatea	Yes (genus)	NV	Confined to northern portion of North Island
Metrosideros bartlettii	Rātā moehau, Bartlett's rātā	Yes (species)	NC	Confined to three forest remnants in Te Paki, North Island
Metrosideros carminea	Carmine rātā	Yes (species)	NV	North Island from Te Paki to Taranaki
Metrosideros colensoi	Rātā	Yes (species)	NV	Common on limestone areas on North and South Islands
Metrosideros diffusa	White rātā	Yes (species)	NV	Widespread throughout North and South Islands and Stewart Island
Metrosideros excelsa	Pōhutkawa	Yes (species)	NV	North Island from North Cape to Taranaki. Southern limit unknown as it is widely planted

Scientific Name	Common Name	Confirmed myrtle rust host) (Genus/species)	Conservation Status	Distribution
				throughout New Zealand
Metrosideros fulgens	Climbing rātā	Yes (species)	NV	North Island, west coast of South Island, and Three Kings Islands north of Cape Reinga
Metrosideros kermadecensis	Kermadec pōhutukawa	Yes (species)	NC	Endemic to Kermadec Islands
Metrosideros parkinsonii	Parkinson's rātā	Yes (genus)	NV	Limited distribution on North and South Islands
Metrosideros perforata	Akatea	Yes (species)	NV	Widespread across North and South Islands
Metrosideros robusta	Northern rātā	Yes (species)	NV	North and South Islands
Metrosideros umbellata	Southern rātā	Yes (genus)	NV	North, South, Stewart and Auckland Islands
Neomyrtus pedunculata	Rōhutu	No	NC	North, South and Stewart Islands
Syzygium maire	Swamp maire, maire tawake, waiwaka	Yes (species)	NC	North Island to top of South Island. Scarce or absent over large parts of former range

* Not endemic to New Zealand
* Conservation status: NC – Nationally Critical, NE – Nationally Endangered, NV – Nationally Vulnerable, Dec – At Risk Declining

Conservation efforts for the world's most threatened plant species are hindered by gaps in fundamental biological information (Cavender *et al.*, 2015) and their seed biology

(Pritchard *et al.*, 2014). These gaps include knowledge about tree taxonomy and phylogeny, reproductive ecology, seed biology and distribution information (Cavender *et al.*, 2015). Similarly, despite the high risk of extinction to New Zealand Myrtaceae species, limited information has been published on the ecology, breeding biology and population dynamics, hampering conservation efforts. Seed production capacity, seed quality, ability to germinate and seed dormancy are often considered the most important aspects for conserving rare and threatened species (Emery & Offord, 2019). The quality of mature seeds is reflected in the results of germination tests although germination alone does not always reflect the viability of a seed collection due to unfavourable conditions, dormancy, or both (Hay & Probert, 2013; Liu *et al.*, 2020). It is estimated that up to 75% of species in temperate broadleaved evergreen forests have some form of dormancy (Baskin & Baskin, 2014), and lack of knowledge on germination is one of the major impediments to the potential use of germplasm of wild species for reintroduction (Hay & Probert, 2013; Liu *et al.*, 2020). Knowledge of seed germination traits is an essential component of long-term seed storage (Lin *et al.*, 2021). Similarly, germination testing to monitor seed viability for seeds in storage is an essential part of effective management of seed bank collections (Hay & Probert, 2013). It is furthermore critical to identify emergence of healthy radicles versus seeds developing into normal seedlings that can produce cotyledons and a normal plantlet. Abnormal seedlings (e.g. those that produce cotyledons without a radicle) are not considered capable of continued growth due to damage, deformation or decay (Liu *et al.*, 2020).

1.2 *Ex situ* plant conservation

The pressing environmental crises of climate change and biodiversity loss can no longer be addressed through *in situ* conservation alone and it is now widely accepted that an integrated conservation approach is needed (Fig. 1.1) (Cavender *et al.*, 2015; Guerrant *et al.*, 2004; Wyse & Dickie, 2017). *Ex situ* conservation, the conservation of genetic resources and germplasm outside of their natural habitat, includes living collections/orchards, conventional seed banking and storage of seeds, embryos, pollen, or other tissues at cryogenic temperatures using liquid nitrogen (Griffith *et al.*, 2014; Wyse *et al.*, 2018). The urgency of *ex situ* conservation strategies to prevent extinction of species susceptible to myrtle rust has been highlighted in Australia (Berthon *et al.*, 2018; Sommerville *et al.*, 2020) and New Zealand (Nadarajan *et al.*, 2021; Van der Walt *et al.*, 2021b). Botanic gardens globally have adopted plant conservation as their main mission, with an increased focus on *ex situ* conservation (Raven & Havens, 2014). The main driver for this conservation focus is the Global Strategy for Plant Conservation (GSPC), a 16 outcome-orientated strategy originally adopted by the Conference of the Parties to the Convention on Biological Diversity at its sixth meeting (COP-2) in 2002 (Sharrock, 2020). Target 8 of the GSPC is particularly relevant to botanic gardens. This target states "at least 75% of known threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes". Seed conservation is an important strategy for *ex situ* conservation of seed-bearing species. Although the core activity of botanic gardens remains curating living collections, over the past two decades many botanic gardens have established seed banks and it is estimated that 45 000 – 55 000 taxa are now in conservation-based genebanks (Mounce *et al.*, 2017; O'Donnell & Sharrock, 2017). Within the botanic gardens community, there are some of the largest native seed banks including Royal Botanic Gardens Kew's Millennium Seed Bank (United Kingdom), the Germplasm Bank of Wild Species (China) and Royal Botanic Garden Sydney's PlantBank (Australia) (O'Donnell & Sharrock, 2017).

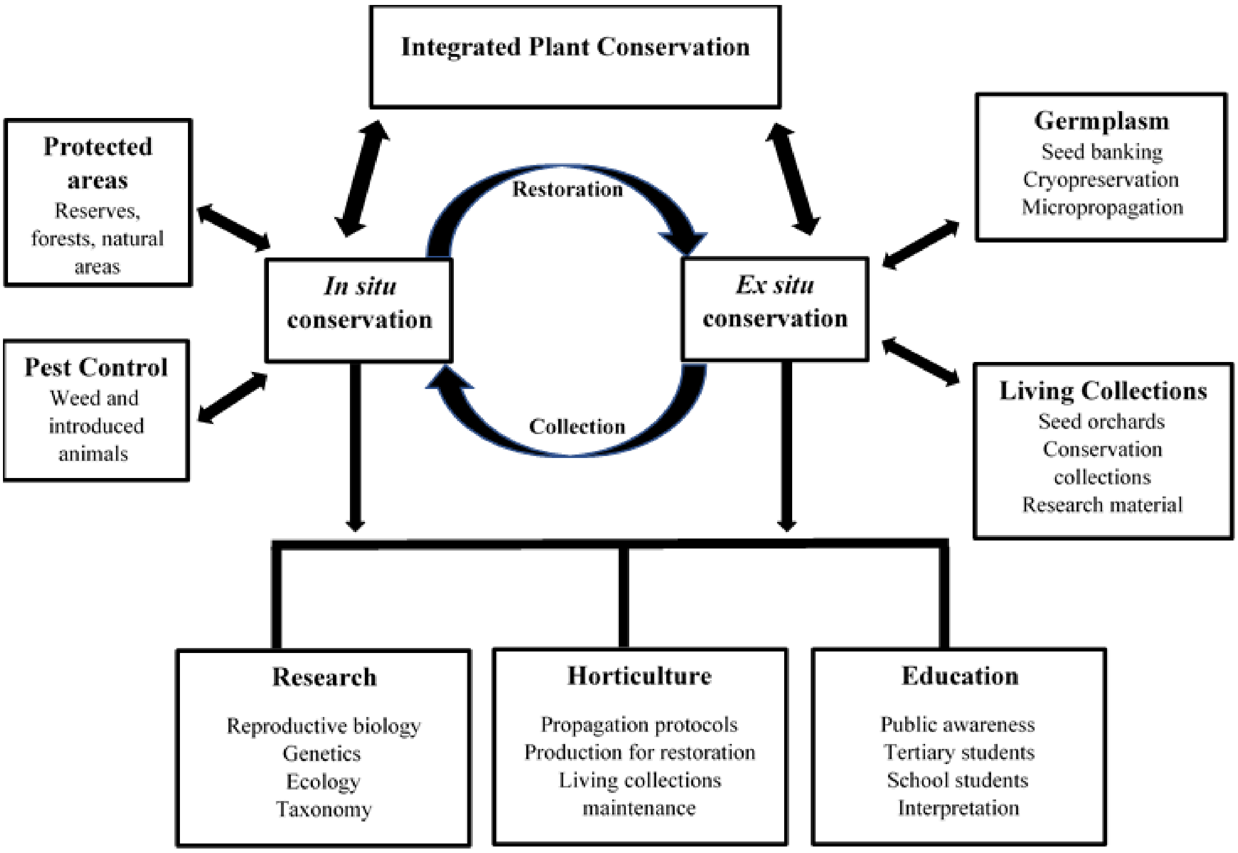

Figure 1.1: Integrated plant conservation in which *in situ* and *ex situ* conservation are combined to support species survival (adapted from Guerrant *et al.*, 2004).

1.3 Seed storage classification and long-term storage

Seed bearing species, in most cases, can be divided into one of two categories based on seed storage behaviour: orthodox (desiccation tolerant) or recalcitrant (desiccation sensitive) (Hay & Probert, 2013).

1.3.1 Orthodox seed

Originally the hypothesis was that 90% of the flowering plant families, including food and agricultural crops, produce seeds that are orthodox (Hay & Probert, 2013; Wyse & Dickie, 2017). Orthodox seeds are characterised by the ability to tolerate desiccation for prolonged periods (Walters *et al.*, 2013). Desiccation tolerance is defined as the ability of plant material to dry to equilibrium with atmospheric relative humidity (eRH) and resume normal functioning when it is rehydrated (Walters, 2015). This complex desiccation mechanism relies on several factors including intracellular physical characteristics, decrease in metabolism, antioxidant systems and the accumulation of protective molecules (late embryogenic abundant proteins – LEAs) and formation of an intracellular glassy state (Berjak & Pammenter, 2008; Berjak & Pammenter, 2013). For orthodox species the most effective way to conserve the seed is through conventional seed storage during which seeds are dried to low moisture content (MC) (3–7%) and stored at c. -20°C (MSB, 2015). The longevity of seeds in conventional storage at Kew Millennium Seed Bank (MSB) is predicted to range from 30 to 6 000 years (see Probert *et al.*, 2009 and references within). However, dry seeds are also not immortal and life spans during storage range from short, intermediate and long (Berjak & Pammenter, 2013).

1.3.2 Recalcitrant seed

It is estimated that 33% of tree species globally produce seeds that are sensitive to drying (recalcitrant) and therefore not amenable to conventional seed banking (Ballesteros *et al.*, 2021). Seed from recalcitrant species remain metabolically active after shedding and this metabolic status continuously changes as the seed enters germination resulting in a post-harvest lifespan of days to months (Berjak & Pammenter, 2008). For recalcitrant species, the only long-term germplasm storage options are cryopreservation, storage of plant material in liquid nitrogen, or through living collections (Walters & Pence, 2021). Although significant progress has been made in the cryopreservation of crop species, research into wild plant cryopreservation is limited (Werden *et al.*, 2020). In addition to this, wild species are highly variable in their capacity to survive cryopreservation and the lack of biological information makes cryopreservation protocol development even more challenging (Coelho *et al.*, 2020).

Recalcitrant seeds generally have large, fleshy fruit with thick layers covering or surrounding the embryo, a mechanism to restrict, rather than avoid, water loss (Xia *et al.*, 2014). This survival mechanism however also prevents rapid desiccation and cooling, two essential steps to eliminate ice crystallization during the cryopreservation process (Walters *et al.*, 2013). The surgical removal of zygotic embryos from large-seeded recalcitrant species was first applied in the cryopreservation of *Hevea brasiliensis* (Normah *et al.*, 1986) and has become the preferred method to conserve genetic diversity in recalcitrant seeded wild species. However, excision is also associated with a burst of Reactive Oxygen Species (ROS) which in some species result in shoot meristem necrosis (Berjak *et al.*, 2011; Whitaker *et al.*, 2010). This initial generation of ROS is amplified during cryopreservation and the cryopreservation process exposes plant material to a number of stresses which could result in morphological and cytological variations (Walters, 2015). The success of the cryopreservation protocol therefore depends on manipulation of all the steps in the process to minimise accumulative impacts, with every step of the cryopreservation process optimised for each species and type of material (Fuller *et al.*, 2004). Despite the low or lack of tolerance to desiccation in recalcitrant species, it is essential that excess moisture is removed from cells to prevent the formation of lethal ice crystals during freezing (Fuller *et al.*, 2004; Reed, 2008). The vitrified state can be achieved using several methods applied singularly or in combination, all of which have advantages and disadvantages. A summary of the methods are provided in Table 1.2, based on Benson (2008) and Naidoo (2010).

1.3.3 Intermediate seed

Studies into seed behaviour of wild species have increased since the 1990's, resulting in the realization that the orthodox and recalcitrant categories were too restrictive, which gave rise to the intermediate category (Ellis *et al.*, 1991). By the mid 1990's more than 40 genera containing 60 species were considered to be of intermediate storage behaviour (Crane *et al.*, 2003). Rapid aging has also been detected in 26% of seed accessions from wild species stored under conventional conditions at KMSB (Probert *et al.*, 2009). At the same time, Davies *et al.* (2020) found that none of the seed from 22 European tree species tested exceeded medium-term storage (< 20 years). Significant ageing was also detected within 25 years in dry seeds and fern spores stored at cryogenic temperatures (Ballesteros *et al.*, 2019). These studies suggest that the number of angiosperms that are amenable to storage at -18 to -20°C might be less than previously predicted and urgent work is now required to determine storage conditions necessary to maximise their longevity (Merritt *et al.*, 2014). Although not yet fully understood, this intermediate storage behaviour could be due to a) tolerance to desiccation is higher than those in recalcitrant seeds but lower than orthodox, b) there is inconsistent longevity responses over a range of temperatures and c) seeds have short lifespans irrespective of the water content or storage temperature (Walters, 2015).

<u>Desiccation tolerance/sensitivity</u>

All seeds have threshold water contents and drying below the minimum water content will impact survival. The water content ranges for the thresholds of orthodox and recalcitrant species are distinct and originally based on people's inability to usefully extend seed storage life by drying (Roberts, 1973; Walters, 2015). Water threshold is considered an indirect measure of water stress, and variation in threshold among seed categories is an indication of the variation in the amount of stress a seed can tolerate due to desiccation (Walters, 2015). In contrast to the recalcitrant seeds, which have high fluid content, orthodox seeds accumulate dry matter reserves during embryo development and maturation (Farrant *et al.*, 1997; Leprince *et al.*, 2017) and in the process form a more solid cellular matrix (Walters, 2015). In addition to cessation of metabolism, molecular movement is impeded and the structure within the cellular matrix becomes fixed and able to support its own weight, thereby forming a solid also known as a glass (Leprince *et al.*, 2017; Walters, 2015). Cytoplasm becomes glassy when dried to 0.06-0.12 H_2O g DW $^{-1}$ (Ballesteros *et al.*, 2020; Ballesteros & Walters, 2011) and glasses in orthodox seeds are comprised of complex materials resulting in transition between fluid and glass over a broad range of temperature and moisture ranges (Walters, 2004). The hypothetical argument is therefore that short-and long-lived orthodox seeds can be quantitatively distinguished by the glass relaxation rates, with the longest surviving seed having the slowest relaxing glasses (Walters, 2015).

<u>Freezing sensitivity</u>

Sommerville *et al.* (2021) found that 24% of the desiccation tolerant Australian rainforest species tested to date were freezing sensitive or short-lived in storage at -20°C. Variables influencing seed longevity can be complex and may relate to aspects such as embryo size, seed mass, seed coat, cellular composition, taxonomy, genotype, and the environment of the maternal plant (Probert *et al.*, 2009; Walters, 2004). Of these variables, seed oil content is often indicated as a key predictor of intermediate seed storage physiology (Crane *et al.*, 2003). Lipids, particularly triacylglycerols, have high crystallization and melting temperatures thereby influencing the stability of glasses (Crane *et al.*, 2003; Mira *et al.*, 2019; Probert *et al.*, 2009). Triacylglycerols (TAG) accumulate into lipid bodies during seed development and molecules within the TAG phase can retain fluidity resulting in crystallization during low temperature storage (Crane *et al.*, 2006; Walters, 2015). The properties of TAG lipids have been shown to directly contribute to differences among species susceptibility to damage when exposed to -18°C (Crane *et al.*, 2003). For *Cuphea* species it was found that those species containing lauric acid (C_{12}) and/or myristic acid (C_{14}) in proportions of more than 58% of the total fatty acids, were susceptible to damage when exposed to -18°C (Crane *et al.*, 2003). Additionally, lipid crystallization occurs in *Cuphea* species at temperatures ranging from +16°C to -59°C (Crane *et al.*, 2003). Freeze sensitivity in tropical wild seeds has also been reported in Hawaii (Chau *et al.*, 2019) and Brazil (Mayrinck *et al.*, 2019). Further research is required into alternative storage temperatures and moisture contents for seed with intermediate storage behaviour due to desiccation or low temperature sensitivity.

Desiccation sensitive seeds often have different morphological and ecological characteristics compared to those seeds generally tolerant to desiccation. For example, desiccation sensitivity has been found more frequently in species growing in moist habitats, in seeds dispersed during the wet season or fleshy fruited seeds (Daws *et al*., 2005; Tweddle *et al*. 2003). However, Sommerville *et al*. (2021) found that tree habit, habitat and fleshy fruit did not make reliable predictors for the purpose of seed banking in Australian rainforest species.

1.4 Living collections for Plant Conservation

Conventional seed banking, although the most effective way to capture genetic diversity, has limitations and cannot be applied to all plant species. "Exceptional species" produce seeds that are not amenable to conventional methods or they do not produce seed at all (Pence, 2013). For these species *ex situ* conservation options are either cryopreservation (discussed previously) or living collections (Griffith *et al.*, 2020; Hoban *et al.*, 2020). Living collections form the core of botanic gardens and in addition to traditional roles in taxonomy, systematics, and teaching, living collections are relied on today for scientific and conservation purposes (Dosmann, 2006; Oldfield, 2009; Westwood *et al.*, 2021). Several globally threatened plant species are widely cultivated in botanic gardens and nurseries, often outnumbering wild stocks (Asmussen-Lange *et al.*, 2011). These living collections are critical for an integrated plant conservation approach, but their conservation contribution is highly dependent on the level of genetic diversity captured (Backs *et al.*, 2021; Westwood *et al.*, 2021). The suitability of *ex situ* material for recovery of threatened species has been found to be limited in some cases (Abeli *et al.*, 2020; Ensslin *et al.*, 2015) due to limited specimens (Griffith *et al.*, 2020; Hoban *et al.*, 2020) low genetic diversity (Christe *et al.*, 2014), low or no seed production (Cochrane, 2004) or negative consequences due to adaptations to *ex situ* conditions (Horiuchi *et al.*, 2020). Spontaneous hybridisation between morphologically similar congenerics can also occur in living collections, further eroding the suitability of progeny for conservation (Maunder *et al.*, 2004; Volis, 2017). Despite this, re-establishment or de-extinction of plant species has been achieved even in cases where founder populations were limited (Hitchcock *et al.*, 2020; Rumsey & Stroh, 2020) using various methods including micropropagation and cross-pollination of in living collections (Cibrian-Jaramillo *et al.*, 2013; Hyvärinen, 2020).

1.4.1 Pollen conservation

Cross-pollination is a common conservation strategy for endangered plants in botanic gardens and living collections and has been successfully applied for example in cycads (Iglesias-Andreu *et al.*, 2017), orchids (Del Vecchio *et al.*, 2019), trees, both angiosperms (Chen *et al.*, 2016) and gymnosperms (Vovides, 1997), and herbaceous species (Sudarmono, 2016). For cross-pollination to be successful, knowledge of the species reproductive biology, which includes pollinator abundance, pollen viability and breeding system, is essential particularly for species with small populations or limited founders (Chen *et al.*, 2016).

Where cross-pollination is conducted as part of an overall conservation plan, it is important that techniques are developed to store and test pollen viability to maximize chances of successful pollination and seed production (Towill, 2004; Wheeler & McComb, 2006). Pollen is becoming increasingly valuable in the conservation of critically rare species, in the restoration of trees and for cross-pollination of individuals which are isolated due to geography or asynchronous flowering (Towill, 2004). For example, pollen conservation of American chestnut trees (*Castanea dentata*: Fagaceae) is now providing options to restore genetic diversity to new chestnut lines which are resistant to chestnut blight (Fernando *et al.*, 2006; Walters & Pence, 2021). Pollen storage methods include conventional methods during which pollen is stored at room temperature, 5°C (fridge) or sub-zero temperatures ranging from -18°C (freezer) to -196°C (liquid nitrogen) (Araujo *et al.*, 2020). One of the essential steps in pollen conservation is the assessment of pollen viability through staining or *in vitro* germination, although germination media and incubation times need to be optimised for each species (Cruzatty *et al.*, 2020; Page *et al.*, 2006). The ability to extend pollen longevity through short- or long-term storage can be used to overcome temporal and geographical separation of flowering plants (Nadarajan *et al.*, 2018; Page *et al.*, 2006) and has been instrumental in the conservation of threatened species such as *Hymenoxys acaulis* var. *glabra*

(Asteraceae), *Brighamia insignis* (Campanulaceae) and *Pittosporum halophilum* (Pittosporaceae) (DeMauro, 1993; Towill, 2004). Although the creation of pollen banks is widely used in crop breeding, it is an underutilized tool for plant conservation and there is an urgent need for the development of species-specific pollen collection and storage protocols (Kormuťák *et al.*, 2019).

It is expected that *ex situ* plant collections of wild species will become increasingly important to prevent species extinction and aid in habitat restoration or reintroduction (Hay & Probert, 2013; Merritt *et al.*, 2014). It is therefore vital that collections of wild species are effectively managed and sufficient viable seeds are available. Although living collections is a valuable conservation tool for exceptional species, it is expected that germplasm in banks will survive several decades and outlive their counterparts in living collections (Walters & Pence, 2021).

Table 1.2: Summary of main cryopreservation methods and examples of species for which the method has been tested for zygotic embryo cryopreservation

Vitrification Method	Description	Advantages	Disadvantages	Examples of species	Post-cryo survival (%)	Post-cryo growth	Reference
Evaporative desiccation	Rapid/flash desiccation Slow desiccation Desiccation over nitrogen gas	Simplest cryopreservation method	Desiccation tolerant species are more amenable to this method Uneven drying of EAs	*Camellia sinensis*	95%	Seedling similar to phenotype	Chandel *et al.* (1995)
				Ekebergia capensis	80%	No shoots developed	Peran *et al.* (2006)
				Aesculus hippocastanum	80-100%	Abnormal and stunted growth	Wesley-Smith *et al.* (2001)
				Trichilia dregeana	0%	Callused	Whitaker *et al.* (2010)
				Landolphia kirkii	93%	4 week delay in growth	Pammenter *et al.* (1991)
Chemical desiccation through cryoprotective agents (CPA)	Use of Plant Vitrification Solutions PVS2 & PVS3 Applied through conventional vitrification or vacuum infiltration vitrification	Highly efficient for cryopreservation of vegetative shoots from many species. Easier to apply uniformly to uneven sized plant material such as EAs	Method is labour intensive CPAs can be toxic to some species or material thus requiring more research to optimise exposure time and method PVS can devitrify and form ice crystals during warming	*Theobroma cacao*	< 20%	No roots or shoots after freezing only callus	Pence (1991)
				Ekebergia capensis	30%	Not reported	Bharuth and Naidoo (2020)
				Syzygium maire	0%	None	Van der Walt *et al.* (2021a)
Desiccation-cryoprotection	A combination of rapid/slow desiccation and cryoprotection	Additional moisture removal possible from highly recalcitrant EAs	Labour intensive CPA can be toxic	*Boophone disticha* *Haemanthus bakerae* *H. coccineus* *Crinum bulbispermum* *Scadoxus puniceus*	30% 30% 35% 15% 55%	Stunted growth	Sershen *et al.* (2008)

Vitrification Method	Description	Advantages	Disadvantages	Examples of species	Post-cryo survival (%)	Post-cryo growth	Reference
Encapsulation-vitrification/ Encapsulation-Dehydration	EAs are encapsulated in a sodium alginate bead before dehydration through vitrification using either physical dehydration or CPAs	Higher survival rates have been recorded in EAs not amenable to any other method	Labour intensive Slower cooling rates of encapsulated beads in cryovials	*Ekebergia capensis*	80%	No shoots	Peran *et al.* (2006)
				Ilex species	3-37%	Not reported	Mroginski *et al.* (2011)
				Ekebergia capensis	80%	No shoots	Peran *et al.* (2006)
				Ilex species	3-37%	Not reported	Mroginski *et al.* (2011)
				Syzygium maire	< 20%	None	(Nadarajan *et al.*, 2021)

1.5 Rationale for the present study and species selection

The present study aims to investigate the *ex situ* conservation options for five nationally critical Myrtaceae species in New Zealand using the three main methods of *ex situ* conservation: conventional seed banking, cryopreservation and living collections. Little to no information has been published on the seed germination and post-harvest behaviour of News Zealand's fleshy fruited Myrtaceae species; *Lophomyrtus bullata, L. obcordata, Neomyrtus pedunculata* and *Syzygium maire* (Sommerville *et al.*, 2021). The two genera *Lophomyrtus* and *Neomyrtus* are endemic to New Zealand and are represented by three species, *L. bullata, L. obcordata* and *N. pedunculata* with both *Lophomyrtus* species highly susceptible to myrtle rust. Our pilot studies found that *L. bullata* seed loses viability during conventional storage while no research has been done on the germination or storage physiology of *Neomyrtus*. Outcomes from this study will identify suitable long-term storage options for *Lophomyrtus* and *Neomyrtus* which will enable seed conservation as an option to mitigate the impacts of myrtle rust.

Syzygium is of global importance with at least 24 species used for medicine or food (Cock, 2012; Hardman, 2017; Shü *et al.*, 2011; Van Wyk *et al.*, 1997). All *Syzygium* species tested to date produced recalcitrant seed and suffer from chilling injury when stored at low temperatures (Abbas *et al.*, 2003; Berjak & Pammenter, 2008; Nagendra *et al.*, 2019; Shü *et al.*, 2011) with seed remaining viable for a maximum of six months (Anandalakshmi *et al.*, 2005; Coelho de Araújo *et al.*, 2008; Van der Walt *et al.*, 2021b). To date, successful cryopreservation of *Syzygium* species is limited to one published study which had limited success in the cryopreservation of *S. francissi* shoots (Shatnawi *et al.*, 2004). Outcomes from the present study will contribute to fundamental knowledge for the cryopreservation of *Syzygium* species globally, and through this improve germplasm conservation prospects.

Concerns about low genetic diversity, inefficient pollination and low seed production in rare trees held in *ex situ* living collections have been raised in many studies (Enslin *et al.*, 2011; Griffith *et al.*, 2020; Tang *et al.*, 2020; Westwood *et al.*, 2021), including *Metrosideros bartlettii* (Myrtaceae), one of New Zealand's rarest trees (Drummond *et al.*, 2000). *Metrosideros bartlettii* is classified as nationally critical and the population, which is confined to the northern corner of North Island, consists of less than 14 individuals. The remaining individuals are threatened by introduced pests such as the brushtail possum and myrtle rust while reproduction failure is often cited as a reason for population decline (Drummond *et al.*, 2000; Melesse, 2017). *Metrosideros bartlettii* is found in cultivation throughout New Zealand, providing an opportunity to study the pollination biology of the species. This study will investigate the pollination system of *M. bartlettii* using DNA sequenced trees in cultivation. Knowledge of pollen biology for each tree as well as short and medium-term pollen storage options will provide an essential tool for the integrated conservation strategy of *M. bartlettii.*

1.6 Research aims

The importance and urgency of effective *ex situ* conservation of New Zealand Myrtaceae has been highlighted above and this thesis aims to identify suitable conservation options for five critically endangered species, four fleshy fruited species and one species with reproduction failure. The goal is to enable the conservation of these species by determining the optimal protocols for seed collection, germination, long-term storage of seed, pollen or embryos and cross-pollination. There is currently limited or no published information on the fruit and seed morphology, seed biology or seed storage behaviour for *Lophomyrtus bullata, L. obcordata, Neomyrtus pedunculata* or *Syzygium maire.* This study will provide novel information by comparing desiccation and freezing sensitivity of *Lophomyrtus* species and relate this to seed composition and thermal properties of the water and lipids. With no published information on *S. maire*, this study will have direct applicable conservation outcomes by determining protocols for seed collection, short-term storage and propagation. In-depth investigations into cryopreservation of zygotic embryos of *S. maire* is novel not only for *S. maire* but for the entire genus which comprises of more than 1,136 species globally. Using living collections of *M. bartlettii*, the study will be the first to test the hypothesis that *M. bartlettii* can hybridise with co-flowering *Metrosideros* species. Together with the confirmation of the species' breeding system, this information will have a significant impact on the management of *ex situ* collections to ensure genetically pure seed for conservation and restoration. The study will provide novel and fundamental information for the conservation of Myrtaceae species in the Pacific Region.

The specific objectives of this study were to:

1. Determine the pollination strategy of *M. bartlettii*, describe cross-pollination treatments and develop pollen conservation strategies for short-, medium- and long-term storage of pollen.
2. Describe fruit, seed, and embryo morphology as well as germination protocols for *Lophomyrtus bullata, Lophomyrtus obcordata, Neomyrtus pedunculata* and *Syzygium maire*.
3. Compare seed storage physiology between *L. bullata* and *L. obcordata* through lipid composition, desiccation tolerance, the combined effect of desiccation and freezing, long-term storage of desiccated seed and investigating thermal behaviour of free water and lipids in seeds at various moisture contents.
4. Investigate the suitability of conventional seed banking for *N. pedunculata* by establishing germination and seed viability assessments, desiccation tolerance/sensitivity and tolerance to freezing.
5. Investigate cryopreservation options for *S. maire* and evaluate the biochemical, physiological, and physical effects of various cryopreservation steps, with and without freezing, on embryo viability and plantlet formation.

1.7 Structure of the thesis

The structure of this thesis is based on various *ex situ* conservation methods and their applicability to the conservation of five highly threatened Myrtaceae species in New Zealand. The thesis begins with an overall introduction (chapter 1), experimental chapters (2 – 6) and overall discussion and conclusion (chapter 7). The research has been divided into three categories represented by five experimental chapters: (a) using living collections for the conservation of *M. bartlettii* (chapter 2): (b) comparing seed storage physiology *L. bullata, L. obcordata* and *N. pedunculata* (chapter 3): and (c) desiccation sensitivity and cryopreservation of *S. maire* (chapters 4 – 6). A more detailed description of the thesis structure is provided below:

Chapter 1

This chapter provides a literature review on *ex situ* conservation of Myrtaceae in New Zealand and identifies gaps in knowledge. It furthermore describes the aims and structure of the thesis in general.

Chapter 2

***Ex situ* conservation of *Metrosideros bartlettii* (Bartlett's rātā, rātā moehau), a critically endangered tree, endemic to New Zealand**

This chapter describes the use of living collections as an *ex situ* conservation method. It focusses on one of New Zealand rarest trees, *M. bartlettii* which is suspected to be self-incompatible. Using genetically sequenced trees in cultivation, the reproductive output was tested for various pollination treatments, including the potential to hybridise with co-flowering *Metrosideros* species. Fresh pollen viability was determined for various individual trees while the impact of desiccation and storage temperature on pollen viability was investigated to inform effective short-, medium- and long-term storage of pollen.

This work was published in the journal Pacific Conservation Biology and a poster was developed as a conservation tool for the species.

Chapter 3

The seed storage physiology of *Lophomyrtus* and *Neomyrtus*, two threatened endemic genera from New Zealand

This chapter investigates seed banking and evaluates the suitability of this method for the long-term seed storage of *L. bullata, L. obcordata* and *N. pedunculata.* The fruit, seed, and embryo morphology of the three species are described while the biochemical composition of *L. bullata* and *L. obcordata* seed is analysed to provide fundamental data to inform seed storage. Seed viability through radicle emergence and using tetrazolium chloride (TZ) staining of fresh, desiccated, and frozen seed are compared to determine if there is any desiccation and/or freezing sensitivity. Lastly, differential scanning calorimetry (DSC) scans are used to establish the thermal transitions of ice and lipids in fresh and desiccated seed of all three species.

Chapter 4

Phenology, fruit morphology and seed characteristics of *Syzygium maire* (Myrtaceae), a threatened endemic New Zealand tree

The phenology, fruit morphology and seed characteristics of *S. maire* are described from various populations as this forms the basis to confirm that the seeds are desiccation sensitive. The aim of the chapter is to address information gaps on the fruit development, germination, and short-term storage of *S. maire* seeds.

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Chapter 5

Oxidative stress, ultrastructure modification and physiological impacts of rapid desiccation of *Syzygium maire* zygotic embryos

This chapter investigates cryopreservation options for *S. maire* by using rapid desiccation of zygotic embryos. Although *S. maire* embryos are recalcitrant (chapter 4), excess moisture must be removed to prevent the formation of ice crystals during freezing. The chapter focusses on the use of desiccation to cryopreserve naked embryos. Additionally, it investigates the reasons for viability loss during desiccation by quantifying oxidative stress and assessing damage markers to determine extent of membrane damage during desiccation. Using DSC, we determine the size of thermal transitions to establish if ice crystals are present and compare the size of the enthalpy of melt at various moisture contents. Finally, ultrastructural observations are made of fresh, desiccated, and desiccated-frozen cells to investigate structural modification at each step.

This work was published in *Plants* (2022) 11, 1056. DOI 10.3390/plants11081056.

Chapter 6

The evaluation of droplet-vitrification, vacuum-infiltration vitrification, and encapsulation-dehydration for the cryopreservation of *Syzygium maire* zygotic embryos

The results from chapter 4 and 5 confirmed that seeds and zygotic embryos from *S. maire* are recalcitrant and therefore the only long-term germplasm storage option for this species is through cryopreservation. This chapter investigates the use of encapsulation-dehydration as a cryopreservation method for *S. maire* zygotic embryos. Additionally, the impacts of the cryoprotectant, Plant Vitrification Solution 2 (PVS2), on the cryopreservation of *S. maire* zygotic embryos are investigated. Two methods of applying PVS2 are tested, droplet vitrification (DV) and droplet vacuum infiltration vitrification (DVIV). The effect of applying PVS2 through these methods are evaluated in terms of embryo germination and plantlet formation while the effect of the methods on water content and phase behaviour is examined using differential scanning calorimetry (DSC).

Parts of this work has been published in CryoLetters 42(4), 202-209.

Chapter 7

This chapter provides an overview of the results from experimental chapters (2 - 6) and includes recommendations for future work. This chapter also provides some concluding remarks which includes a short analysis of problems encountered and their relevance to *ex situ* plant conservation.

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Chapter 2.

Ex situ conservation of *Metrosideros bartlettii* (Bartlett's rātā, rātā moehau), a critically endangered tree, endemic to New Zealand

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Abstract

Metrosideros bartlettii is one of the most threatened trees in New Zealand and with less than 14 individuals spread across three populations, the species is at high risk of extinction. Despite reproductive failure being identified as one of the factors contributing to population decline, little is known about its pollination biology. Using trees in cultivation, reproductive output was tested for autonomous self-pollination, self-incompatibility, interspecific compatibility (hybridisation) and apomixis. In addition, the viability of fresh pollen was determined for each tree and the impact of desiccation and storage temperature (5°C, -18°C and -196°C) on pollen viability assessed. *Metrosideros bartlettii* was found to be highly self-incompatible with seed formed via autonomous self-pollination having low viability. Hybridisation with *M. excelsa,* another native species commonly found in cultivation, produced viable seeds, but seedlings failed to survive. Pollen viability differed significantly among trees, and pollen stored at -18°C and -196°C retained viability after six months. This study has confirmed cross-pollination should be the preferred pollination method to increase seed production and supplement wild populations. Long-term storage of pollen at the temperatures determined here will help to overcome challenges in cross-pollination of trees that are spatially and geographically isolated. Overall, results from this study will guide the use of these *ex situ* collections to produce viable seed for seed banking or future restoration efforts.

Keywords: botanic garden, breeding system, hand pollination, hybridisation, integrated conservation, long-term storage, myrtle rust, pollen.

2.1 Introduction

It is estimated that more than 26% of tree taxa are threatened with extinction due to forest clearing for agriculture, logging, climate change and the global spread of pathogens (GTA, 2021; Sharrock, 2020). Similar trends have been recorded for New Zealand where half of the native vascular plants are at risk of extinction (de Lange, 2018). Native trees in New Zealand are threatened by large scale deforestation, invasive pest animals and weeds. More recently impacts from fungal pathogens such as myrtle rust (*Austropuccinia psidii*) resulted in all 29 Myrtaceae species classified as conservation concern (de Lange, 2018). One of these species is the critically endangered *Metrosideros bartlettii* (Bartlett's rātā, rātā moehau) (de Lange, 2014). Despite its 30 m height, this emergent endemic forest tree was first recorded in 1975, with a second locality confirmed in 1984 (Dawson, 1985). It is unlikely that *M. bartlettii* was ever common, and extensive surveys conducted in the 1990's found only 34 individuals in three populations located in the far north of New Zealand (Fig. 2.1). By 2016, 14 individuals in three localities remained in the wild (de Lange, 2014). Although the biggest historical decline of *M. bartlettii* was due to land use changes following settlements, more recent reasons for population decline include reproductive failure and devastating effects of the introduced brushtail possums (*Trichosurus vulpecula*) on buds, flowers and young shoots (de Lange, 2014). *Metrosideros bartlettii* has a high probability of extinction due to genetic, demographic, and environmental stochasticities, and urgent conservation measures are needed to increase the population size and genetic diversity (Melesse, 2017). Unfortunately, despite flowering prolifically fruit/seed set is low, and seedlings are rarely found in the wild (Drummond *et al.*, 2000). Therefore, conservation of *M. bartlettii* requires urgent research focusing on its reproductive biology and seed ecology to understand the lack of recruitment in wild populations.

Several threatened plant species are widely cultivated in botanic gardens and nurseries globally, often outnumbering wild stocks (Asmussen-Lange *et al.*, 2011). This is also the case for *M. bartlettii* which can be found in public and private gardens throughout New Zealand. However, the suitability of *ex situ* material for recovery of threatened species has been limited in some cases (Abeli *et al.*, 2020; Ensslin & Godefroid, 2020) due to low genetic diversity (Christe *et al.*, 2014), low or no seed production (Cochrane, 2004) or negative consequences due to adaptations to *ex situ* conditions (Horiuchi *et al.*, 2020). Spontaneous hybridisation between morphologically similar congenerics can also occur in *ex situ* collections, further eroding the suitability of progeny for conservation (Lozada-Gobilard *et al.*, 2020a; Volis, 2017). Despite this, re-establishment or de-extinction of plant species has been achieved where founder populations were limited (Abeli *et al.*, 2020; Hitchcock *et al.*, 2020) using methods such as micropropagation and cross-pollination (Cibrian-Jaramillo *et al.*, 2013; Hyvärinen, 2020). Cross-pollination is a common conservation strategy for endangered plants in botanic gardens and private collections and has been successfully applied in cycads (Iglesias-Andreu *et al.*, 2017), orchids (Del Vecchio *et al.*, 2019), trees (Chen *et al.*, 2016) and herbaceous species (Sudarmono, 2016). For cross-pollination to be successful, knowledge of the species reproductive biology, pollen viability and breeding system, is essential particularly for species with small populations or limited founders (Chen *et al.*, 2016).

Where cross-pollination is conducted as part of an overall conservation plan, it is important that techniques are developed to store and test pollen viability to maximize chances of successful pollination and seed production (Towill, 2004; Wheeler & McComb, 2006). *In vitro* germination of pollen can provide an effective method for viability assessment, although the germination media and incubation time need to be optimized for each species (Cruzatty *et al.*, 2020; Page *et al.*, 2006). The ability to extend pollen longevity through short- or long-term storage can be used to overcome temporal and geographical separation of flowering plants (Nadarajan *et al.*, 2018; Page *et al.*, 2006) and has been used in the conservation of threatened species such as *Hymenoxys acaulis* var. *glabra, Brighamia insignis* and *Pittosporum halophilum* (DeMauro, 1993; Towill, 2004). Although the creation of pollen banks is widely used in crop breeding, it is an underutilized tool for plant conservation and there is an urgent need for the development of species-specific pollen collection and storage protocols (Kormuťák *et al.*, 2019).

The current study was undertaken to gain knowledge of the reproductive biology of *M. bartlettii* by using trees in cultivation for which the source population is known. The specific objectives of the study were to: 1) gain insight into the breeding system and reproductive success of selected individual trees in cultivation, 2) determine seed characteristics, seed germination and seedling establishment, 3) compare pollen viability between trees and establish short-, medium- and long-term pollen storage protocols and, 4) advise feasible conservation actions for *M. bartlettii* which can be applied to plants in cultivation and in the wild.

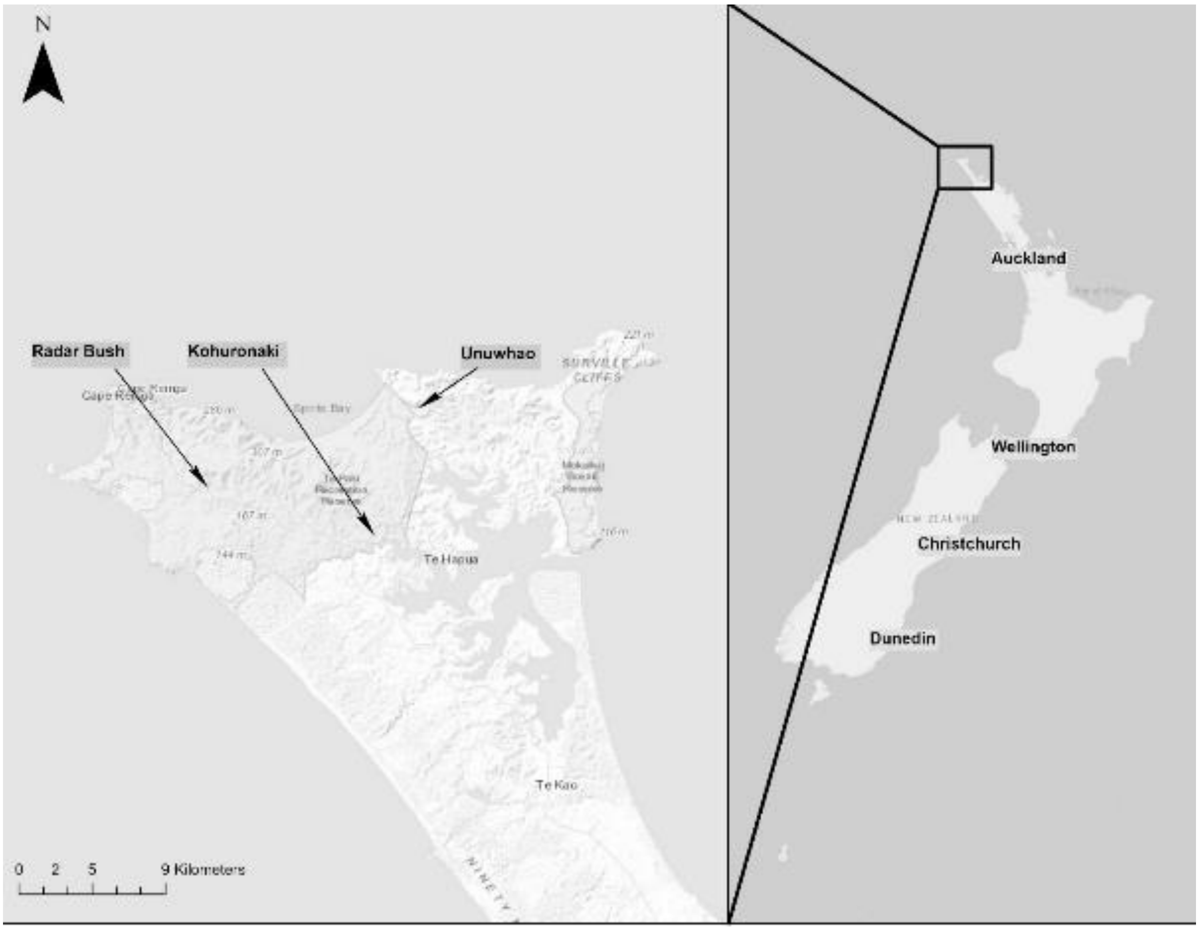


Figure 2.1: Locations of the three populations of *Metrosideros bartlettii* in the far north of New Zealand

2.2 Materials and Methods

2.2.1 Study trees

All the trees used in this study are in cultivation, both at botanic and private gardens, in Wellington and Auckland, New Zealand. The trees were selected based on presence of flowers, known source population (through DNA sequencing), accessibility, and landowner permission. A total of seven individual trees, representing two wild populations (Kohuronaki and Radar Bush), were used in this study (Table 2.1).

Pollination studies were conducted during the flowering period for *M. bartlettii* in November/December of 2017 and 2019, with capsules, seed set, and seed viability assessed in March/April of 2018 and 2020.

Table 2.1: Source population, *ex situ* locality, tree code of cultivated trees used to determine the breeding system of *Metrosideros bartlettii*. Pollination treatments performed included Autonomous self (AS), apomixis (APO), hand-self (HS), natural (N), pollination with a different source population (×DS), pollination with different individual from same source population (×SS), hybridised with *Metrosideros excelsa* (HY)

Source population	*Ex situ* locality	Tree code	Pollination treatments
Kohuronaki	Ōtari Botanic Garden -Offshore Island garden, Wellington	ŌOI	AS, APO, HS, N, ×DS, ×SS
Kohuronaki	Ōtari Botanic Garden - Alpine Lawn, Wellington	ŌAL	HY
Kohuronaki	Te Aro, Wellington	TAW	AS, HS, N, ×DS, ×SS
Radar Bush	Thomas Building, The University of Auckland	TUA	None (Pollen donor in 2017)
Radar Bush	Waiwhetū, Lower Hutt	WLH	AS, HS, N, ×DS, ×SS
Radar Bush	Entrance to Percy's Reserve, Lower Hutt	PLH	None (Pollen donor in 2019)

2.2.2 Flower development and pollen morphology

Ten flower clusters containing flowers in bud were randomly tagged among the two trees at Ōtari Native Botanic Garden and Wilton's Bush Reserve (Ōtari). Flower development from bud to pollen shed was monitored daily and changes in the position of the style and stamens were recorded. Flowers were considered mature when all stamens were shedding pollen. Scanning electron microscopy (SEM) and light microscopy (LM) were used to examine the morphology of fresh pollen and stigmas. For SEM observations, pollen and stigmas were preserved in ethanol (70%). At the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North) samples were critical point dried, sputter-coated with gold and observed and photographed using a Cambridge 250 SEM.

2.2.3 Breeding system

Pollination experiments were applied to randomly selected inflorescences on four trees (Table 2.1), these experiments included autonomous self-pollination (AS), apomixis (APO), hand self-pollination (HS), natural pollination (N), pollination with different source population (×DS), pollination with a different individual from the same source population (×SS) and hybridisation with *Metrosideros excelsa* (HY). These treatments tested for autonomous self-pollination, self-incompatibility, interspecific compatibility (hybridisation) and apomixis (asexual reproduction in which seeds are produced from unfertilized ovules). Natural pollination, in which no flower manipulation was applied, represented the control. For pollination experiments, three to six flowering branches were randomly assigned to a treatment and marked with flagging tape. At each branch, floral buds were counted and then covered with a fine mesh bag to prevent introduction of any foreign pollen (Neal & Anderson, 2004). Before anthesis, flowers assigned to hand pollination (e.g. HS, ×DS, ×SS and HY) and apomixis treatments were emasculated using tweezers and bagged again until the style had straightened indicating that the flower reached maturity (Fig. 2.2). Flowers assigned to test for apomixis remained bagged until floral senescence. In other treatments, a clean paint brush was used to transfer pollen between mature flowers on the same tree (self-pollination), between flowers on different trees of the same species (cross-pollination) or between flowers of different species (hybridisation) (Table 2.2). The process was repeated for three to five days later. Flowers remained bagged until the stigma wilted, which was approximately three weeks after pollination. Capsules were collected at maturity (between March and April). The outcome of each treatment was assessed by evaluating fruit set (ratio of capsules to pollinated flowers) and capsule size (length × width). Capsule size was also measured for 15 capsules produced outside pollination of treatments. The viability of the pollen used in each treatment was verified as per methodology described below.

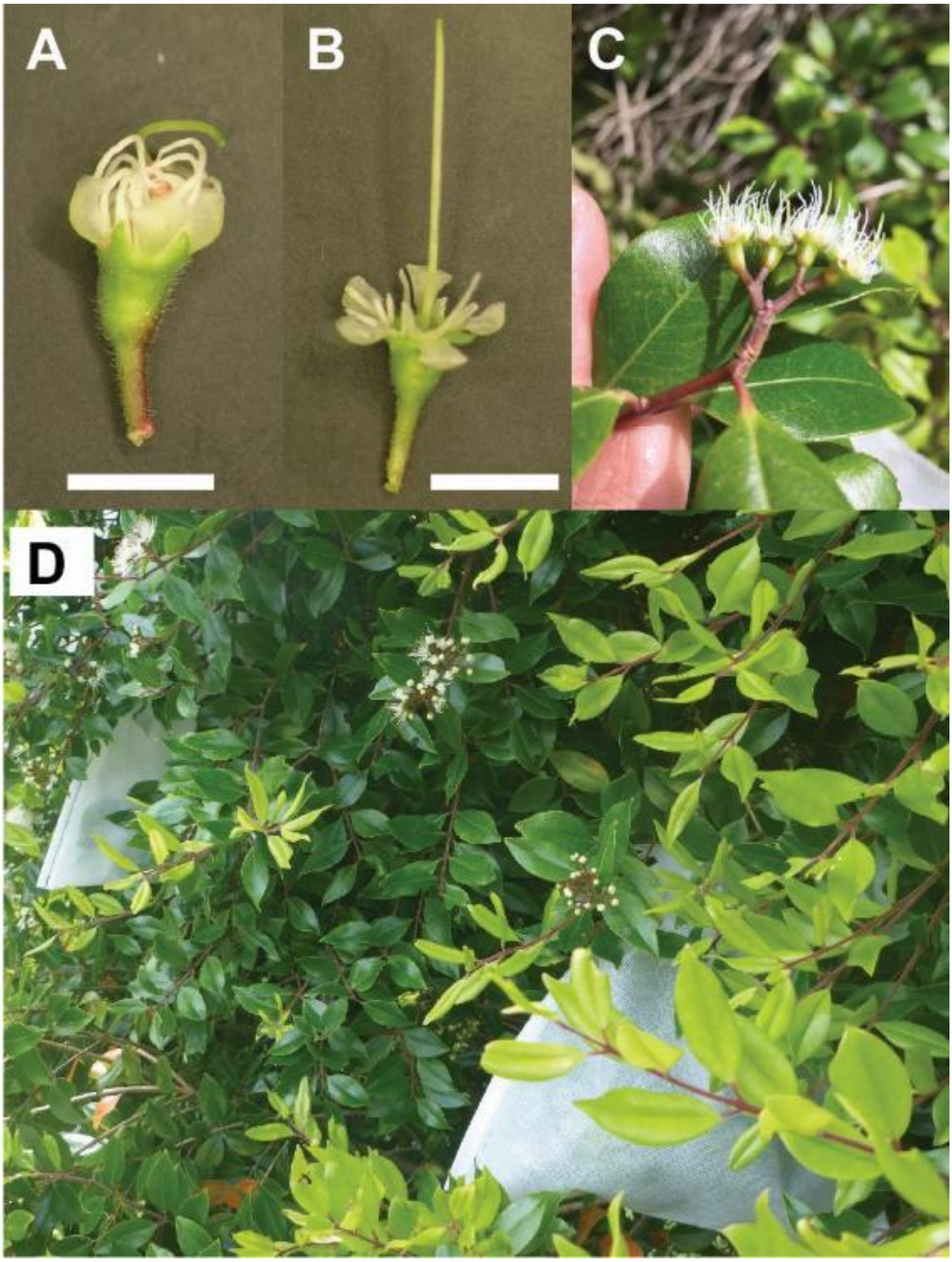


Figure 2.2: *Metrosideros bartlettii* flower ready for emasculation (A), emasculated flower (B) and emasculated flowers on the tree before pollination (C). Flowers in various pollination treatments were covered with a fine mesh nylon bag (D).

Table 2.2: Description of pollination treatments, number of flowers and pollen source used in each pollination treatment applied to determine the breeding system for *Metrosideros bartlettii*. AS: Autonomous self-pollination. APO: Apomixis. HS: Hand self-pollination. HY: hybridised with *Metrosideros excelsa*. N: Natural pollination. ×SS: pollination with different individual from same source population. ×DS: pollination with a different source population.

Pollination treatment	**Number of flowers**	**Flowers emasculated**	**Pollen source**	**Flowers bagged**
AS	1007	No	Same tree/flowers	Yes
APO	20	Yes	None	Yes
HS	206	Yes	Same tree/flowers	Yes
HY	43	Yes	*Metrosideros excelsa*	Yes
N	1319	No	Open	No
×SS	420	Yes	Different tree from the same source population	Yes
×DS	983	Yes	Different tree from a different source population	Yes

2.2.4 Seed viability, germination potential and seedling development

The percentage of viable seed per treatment was determined by germinating 200 randomly selected seeds. In cases where no germination was observed, remaining seed from the treatment was scanned for seeds with a developed embryo using a stereo microscope (Fig. 2.3). If seeds containing an embryo (filled) were found, these were used to assess germination potential and seedling establishment.

Figure 2.3: *Metrosideros bartlettii* seed were classified as empty (embryo-lacking) or filled (containing an embryo). Red arrows indicate filled seed.

Seeds were germinated in 90 mm Petri Dishes containing 7% water agar using an incubator set at 15/25 °C with a 16 h dark/8 h light cycle. To limit infection by fungi or bacteria, seeds were surface sterilised by washing in 5 g/L sodium dichloroisocyanurate (NaDCC) for 20 min followed by three rinses in sterile distilled water. Radicle protrusion of at least 2 mm was the criterion for germination, with seedling establishment classified as the development of cotyledons. Germination was scored three times a week for six weeks which is the maximum time needed for seed germination. Non-germinated seeds were inspected under a stereo microscope to determine if they contained an embryo. To assess seedling survival and development, germinated seeds were transferred to a petri dish containing soil collected from the forest at Ōtari and returned to the incubator. After six weeks in soil, seedling size was visually compared and then seedlings planted in pots containing commercial seed raising mix soil and transferred to the nursery at Ōtari.

2.2.5 Self-incompatibility

The index of self-incompatibility (ISI) was calculated based on Schmidt-Adam *et al.* (1999) and Koelling *et al.* (2011). Individuals with an ISI of 0.2 or less were considered self-incompatible:

$$\text{ISI} = \frac{\text{Percentage of viable seeds per capsule after self-pollination}}{\text{Percentage of viable seeds per capsule after cross-pollination}}$$

For ISI calculation only AS, HS and ×DS treatments were considered

2.2.6 Pollen viability, desiccation, and storage

Fresh pollen viability was assessed for four trees (ŌOI, PLH, TAW and WLH) in November 2019 and for two trees (ŌOI and PLH) in November 2020 (Table 2.1). Pollen collected from ŌOI in November 2020 was used to investigate the effect of desiccation and temperature on short- and medium-term storage of pollen. A clean paint brush was used to collect pollen from the anthers under a stereo microscope. Pollen viability was assessed through *in vitro* germination on semi-solid Brebaker & Kwack (BK) pollen medium (Brewbaker & Kwack, 1963) consisting of 300 mg/L $Ca(NO_3)_2.4H_2O$ (hydrated calcium nitrate), 200 ppm $MgSO_4.7H_2O$ (hydrated magnesium sulphate), 150 ppm boric acid, 100 ppm KNO_3 (potassium nitrate) supplemented with 20% (w/v) sucrose. Pollen was considered germinated when the pollen tube length was greater than the diameter of the grain. To screen for desiccation sensitivity, freshly collected pollen was desiccated at 5°C for three days over a solution of lithium chloride (g/L) generating a relative humidity of ~15% (Gold & Hay, 2014). The impact of desiccation and storage temperature was assessed by storing non-desiccated and desiccated pollen at 20°C (room temperature), 5°C, -18°C and -196°C. Frozen pollen was thawed at room temperature for two hours before slow rehydration in a high humidity chamber for four hours at 25°C. Pollen was then germinated *in vitro* for 72 h at 25°C to assess its viability. All treatments consisted of five replicates containing a minimum of 100 pollen grains.

2.2.7 Statistical analysis

Differences in seed set, capsule size and seed viability between treatments, as well as variables associated with pollen viability between trees and across storage treatments, were analysed using a one-way *ANOVA followed by pairwise comparisons using Fisher's LSD (normally distributed data) or Kruskal Wallis test (data not normally distributed). Since ANOVA does not assume equal sample sizes provided variance between samples are not big, the unequal sample sizes were managed through minimal differences in variance between treatment A general linear model (GLM) was used to analyse the pollen viability affected by three variables (time in storage, desiccation, and temperature) and their interactions. A two-way ANOVA was used to investigate the relationship between desiccation and storage temperature on pollen viability. Arcsine square root transformation was used to normalise data where needed. Results are presented as the mean ± one standard deviation. All statistical analysis was conducted using XLStat Software version 1.3 (2021) and SAS/STAT version 14.2.

* Equal sample size is not one of the assumptions made in an ANOVA although unequal sample sizes in the pollination treatments might have reduced the statistical power of the test. ANOVA is considered robust to moderate departures from the equal variance assumption and treatments did not violate the variance assumption.

2.3 Results

2.3.1 Flower development and fruit development

The flowering period for *M. bartlettii* in cultivation lasts approximately for three weeks. During the three-year observations (2017, 2019 and 2020), the trees flowered from mid-November until the first week of December. Trees across Wellington were slightly out of sync; those in private gardens (TAW and WLH) were in peak flowering between 19 and 29 November, while the trees at Ōtari (ŌOI and ŌAL) were lagging 7–10 days behind, with flowers fully open from 29 November until 10 December. Flower development from bud to the start of pollen shed was completed in five days (Fig. 2.4). The style emerged from the flower bud on day 1, approximately 12–24 hours before the anthers. At the end of day 2, the style and anthers were all erect, but pollen shed was only initiated on day 4. Flowers wilted approximately seven days after pollen shedding was first recorded.

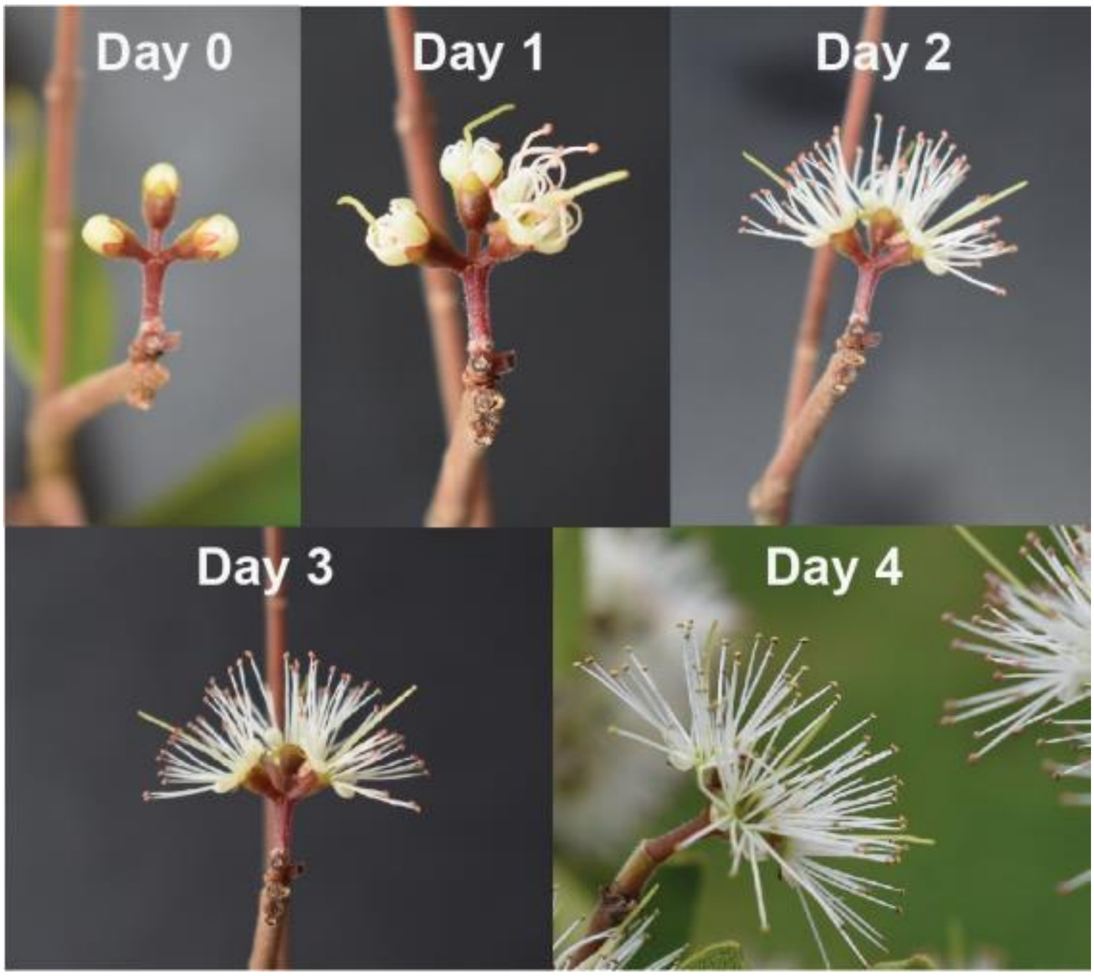


Figure 2.4*:* Flower development in *Metrosideros bartlettii* from bud stage on day 0 to pollen shed on day 4.

The complete reproductive event, from buds to seed dispersal, takes five months. Initial capsule development is visible four weeks after pollination (mid-December) and mature capsules split open to release seed late-March or early-April.

Despite using more than 1,100 flowers in pollination treatments on the TAW tree, fruit set was only 2%. This contrasted with WLH in which all treatments resulted in similar fruit set (Fig. 2.5A). In 2018, ŌOI produced capsules on ×DS treatments exclusively (Fig. 2.5C) but in 2020, the same tree produced capsules on all treatments except for N, although capsules were also formed outside pollination treatments (Fig. 2.5B). Pollination treatment had a significant impact on capsule size ($p < 0.001$) with the biggest capsules (17.2 ± 4.2 mm) obtained by HY while the smallest capsules were by AS treatments (10.8 ± 2.8 mm) (Fig. 2.5D). APO treatments did not produce any capsules in 2018.

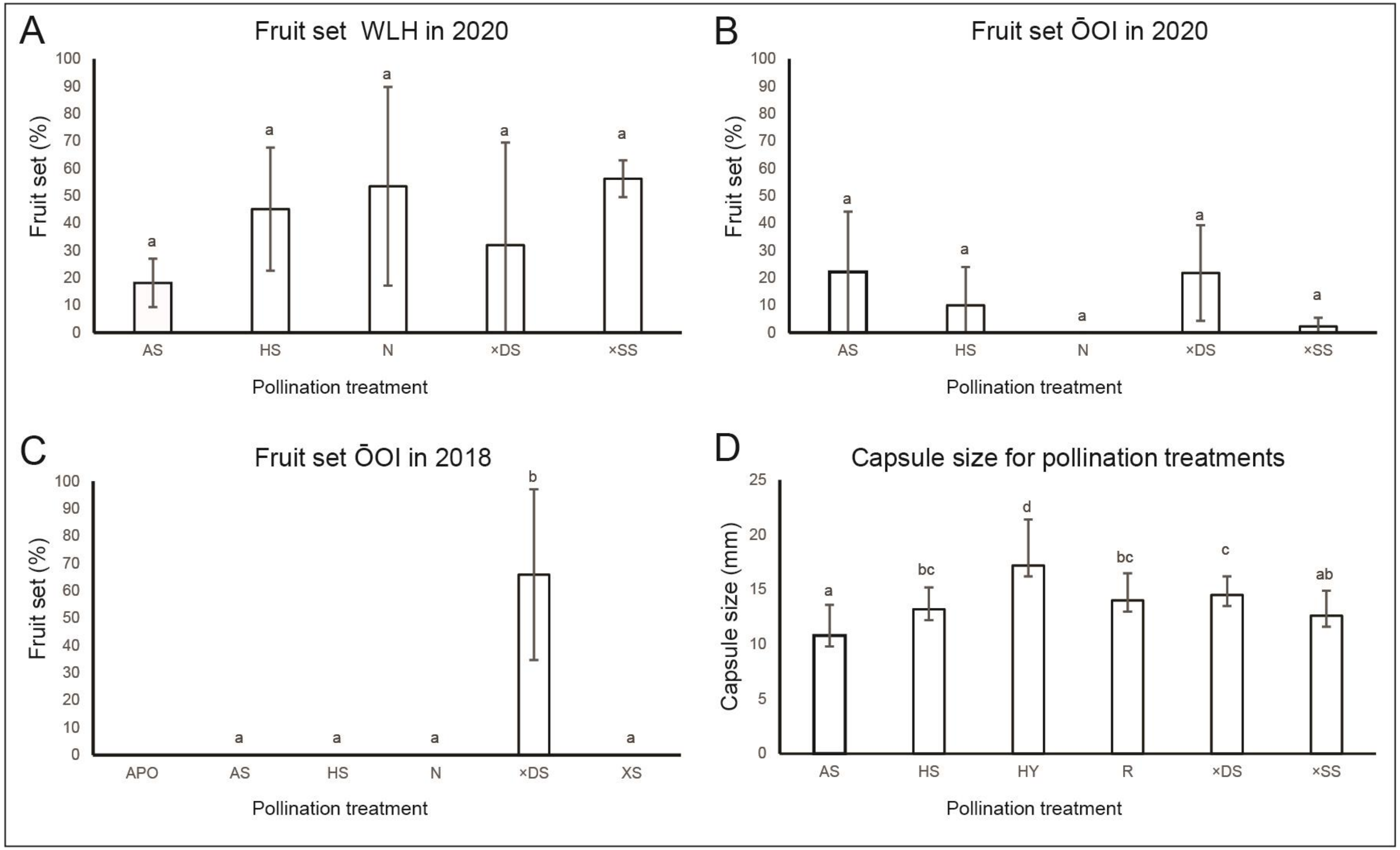


Figure 2.5: Fruit set (A–C) and capsule size (D) from various pollination treatments for two trees (WLH and ŌOI) in 2018 and 2020. Autonomous self (AS), apomixis (APO), hand self (HS), natural (N), pollination with a different source population (×DS), pollination with different individual from same source population (×SS), hybridised with *Metrosideros excelsa* (HY.) Different letters above bars indicate significant difference at $P < 0.05$ based on Fisher's LSD test

2.3.2 *Self-incompatibility*

Metrosideros bartlettii was found to be highly self-incompatible with only one tree (WLH) producing viable seed from self-pollination treatments, but viability of these seeds barely reached 1% (Table 2.3). The ISI score for this tree was 0.06.

Table 2.3: Percentage viable seed for capsules collected in 2018 and 2020. Results are displayed as mean ± SD, letters in each column indicate significant difference at P < 0.05 based on Fishers LSD test.

Tree code and year capsules were collected (source population)	Pollination Treatment	Viable Seed (%)
ŌOI 2018 (Kohuronaki)	AS	n/a
	HS	n/a
	N	n/a
	×DS (Radar Bush - TUA)	21.5 11.9[a]
ŌOI 2020 (Kohuronaki)	AS	0 ± 0[b]
	HS	0 ± 0[b]
	×DS (Radar Bush - WLH)	0 ± 0[b]
	×SS (Kohuronaki - TAW)	0 ± 0[b]
ŌAL 2020 (Kohuronaki)	AS	n/a
	HY	0.1 ± 0[b]
	HS	n/a
	N	n/a
	×SS (Kohuronaki – ŌOI)	n/a
WLH 2020 (Radar Bush)	AS	1.0 ± 1.9[b]
	HS	0 ± 0[b]
	N	2.0 ± 2.7[b]
	×DS (Kohuronaki - TAW)	15.6 ± 19.6[a]
	×SS (Radar Bush - PLH)	0.3 ± 1.2[b]
TAW 2020 (Kohuronaki)	AS	n/a
	HS	n/a
	N	n/a
	×DS (Radar Bush - WLH)	0 ± 0[b]
	×SS (Kohuronaki - ŌOI)	0 ± 0[b]

2.3.3 *Seed viability, germination potential and seedling development*

Overall seed viability was low with the highest viability (21.5 ± 11.9%) observed in seeds produced by ×DS treatments (Table 2.3, ŌOI). Of the 43 flowers used for HY in ŌAL, 11.6% produced capsules although seed viability was very low (<1%) despite the presence of viable pollen on *M. excelsa* (data not included). Capsules from ×SS treatments did not contain any viable seed.

Mean germination of filled seeds was highly variable ranging from ~20% for seeds from randomly selected capsules (those outside of the pollination treatments) to ~93% in ×DS treatments (Fig. 2.6A). All seedlings transferred to soil survived (Fig. 2.6B) but after six weeks in soil, seedlings from randomly selected capsules were smaller than those from ×DS treatments (Fig. 2.6C). Seedlings from ×DS treatments established successfully in nursery conditions (Fig. 2.6D) but those from randomly selected capsules failed to survive in nursery conditions. Although germination rate was similar across treatments, seedlings from the HY treatments failed to survive for longer than four weeks *in vitro* compared to high survival from other treatments.

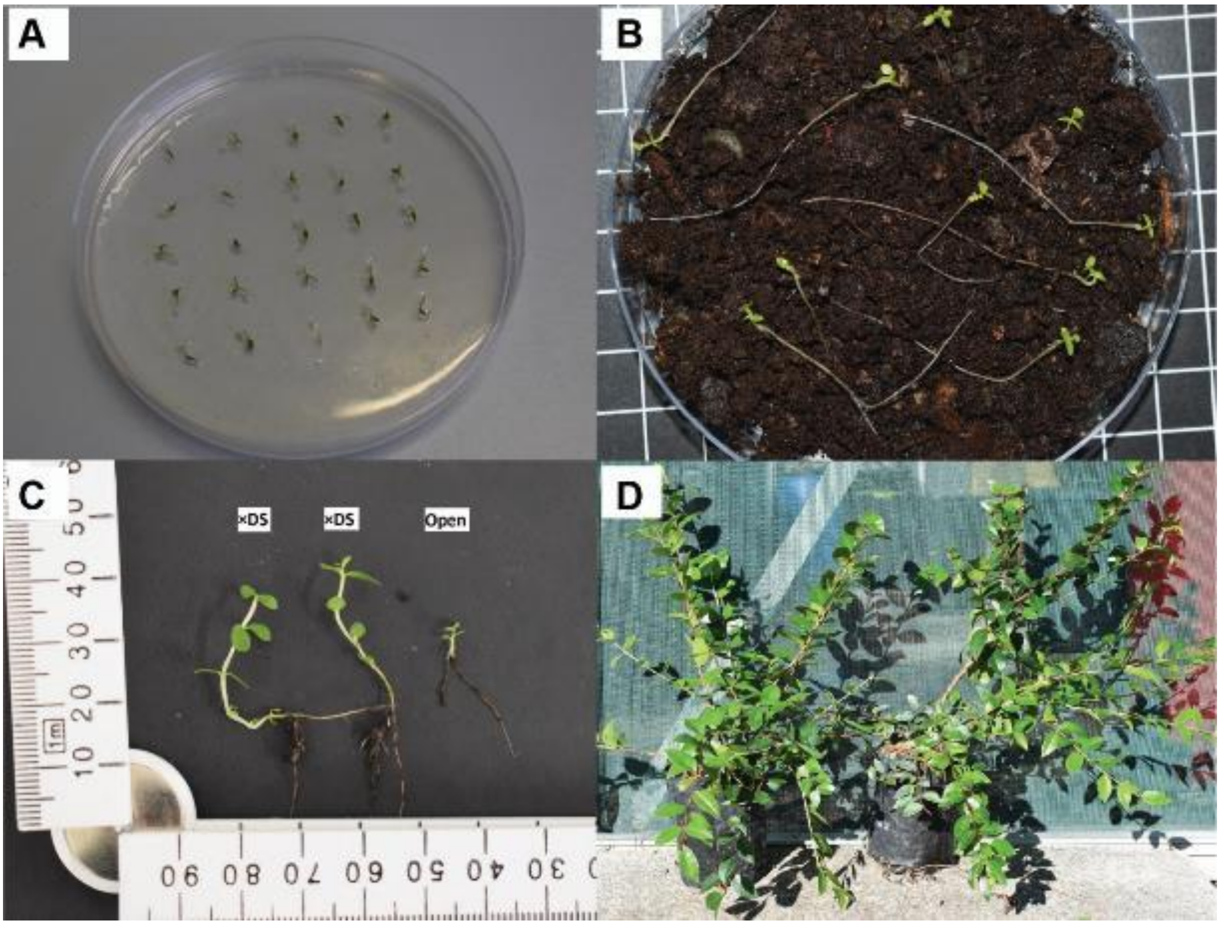
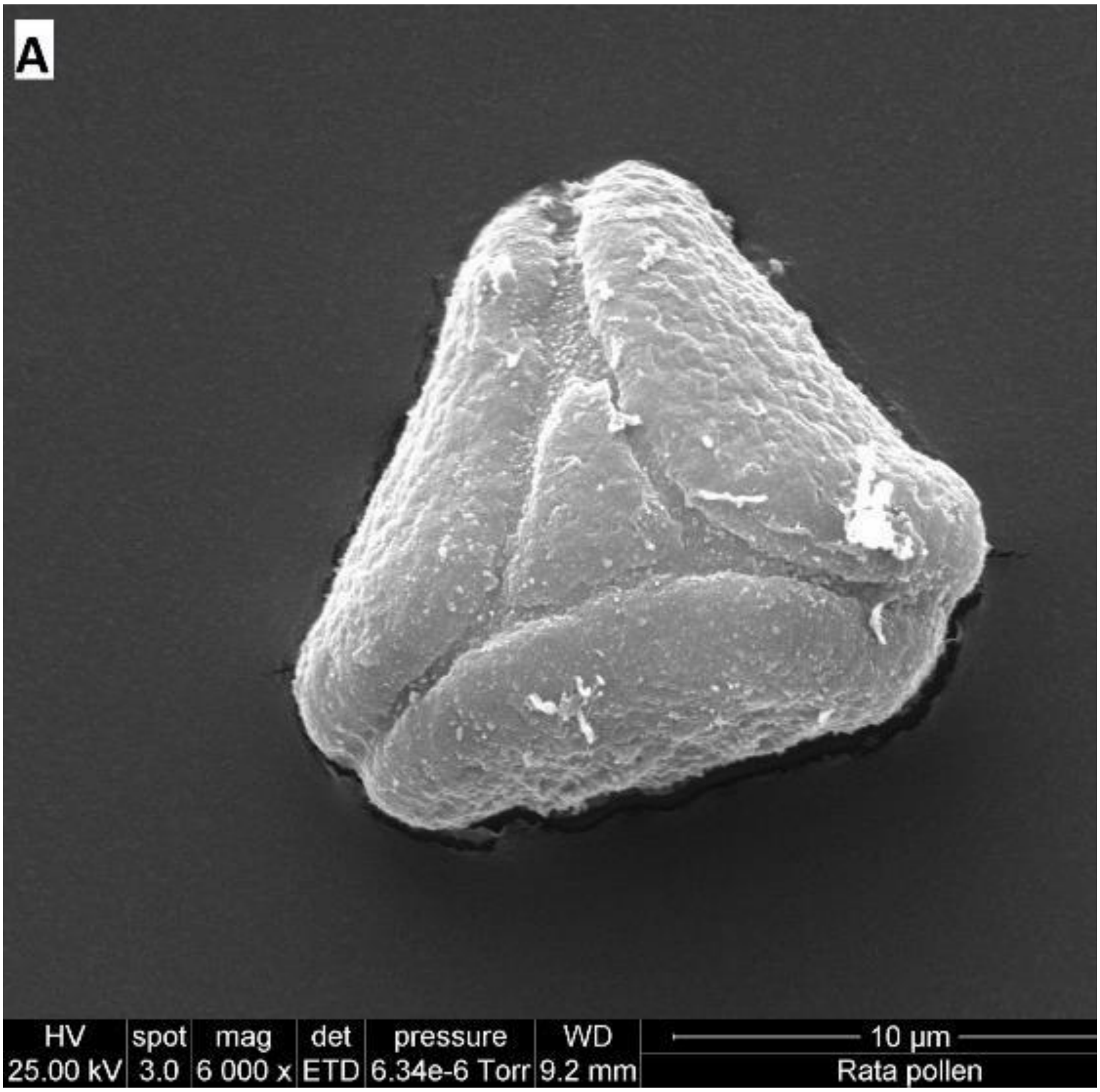


Figure 2.6: Germination of embryo-containing seeds of *Metrosideros bartlettii* formed after cross-pollination using pollen from a difference source population (×DS) and posterior seedling development. Seed germination on agar after 7 days (A). Six-week-old seedlings transferred to soil in petri dishes (B). Differences in size between seedlings formed by ×DS and open pollination (C) and three-year-old plants from ×DS pollination treatments established in nursery conditions (D).

2.3.4 Pollen viability, desiccation, and storage

Metrosideros bartlettii pollen grains are tricolpate and ~ 30 µM in size (Fig. 2.7A). Stigmas collected at time of anthesis were covered with *M. bartlettii* pollen (Fig. 2.7B) including germinating pollen (Fig. 2.7C). Pollen viability in 2019 differed significantly between individual trees with the highest viability recorded in PLH (78%) and WLH (74%) and lowest viability associated with TAW (50%). Viability also differed significantly between years for the same tree with higher viability associated with pollen collected in 2019 for both ŌO1 (58%) and PLH (78%) compared to 2020 when viability was 37% and 67% respectively.

Desiccating pollen to ~15% eRH did not significantly impact viability (P = 0.848) but pollen stored at room temperature (20°C) lost all viability within seven days irrespective of desiccation treatment (Fig. 2.8A). No viability loss was recorded in desiccated pollen stored for six months at –18°C (42%) and –196°C (43%) compared to very low viability for pollen stored at 5°C (Fig. 2.8B & C).



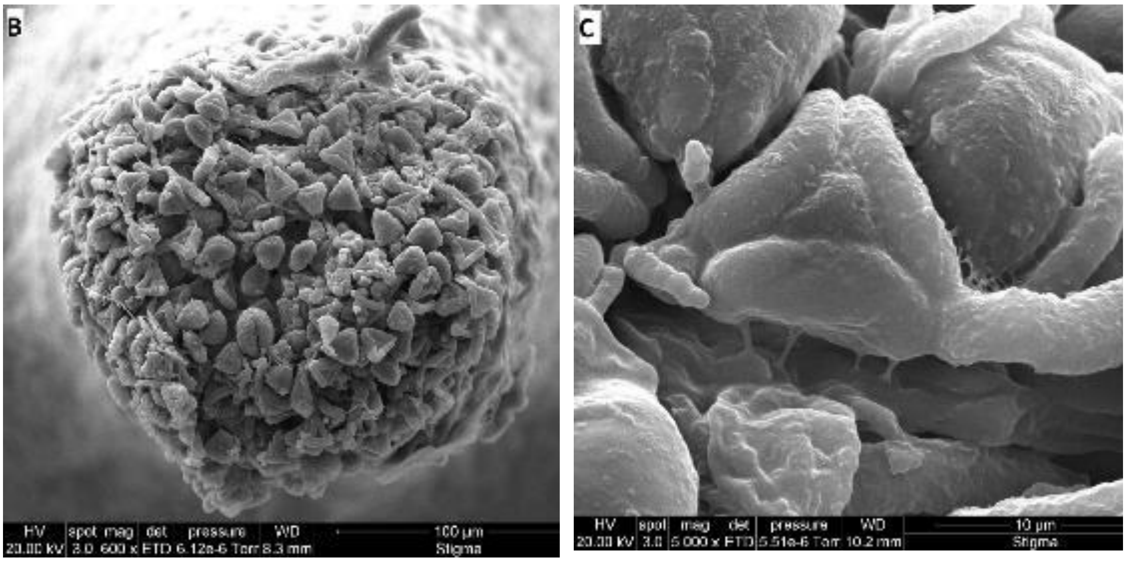


Figure 2.7: *Metrosideros bartlettii* pollen grain (A), with the stigma covered with pollen (B) including germinating pollen grains (C).

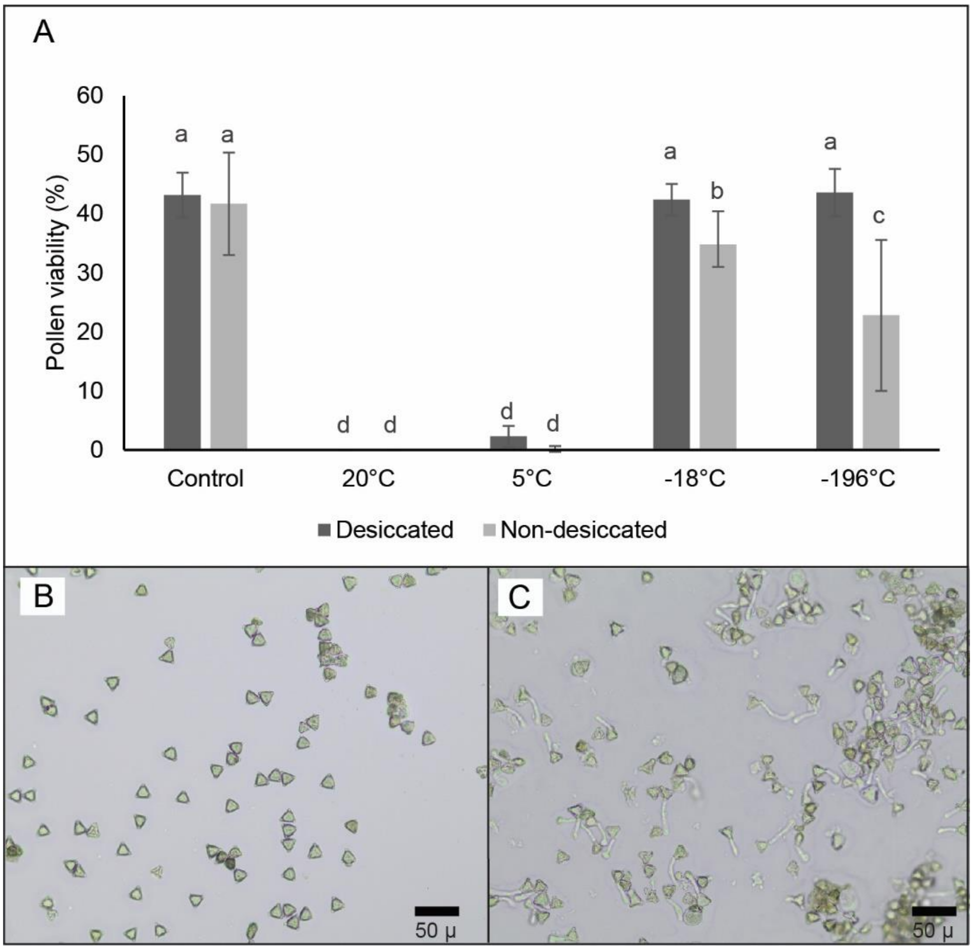


Figure 2.8*:* Viability of non-desiccated and desiccated pollen of *Metrosideros bartlettii* stored for six months at 20°C, 5°C, -18°C and -196°C (A) and *in vitro* germination experiments of non-desiccated pollen stored at 5°C (B) and desiccated pollen stored at -196°C (C) after six months storage. Control indicates fresh viability of non-desiccated and desiccated pollen. Different letters above bars indicate significant difference at $P < 0.05$ based on Fishers LSD test.

2.4 Discussion

This study has demonstrated that *M. bartlettii* is a highly self-incompatible tree where the production of viable seeds is significantly increased by cross-pollination with pollen sourced from trees from a different population. This finding is not unexpected as self-incompatibility is common in Myrtaceae and it has been previously reported in other *Metrosideros* species in New Zealand and overseas (Carpenter, 1976; Cause *et al.*, 2013; Schmidt-Adam *et al.*, 1999). In all known examples, however, a mosaic of self-compatibility and partially self-incompatible individuals occur within the same population. The differences in self-compatibility observed here between the trees TAW and WLH may be an indication that this mosaic also occurs in *M. bartlettii*. In the case of *M. collina*, a species from Hawai'i, differences in self-compatibility are also linked with differences in flower colour (Carpenter, 1976). Such flower colour polymorphism has not been reported in *M. bartlettii* and in this study, no other noticeable morphological differences were noted between trees, but this study included only a small number of individuals and they may not reflect the morphological diversity occurring in natural populations. Future studies should investigate morphological diversity of each of the 14 remnant trees and their mating system as a bias towards self-incompatibility, along with the lack of suitable pollinators to move pollen between compatible trees, may be responsible for the poor recruitment reported for this species (Drummond *et al.*, 2000).

Fertility in *Metrosideros* species is generally very low and this appears to be genetically determined rather than caused by pollinator or resource limitation (Schmidt-Adam *et al.*, 1999). It is likely that low fertility, along with self-incompatibility, may help to promote heterozygosity within populations by favouring the development of ovules fertilised by only a distinct genotype. It is known that outcrossing is paramount to the reproductive success in *M. excelsa,* with progeny originating from self-pollination unlikely to survive in natural populations (Schmidt-Adam *et al.*, 2009). As with other Myrtaceae species, flower development in *M. bartlettii* is characterised by a temporal separation of stigma receptivity and pollen shed (i.e. dichogamy) a well-known mechanism to prevent self-pollination (Beardsell *et al.*, 1993). In this study, most of the pollination treatments applied to *M. bartlettii* triggered capsule formation, but seed viability was greater than 15% only in those treatments that used pollen from a different source tree (Table 2.3). This outcome was however not consistent as the ŌOI tree failed to produce viable seeds in the second flowering season (Table 2.3) even though the pollen donor was from a different source population. The pollen donors for ŌOI were however different trees in 2017 (TUA) and 2020 (WLH). Meanwhile, the tree TAW also failed to produce viable seeds after using pollen from different source population (Table 2.3). Since none of these trees produce viable seeds by any other treatment in that season, it is possible that resource limitation or environmental conditions, rather than pollination treatment, affected the production of viable seeds. These hypotheses are supported by the results from ×DS treatments on ŌOI in 2018, which produced the highest percentage viable seed (21.5%) in this study (Table 2.3). An alternative explanation is that some of these trees are genetically too similar for effective pollination and future genetic studies will be useful to determine this. Finally, a somehow unexpected result was that *M. bartlettii* also readily produced capsules outside of ×DS treatments (i.e. N and AS treatments, as well as outside of treatments), but the seed viability associated with these capsules was very low (<1.6%) and seedlings failed to survive under nursery conditions. It is likely these seeds have originated by hybridisation. We have experimentally confirmed hybridisation between *M. bartlettii* and *M. excelsa* results in capsule formation, but these seeds have very low viability (Table 2.3). Birds and insects (honeybees, bumble bees, native solitary bees, and flies) were commonly observed feeding on *M. bartlettii* and these may facilitate transport of pollen from other species of *Metrosideros* flowering within the gardens/neighbourhood. An alternative explanation is that these capsules were formed via self-pollination. Similar scenarios have been reported in many other mass flowering Myrtaceae species in which wind and rain may be responsible for self-pollination, but the production of viable seed is low or unlikely (Beardsell *et al.*, 1993; Griffin, 1980).

This study has confirmed cross-pollination is the only pollination method to increase fruit and obtain viable seeds to supplement wild populations. Hand pollination has been successfully applied to critically endangered tree species such as *Magnolia sinica* a pollinator dependant species in China (Chen *et al.*, 2016) and *Ocotea catharinensis* in Brazil (Montagna *et al.*, 2018). For the critically endangered *Morus boninensis,* controlled crossing between genotypes is now the only way to obtain pure seedlings due to extensive hybridisation with the invasive species *M. accidosa* (Tani *et al.*, 2006). Spontaneous hybridisation in *ex situ* collections can result in maladaptive changes in life-history traits, fitness, and genetic diversity, rendering plant material unsuitable for restoration (Lozada-Gobilard *et al.*, 2020b). Genetic studies have confirmed hybridisation between *Metrosideros* species in New Zealand (Gardner *et al.*, 2004; Melesse, 2017) and, as mentioned earlier, our study found that hybridisation with co-flowering *M. excelsa* produced viable seed. However, seedlings of hybrid origin were less vigorous than those seedlings generated via ×DS treatments and failed to survive beyond four weeks. It should be noted that the hybridisation treatment was limited to a single tree. It is recommended that future studies investigate fruit set, seed viability and seedling vigour from hybridisation using a larger sample size and including *M. robusta* another co-flowering species. This will provide fundamental information for the collection of seed from trees in cultivation and their potential use to reinforced extant wild populations since seedlings obtained from hybridisation will erode the genetic integrity of *M. bartlettii* in the wild

The self-incompatible breeding system, isolation of individuals and out of sync flowering associated with *M. bartlettii* makes the storage of pollen an essential part of its overall conservation strategy. Short- and long-term storage of pollen allows for cross pollination between trees that flower at different times or grow in different areas (Cruzatty *et al.*, 2020). Studies investigating pollen viability and long-term storage for Myrtaceae have mostly focussed on species of commercial interest such as various *Eucalyptus* species (e.g. Wheeler *et al.*, 2006) and *Kunzea pomifera* (Page *et al.*, 2006). Information for *Metrosideros* pollen is limited to two studies, both on *M. excelsa* in cultivation (Nadarajan *et al.*, 2021; Schmidt-Adam *et al.*, 1999). This study has addressed this research need and our findings will guide handling of *M. bartlettii*' s pollen during pollination and inform short-, medium- and long-term storage. For instance, it was found that the viability of pollen kept at room temperature rapidly declines while it remains consistent for up to four weeks when stored in the fridge (5°C) but then it rapidly declines. Desiccation and freezing of pollen are essential to maintain viability for longer than four weeks, with pollen stored in liquid nitrogen producing the best results. Our next step towards developing this technique is to verify moisture content of fresh pollen and measure pollen vigour after long-term storage by evaluating its ability to produce viable seeds. Another unexpected finding was detecting significant variation in fresh pollen viability between trees and years, ranging from 37–78%. Variation in pollen viability across individuals of the same species is not unique to *M. bartlettii* and it has been detected in *Castanea sativa* and *Picea abies* (Beyhan & Serdar, 2008; Nikkanen *et al.*, 2000) where there was up to a 10-fold difference in pollen viability between genotypes. These studies suggest variation in pollen viability among trees indicates a potential for male gametophyte competition, a strategy to maintain genetic diversity. However, this also means some trees are better pollen donors than others and this should be considered when conducting controlled pollination of *M. bartlettii*.

2.5 Conclusion and recommendations for conservation

The continued decline, extremely small population size, reproductive failure and threats from myrtle rust mean that without urgent conservation interventions, extinction of *M. bartlettii* is highly likely (Drummond *et al.*, 2000; Melesse, 2017). We have shown that the breeding system of *M. bartlettii* favours cross-pollination, supporting a previous study that showed that the inbreeding coefficient for *M. bartlettii* is very low (Melesse, 2017). Although cultivated specimens can provide material to support *in situ* recovery, careful consideration should be given to provenance, genetic diversity, and hybridisation risks. The lack of genetic diversity associated with *M. bartlettii* in cultivation may limit contribution to the overall conservation of the species. However, in this study, key individual trees were identified from different source populations that can be outcrossed to increase seed viability and offspring fitness, and therefore can make a significant contribution towards decreasing the risk of extinction. Pollen handling and storage protocols make it possible to overcome reproduction barriers due to different flowering times or distance between trees.

Recommendations for the conservation of *M. bartlettii*:

1. Develop a pollination network that utilizes pollen banks to optimise reproductive output through cross pollination of trees of known provenance/sources.
2. Secure germplasm of the remaining genotypes through living collections and/or cryopreservation.
3. Create a seed bank for seed obtained from wild populations and cross pollination of trees in cultivation.
4. Develop a reintroduction/enhancement program to increase population sizes in the wild.

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Chapter 3.

The seed storage physiology of *Lophomyrtus* and *Neomyrtus*, two threatened endemic Myrtaceae genera from New Zealand

Abstract

Lophomyrtus and *Neomyrtus* are two genera in Myrtaceae family that are endemic to New Zealand. Due to the threat from myrtle rust (*Austropuccinia psidii)* all three species, *Lophomyrtus bullata, Lophomyrtus obcordata* and *Neomyrtus pedunculata,* were classified as nationally critical in 2017. Despite their threat status, there is no published information on the seed germination or seed storage physiology on *Lophomyrtus* or *Neomyrtus*. This study investigated the seed morphology, seed germination requirements as well as tolerance to desiccation, the combined effect of desiccation and freezing, and storage at 5°C, -18°C and -196°C for these species. Fatty acid profiles were compared between fresh seed from *L. obcordata* and *L. bullata* as well as desiccated seed of *L. bullata* stored for 15 months at 5°C and -196°C. Variability in storage behavior between *L. bullata, L. obcordata* and *N. pedunculata* was investigated through differential scanning calorimetry (DSC) by comparing thermal properties of lipids. This study found that *L. obcordata* seed were desiccation tolerant and viability was retained when desiccated seed (15% equilibrated relative humidity) was stored 24 months at 5°C. However, in two of the three *L. obcordata* seed lots, seed viability decreased to less than 13% after storage at -18°C for 12–36 months. *L. bullata* seed was both desiccation and freezing sensitive while *N. pedunculata* was desiccation sensitive. DSC analysis revealed that lipid crystallization in *L. bullata* occurred between -18°C and -49°C and between -23°C and -52°C in *L. obcordata* and *N. pedunculata*. It is postulated that the metastable lipid phase, which coincides with the conventional seed banking temperature (-18°C), could cause the seeds to age more rapidly through lipid peroxidation. It is recommended that seeds of these three species are best stored outside of their lipid metastable temperature ranges i.e. using cryopreservation.

Keywords: cryopreservation, desiccation, differential scanning calorimetry (DSC), lipid, myrtle rust, seed banking, seed physiology, seed storage behaviour, threatened, Tetrazolium Chloride Staining (TZ).

3.1 Introduction

The invasive fungal pathogen *Austropuccinia psidii* (myrtle rust) has become a significant threat to native as well as economically important species in several genera of Myrtaceae worldwide (Beenken, 2017). Myrtle rust, which is native to South America, has spread globally causing extensive damage to nurseries, *Eucalyptus* plantations, commercial guava production and the allspice (*Pimenta officinalis*) industry (see Toome-Heller *et al*., 2020 and references within). Myrtle rust has also impacted Myrtaceae dominated ecosystems, for example in Australia the rust has killed mature trees in forest ecosystems in less than four years (Carnegie *et al*., 2015). Similar ecosystem impacts are predicted for Mexico (Esperón-Rodríguez *et al*., 2018), New Caledonia (Soewarto *et al*., 2018) and South Africa (Roux *et al*., 2015). On a species level, myrtle rust has contributed to the ongoing decline of *Eugenia koolauensis*, a critically endangered Hawaiian species (Chock, 2018; Keir, 2018) and caused local extinctions in formerly widespread Australian species such as *Rhodamnia rubescens* and *Rhodomyrtus psidiodes* as well as *Lophomyrtus* species in New Zealand (Fensham *et al*., 2020; Sommerville *et al*., 2020; Sutherland *et al*., 2020).

Myrteae, a tribe in the Myrtaceae family, includes most of the species with fleshy fruits. The Myrteae tribe in New Zealand is represented by two endemic genera, *Lophomyrtus* and *Neomyrtus* (Lucas *et al*., 2005). *Lophomyrtus* comprises two species: *L. bullata* (Soland. ex A. Cunn.) Burret and *L. obcordata* (Raoul) Burret (Garnock-Jones & Craven, 1996) while *Neomyrtus* is represented by a single species, *N. pedunculata* (Hook.f.) Allan. *Lophomyrtus bullata* (ramarama) occurs throughout suitable forest and shrubland on the North Island as well as the top of South Island, while *L. obcordata* (rōhutu) occurs on both North and South Islands (Woollard *et al*., 2008). Where the species co-occur, the natural hybrid, sometimes referred to as *L.* x ralphii, can become locally dominant resulting in local extinction of *L. bullata* (de Lange, 2021a). *Neomyrtus pedunculata* (rōhutu) occurs on North, South and Stewart Islands where it can be a conspicuous component of the understory. All three species are small trees or shrubs with similar white flowers although *L. bullata* flowers are slightly bigger (12–14 mm) compared to *L. obcordata* and *N. pedunculata* (6–8 mm; Fig. 3.1). Fruits of both *Lophomyrtus* species are fleshy ovate berries, 6–8 mm in size and dark red to black in colour (de Lange, 2021a) (Fig. 3.1B & D). *Neomyrtus* fruits are of similar size (6–8 mm) but yellow to orange in colour (de Lange, 2021b) (Fig. 3.1F).

Lophomyrtus species are valued for their medicinal properties, *L. bullata* leaves are used to treat bruises and cuts while *L. obcordata* bark and fruit are used to treat dysmenorrhoea (Riley, 1994). Fruit of *L. bullata* and *N. pedunculata* are high in anthocyanins and considered a potential functional food in the traditional Maori diet (Cambie & Ferguson, 2003). *Lophomyrtus* species are highly susceptible to myrtle rust with fruit infection and high degree of seedling death likely to result in localised extinction (Smith *et al*., 2020; Sutherland *et al*., 2020). The impact of myrtle rust on *N. pedunculata* is not yet to be known as this species has not been included in any of the susceptibility tests done to date. Due to this threat from myrtle rust, all three species are now classified as nationally critical (de Lange, 2018).

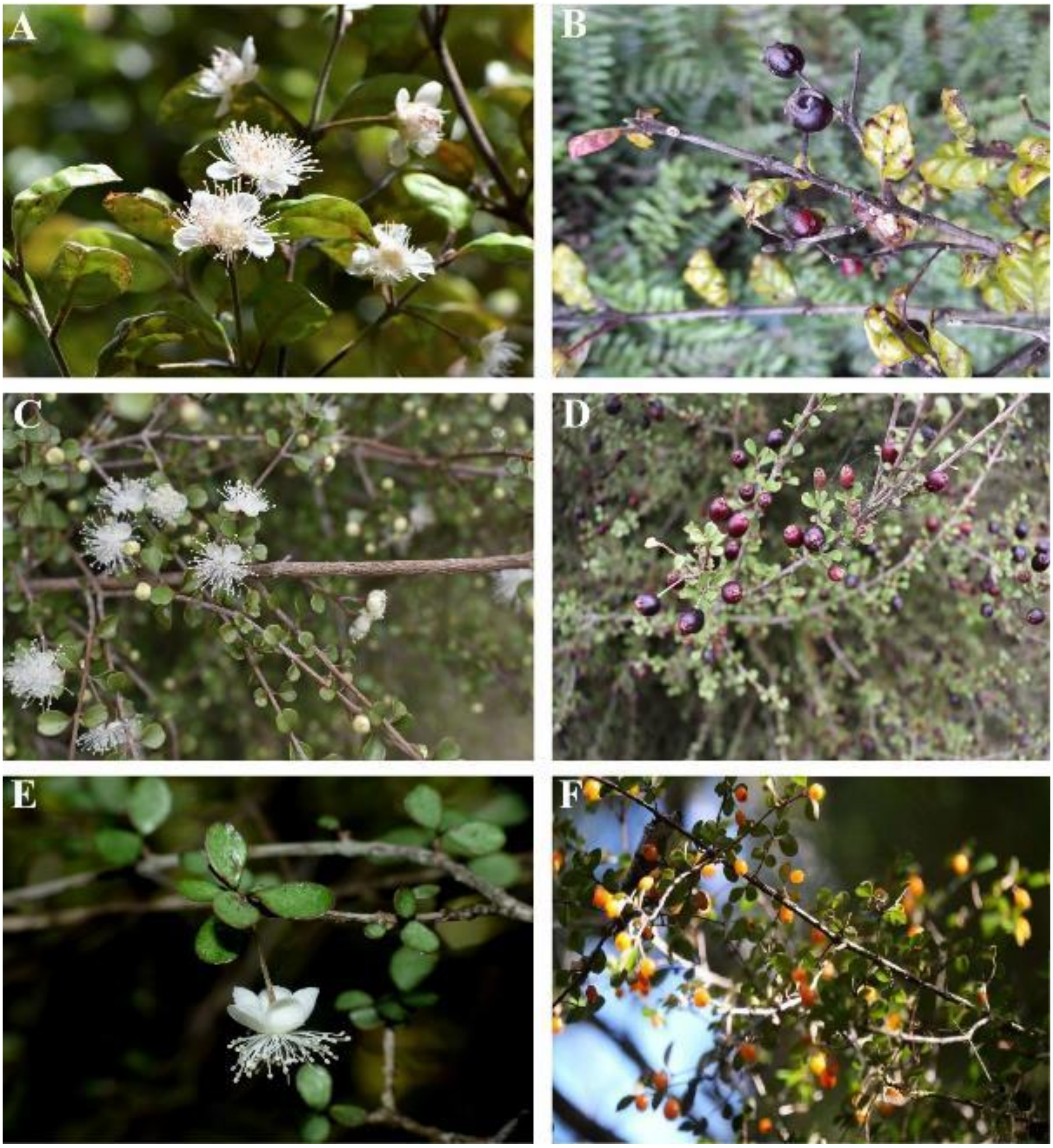


Figure 3.1: Flowers and fruit of *Lophomyrtus bullata* (A & B), *Lophomyrtus obcordata* (C & D) and *Neomyrtus pedunculata* (E & F). Photo credits: *L. bullata* flower: D. Townsend. *N. pedunculata* flower: H. Lurling and fruit: B. Williamson.

The urgency of *ex situ* conservation strategies to prevent extinction of species susceptible to myrtle rust has been highlighted in Australia (Berthon *et al.*, 2018; Sommerville *et al.*, 2020) and New Zealand (Nadarajan *et al.*, 2021; Van der Walt *et al.*, 2021). *Ex situ* conservation methods include maintenance of cultivated plants as living collections, tissue cultured plants in *in vitro* collections, conventional seed banks (seed dried to 15% relative humidity and stored at -18°C according to FAO (2014)) and cryopreservation i.e. storage of seeds, embryos, pollen or vegetative material at cryogenic temperatures *i.e.* -136°C to -196°C using vapor or liquid phase of liquid nitrogen (Wyse *et al.*, 2018). The most suitable *ex situ* conservation method will largely depend on the seed storage classification of the species. Seed bearing species, in most cases, can be found along a continuum of storage behaviour which includes a) orthodox (desiccation and freezing tolerant, b) recalcitrant (desiccation sensitive) or c) intermediate (sensitivity to desiccation, freezing or both) (Hay & Probert, 2013; Merritt *et al.*, 2021). Seed banking is often considered the most efficient form of *ex situ* conservation, but this method is only applicable to seeds that can survive desiccation and freezing (orthodox) (Sommerville *et al.*, 2020; Walters, 2004). The hypothesis is that 90% of the flowering plant families, including food and agricultural crops, produce seeds that are orthodox (Hay *et al.*, 2013; Wyse & Dickie, 2017). However, there is an increasing recognition of variation in desiccation responses within and among seed categories, and that desiccation stress and response should not be treated as qualitative features (Berjak & Pammenter, 2008; Walters, 2015).

Research on seed storage behaviour of wild species have increased since the 1990's, resulting in the realization that the orthodox and recalcitrant categories were too restrictive, this gave rise to the intermediate category (Ellis *et al.*, 1991). Walters (2015), indicates that this intermediate storage behaviour could be due to; a) tolerance to desiccation is higher than those in recalcitrant seeds but lower than orthodox seeds, b) there is inconsistent longevity responses over a range of temperatures and c) seeds have short lifespans irrespective of the water content or storage temperature. Studies suggest that the number of angiosperms that are amenable to storage at -18°C might be less than previously predicted and urgent work is now required to determine optimal storage conditions necessary to maximise their longevity (Merritt *et al.*, 2014). This is supported by Sommerville *et al.* (2021), who found that 24% of the desiccation tolerant Australian rainforest species tested to date were freezing sensitive or short-lived in storage at -18°C.

In addition to desiccation and freezing sensitivity, other variables influencing seed longevity can be complex and may relate to aspects such as embryo size, seed mass, seed coat, cellular composition, taxonomy, genotype, and the environment (Probert *et al.*, 2009; Walters, 2004). Of these variables, seed lipid composition, particularly the thermal stability of the lipids at the storage temperature, is a key predictor of intermediate seed storage physiology (Colville & Pritchard, 2019; Crane *et al.*, 2003; Dussert *et al.*, 2001; Mira *et al.*, 2019; Probert *et al.*, 2009). Triacylglycerols (TAG) consist of trihydric alcohol glycerol esterified and usually comprises long-chain fatty acids (C_{14}-C_{22}) and may determine whether dry seeds are damaged when exposed to freezing temperatures (Walters, 1998). Analysis of seed composition, in particular lipid content and their fatty acid compositions, can aid in developing the most appropriate storage protocol for seed. In addition to this, thermal analysis using differential scanning calorimetry (DSC) can characterize the physical properties such as crystallization and melting temperatures of lipids and their stability. Using the DSC, lipid phase changes (solid to liquid and vice versa) and their relevant heat transitions are monitored and analysed as a function of time and temperature (Volk & Walters, 2006). This enables an assessment of seed lipid thermal fingerprints and their physical state stability at stored temperatures (Mira *et al.*, 2019). The range (onset to end) of lipid crystallization and melting temperature ranges can indicate the stability of the lipid phase at a particular temperature at which seeds are stored (Crane *et al.*, 2006).

The aim of long-term seed storage is to retain high seed germination, hence, knowledge on seed germination, including dormancy breaking, is a crucial part of the seed banking process. It is estimated that up to 75% of species in temperate broadleaved evergreen forests has some form of dormancy (Baskin & Baskin, 2014). Assessment of seed storage physiology can be hampered by the inability to germinate seed due to dormancy or lack of data on optimum germination conditions and therefore, research into seed germination should be prioritised for these species (Sommerville *et al.*, 2021). Seed germination tests are the optimal way to monitor the physiological status of the seed, however, in some species results from germination tests could be misleading due to ineffective germination conditions or partially effective dormancy breaking treatments (Liu *et al.*, 2020). For seed in storage, loss of fitness due to aging prior to seed mortality might also influence germination results (Walters *et al.*, 2005).

Another tool to assess seed viability is through biochemical staining using triphenyl tetrazolium chloride (TZ) (Liu *et al.*, 2020). TZ tests are based on the principle that respiring tissue can convert colourless TZ solution to red coloured water-insoluble formazan (Miller, 2010). TZ results however can also suggest that viable cells are present thus indicating that there is potential for ongoing development. However, Quain *et al*. (2009) noted that when correlating TZ results with *in vitro* germination it has been found that plantlets did not contain sufficient cells to survive. It is therefore important to pair TZ- and germination tests to correlate results prior to use in evaluation of seed viability. Another challenge of the TZ test is that protocols are species specific and limited work has been done to determine TZ staining procedures for native shrubs and forbs. Varying conditions of the staining protocol which needs to be optimised include staining time, staining temperature, scarification method, TZ solution concentration and seed pre-treatment (Babineau *et al.*, 2017).

Threatened plant conservation is often hampered by gaps in fundamental biological information and seed biology (Cavender *et al.*, 2015; Pritchard, 2004). To effectively conserve the genetic diversity of seeds, it is important to optimise the development stage at which the fruits or seeds are harvested (Hay & Probert, 1995; Hay *et al.*, 2013). Various aspects of seed physiology are particularly relevant to the long-term storage of seed. This includes an understanding of seed viability and dormancy, the ability of a seed to tolerate desiccation during seed development, seed ageing and how it impacts viability loss and how long seed lots will remain above viability thresholds (Whitehouse *et al.*, 2020). These physiological aspects have been poorly studied for many native New Zealand species, including *Lophomyrtus* and *Neomyrtus* (Sommerville *et al*., 2017; SID, 2021). This lack of information is hampering effective *ex situ* conservation of Myrtaceae species threatened by myrtle rust. The objectives of this study were to: 1) understand seed morphology, fruit and seed development for *L. bullata, L. obcordata* and *N. pedunculata,* 2) investigate seed storage physiology by testing desiccation sensitivity, the combined effect of desiccation and low temperature exposure and long-term storage of seed, and 3) to understand seed composition and lipid thermal profiles to predict seed storage behaviour and to optimise long-term seed storage protocols for these species.

3.2 Materials and Methods

3.2.1 Plant material

Lophomyrtus bullata, *L. obcordata* and *L. bullata x L. obcordata* fruit was collected between April and June 2018–2021 from various localities in the lower North- and upper South Island. *Neomyrtus pedunculata* fruit was collected from Taranaki in 2021 (Table 3.1). Fruits were transported to the Lions Ōtari Plant Conservation Laboratory in Wellington where they were cleaned and stored at 5°C until further use. Seeds used for non-stratified experiments were not stored prior to experiments.

3.2.2 Fruit development and seed morphology

Lophomyrtus bullata fruit development was studied to identify the optimal stage for seed collection. Fruit at various stages of maturation can be found on *L. bullata* between May and July. To assess the role of development stage on seed moisture content, viability and dormancy, green and red fruits were collected from Maungakotukutuku valley (Wellington Region) in May 2019 (Fig. 3.2). Fruit weight (g), number of seed per fruit, seed moisture content (expressed as g H_2O /g seed dry weight), seed viability (TZ staining test) and seed germination (procedures described below) were compared for each development stage. Seed morphology for *L. bullata*, *L. obcordata* and *N. pedunculata* was described in terms of weight and size of randomly selected seeds. Seed weight was determined as the average weight (mg) of 10 seeds in four replicates weighed on a precision scale (Model: AUW120D, Shimadzu, Japan). To determine seed diameter, digital images were taken of 20 randomly selected seeds per species and measured using LC Micro 2.2 image analysis software (Olympus).

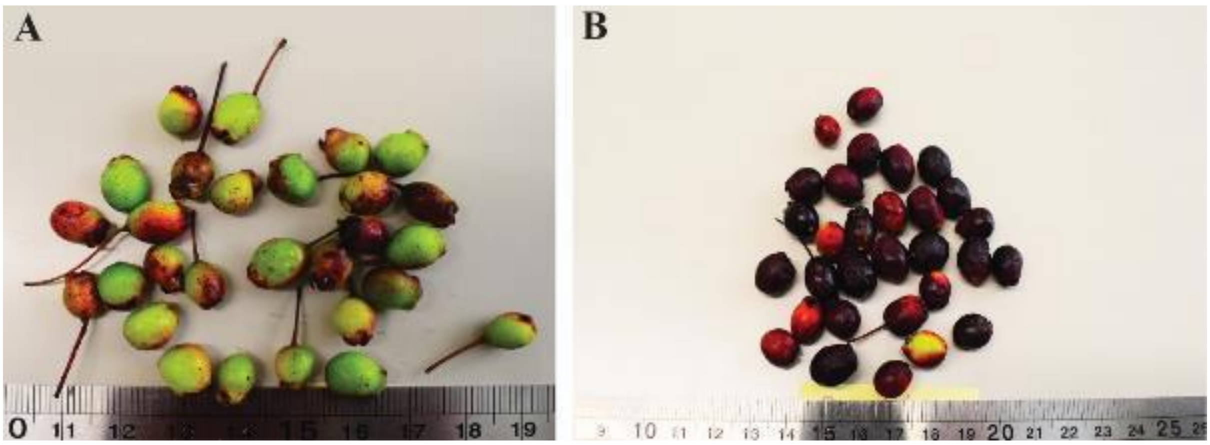


Figure 3.2: Fruit of *Lophomyrtus bullata* was divided into two development stages based on green (A) or red (B) colour of the pulp at time of collection.

Table 3.1: Summary of the *Lophomyrtus bullata*, *L. obcordata*, *L. bullata x L. obcordata* and *N. pedunculata* seed collected between April 2018 and June 2021

Species	Seed Lot	Region	Locality	GPS coordinates (DMS)	Elevation (m.a.s.l.)	Date Collected
L. bullata	Kap 19	Wellington	Maungakotukutuku valley	40°56'58.9"S 175°01'58.7"E	140	May 2019
L. bullata	Kap 21	Wellington	Maungakotukutuku valley	40°56'58.9"S 175°01'58.7"E	140	June 2021
L. bullata	Wrights	Wellington	Wright's Hill Reserve	41°17'34.7"S 174°44'02.3"E	301	June 2018
L. bullata	Butchers	Marlborough	Richmond Forest Park - Butchers Flat	41°17'47.65"S 173°20'29.54"E	210	April 2018
L. bullata x *obcordata*	Skyline 20	Wellington	Skyline Reserve	41°14'17.8"S 174°46'09.5"E	317	May 2020
L. obcordata	Ōtari 21	Wellington	Ōtari native botanic garden	41°16'04.7"S 174°45'23.7"E	101	May 2021
L. obcordata	Ōtari 19	Wellington	Ōtari native botanic garden	41°16'04.7"S 174°45'23.7"E	101	May 2019
L. obcordata	Old Mill Road	Marlborough	Nelson	41°23'22.4"S 173°35'54.63"E	87	April 2018
L. obcordata	Matai	Marlborough	Richmond Forest Park -	41°17'47.65"S 173°20'29.54"E	210	April 2018
N. pedunculata	Tar	Taranaki	Midhirst	39°17'43.7"S 174°11'11.3"E	502	March 2021

3.2.3 Biochemical composition of Lophomyrtus seed

Since information on seed biochemical composition, particularly fatty acids, can aid in developing an appropriate storage protocol for seed, this was analysed for fresh *L. bullata* and *L. obcordata* seed. In addition, fatty acid composition was also analysed for desiccated (~15% eRH) *L. bullata* seed stored for 15 months at 5°C and -196°C. Total solids, crude protein, fat, starch, sugars and fatty acid profiles were analysed for fresh *L. bullata* and *L. obcordata* seed using standard methods of analysis prescribed by AOAC International (AOAC, 2021). The total fat and fatty acid composition was based on Sukhija and Palmquist (1988) with in house modifications and included extraction, purification and esterification followed by gas chromatographic analysis. For fatty acid profiles, 2 g of ground seed was placed in a 50 mL beaker containing 2 mL benzene. After adding 10 mL HCI the solution was incubated at 70–80°C for 40 min. After adding 10 mL anhydrous methanol to the mixture, and once the solution has cooled down, it was poured together with 25 mL ether into the extraction tube of a Mojonnier fat-extractor. Following 1 min of mixing, an additional 25 mL of redistilled petroleum ether was added, and the solution was centrifuged for 20 min at 600 rpm. The ether-fat solution was filtered through a cotton pledget packed before re-extracting 15 mL of ether. After shaking the solution, clear ether was drawn and evaporated slowly before placing the dry fat in an oven at 100°C to constant weight. Samples were then submitted for gas chromatographic analysis (GC2010; Shimidzu, Japan).

3.2.4 Moisture content determination

Moisture contents (MC) for all treatments were determined gravimetrically after drying at 103 ± 1°C for 17 h using the ISTA (2018) method. Values represent the mean MC of ten seeds replicated four times and expressed as g H_2O per g dry matter (g/g).

3.2.5 Tetrazolium chloride staining tests

Tetrazolium tests were conducted to confirm viability for fresh, desiccated, and stored seed of all three species. Imbibed seed were scarified in the proximity of the radicle tip and treated with 1% (v/v) solution of 2,3,5-triphenyl-tetrazolium chloride (TZ) (Sigma Aldrich, NZ). The Eppendorf tubes containing TZ solution and seeds were incubated at 30°C for 48 h in the dark. After the staining period, seeds were washed with distilled water, dissected, and assessed immediately for colour development and uniformity in staining. TZ test results were correlated to germination tests conducted at the same time.

3.2.6 Seed germination test and seedling vigour assessment

Germination trials were conducted in 500 ml Magenta® tissue culture vessels containing 100 ml soil. Seeds were not sterilised before germination experiments. The presence of dormancy and role of cold stratification (5°C) on germination was determined. *L. bullata, L. obcordata* and *L. bullata x L. obcordata* seed were germinated fresh on the day of collection and following dry stratification for four weeks at 5°C. *Neomyrtus pedunculata* seed were either stratified dry (cleaned seed in glass vials) or moist (in fruit pulp) at 5°C for four and 20 weeks before germination. All germination experiments were conducted in an incubator set at alternating temperature of 15/25° C with a 18 h photoperiod. These temperatures were selected based on pilot studies (data not shown). Radicle protrusion of at least 5 mm was the criterion for radicle emergence with germination defined as the opening of cotyledonary leaves. For *Lophomyrtus* species, germination was assessed weekly for 6 weeks, *Neomyrtus* germination was assessed for 8 weeks. At the end of the germination period, non-germinated seeds were dissected to determine the presence of an embryo. Seedling height (mm) and number of leaves were recorded for all germinated seed. Germination results were compared with TZ test results for the same treatment. Each treatment consisted of a minimum of 10 seeds in four replicates.

Table 3.2: Seed germination and dormancy breaking treatments for *Lophomyrtus bullata, Lophomyrtus obcordata, Lophomyrtus bullata x Lophomyrtus obcordata* and *Neomyrtus pedunculata*

Species	**Fresh**	**Dry stratification at 5°C for 4 weeks**	**Wet stratification at 5°C for 4 weeks**	**Wet stratification at 5°C for 20 weeks**
L. bullata	✔	✔	NT	NT
L. obcordata	✔	✔	NT	NT
L. bullata x L. obcordata	✔	✔	NT	NT
N. pedunculata	✔	✔	✔	✔

✔ indicates condition tested
NT indicates conditions not tested

3.2.7 Seed desiccation trial

Prior to long-term storage seed should be desiccated to at least 10–15% relative humidity (eRH) to remove freezable water (Merritt *et al.*, 2021). However, when the desiccation tolerance of a species is not known, a two-step approach can be used in which seeds are desiccated to higher moisture contents (i.e. 30% eRH) followed by desiccation to a lower moisture content i.e. 15% eRH (Hong & Ellis, 1996). Thus, to screen for desiccation sensitivity in *L. bullata* and *L. obcordata*, seeds were desiccated to 15% and 30% eRH in airtight chambers kept at room temperature until they have fully equilibrated. These RH environments were created using lithium chloride (Sigma Aldrich, NZ) salt solution with different concentrations and are commonly applied in seed banking (Gold & Hay, 2014). Seeds were weighed every second day until there is no more change in the weight. Unchanged weights indicated that seeds have equilibrated to the required relative humidity. MC tests were carried out to verify seed moisture prior to further treatment. Desiccated seeds were assigned to a germination and test TZ staining test respectively. To prevent imbibition damage, desiccated seeds were rehydrated in a 100% humidity chamber for 24 h before germination or TZ tests. Due to a limited number of seed in two seed lots for *L. bullata* (Butchers and Wrights) and *L. obcordata* (Matai and Old Mill), viability testing was not conducted following desiccation. Also due to limited seeds in *L. bullata x L. obcordata* and *N. pedunculata* these seeds were desiccated to 15% eRH only.

3.2.8 Combination of desiccation and freezing sensitivity assessment

The combination of seed sensitivity to desiccation as well as low and sub-zero temperatures was evaluated on *L. bullata* (Kap 21) and *L. obcordata* (Ōtari 21) by exposing fresh and desiccated seeds (15% and 30% eRH) to 5°C (fridge), -18°C (freezer) and -196°C (liquid nitrogen) for 14 days. Due to limited number of seed, *N. pedunculata* seeds were desiccated to 15% eRH and exposed to -18°C for 14 days. Viability was assessed through germination and TZ staining tests as described above.

3.2.9 Seed storage trial

The long-term storability was also evaluated for non-desiccated and desiccated seed (15% eRH) stored at 5°C (fridge), -18°C (freezer) and -196°C (liquid nitrogen) for different collections of *L. bullata*, *L. obcordata* and *L. bullata x L. obcordata*. The time in storage ranged from 12 to 36 months (Table 3.2).

Table 3.3: Seed storage trial for *Lophomyrtus bullata*, *L. obcordata* and *L. bullata x obcordata* seed desiccated to 15% eRH and stored at 5°C, -18°C and -196°C for 12 to 36 months. Non-desiccated *L. bullata* and *L. obcordata* seeds were also stored at 5°C for 24 months.

Species	Seed Lot	Storage Temperature				Storage duration (months)
		Non-desiccated	Desiccated	Desiccated		
		5°C		-18°C	-196°C	
L. bullata	Kap 19	✔	✔	✔	✔	24
	Wrights	NT	NT	✔	NT	36
	Butchers	NT	NT	✔	NT	36
L. bullata x obcordata	Skyline 20	NT	NT	✔	✔	12
L. obcordata	Ōtari 19	✔	✔	✔	NT	24
	Old Mill Road	NT	NT	✔	NT	36
	Matai	NT	NT	✔	NT	36

✔ indicates condition tested
NT indicates conditions not tested

3.2.10 Differential Scanning Calorimetry

Thermal behaviour of lipids in seed desiccated to 5% eRH, 15% eRH and 30% eRH were determine using a Perkin-Elmer differential scanning calorimeter (DSC 8500) (Shelton, USA), calibrated for temperature and heat flow with zinc (melting point 419.5°C) and indium (melting point 156.6°C). Seeds (3 seeds per treatment) of *L. bullata*, *L. obcordata* and *N. pedunculata* were hermetically sealed into a large volume (60 µL) stainless steel capsule (sample pan) using an O-ring with the aid of a Perkin-Elmer Universal Crimper (Shelton, USA). Sample weight was measured using a micro-balance (Model: XPR6UD5; Mettler-Toledo, Switzerland), and subjected to calorimetric assessment within 5 min of sample preparation. Samples were cooled from 25°C to -100°C and held for 1 min before rewarming to 25°C at a rate of 10°C min^{-1}. The exothermic- and endothermic-heat changes derived from the crystallization and melt endotherm during the cooling and warming cycles were regarded as lipid transitions. The onset temperature of the transition was determined as the temperature at which the tangent of the sharpest portion of the first peak intersected the baseline. The onset, peak and end temperatures were calculated using PYRIS software. The areas of melt and crystallization peaks were calculated from the area above the baseline and expressed as millijoule (mJ). The enthalpies for these transitions are presented as joule per gram of sample weight (J/g).

3.2.11 *Statistical analysis*

All germination experiments involved a minimum of 10 seeds for each treatment, repeated four times. Germination results and seedling heights were compared using ANOVA, followed by Fisher's protected Least Significant Difference (LSD) test for significantly different means ($P < 0.05$). Correlations between germination- and TZ results were tested using Pearson correlation analysis. For correlation analysis, percentages were transformed (arcsine) to conform to parametric test assumptions. A two-way ANOVA was used to investigate the relationship between desiccation and storage temperature on radicle emergence, germination and seedling vigour (height and number of leaves). Arcsine square root transformation was used to normalise data where needed. ANOVA followed by Tukey's Honest Significant Difference (HSD) test was used to investigate differences in onset of melt temperature, freeze temperature and enthalpy of the melt. All statistical analysis were performed at 0.05 level of significance and results are expressed as mean ± SD. Selection of statistical tests were based on data type (binomial), variance, sample sizes and available packages. Statistical analysis was conducted using XLStat Software version 1.3 (2021) and SAS/STAT version 14.2.

3.3 Results

3.3.1 *Fruit development and seed morphology*

Green fruit of *L. bullata* weighed significantly less (0.29 ± 0.05 g) and contained fewer seed (12 ± 6) compared to red fruit (0.46 ± 0.07 g; 14 ± 5 seeds). Seed moisture content was also slightly higher (0.94 ± 0.5 g/g) for seed obtained from green fruit compared to seed from red fruit (0.74 ± 0.3 g/g). Seed germination coupled with TZ testing indicated seeds from both developmental stages were viable but dormant at time of collection (May 2019). MC of mature seeds collected from the same population (Kap) in May and June (2021) was 0.74 g/g and 0.52 ± 0.04 g/g respectively. *Lophomyrtus bullata* fruit and seed were significantly larger compared to *L. obcordata* and *N. pedunculata* and seed had higher MC c. 0.52 g/g compared to *L. obcordata* (MC c. 0.35 g/g) and *N. pedunculata* (MC c. 0.15 g/g) (Fig. 3.3; Table 3.4).

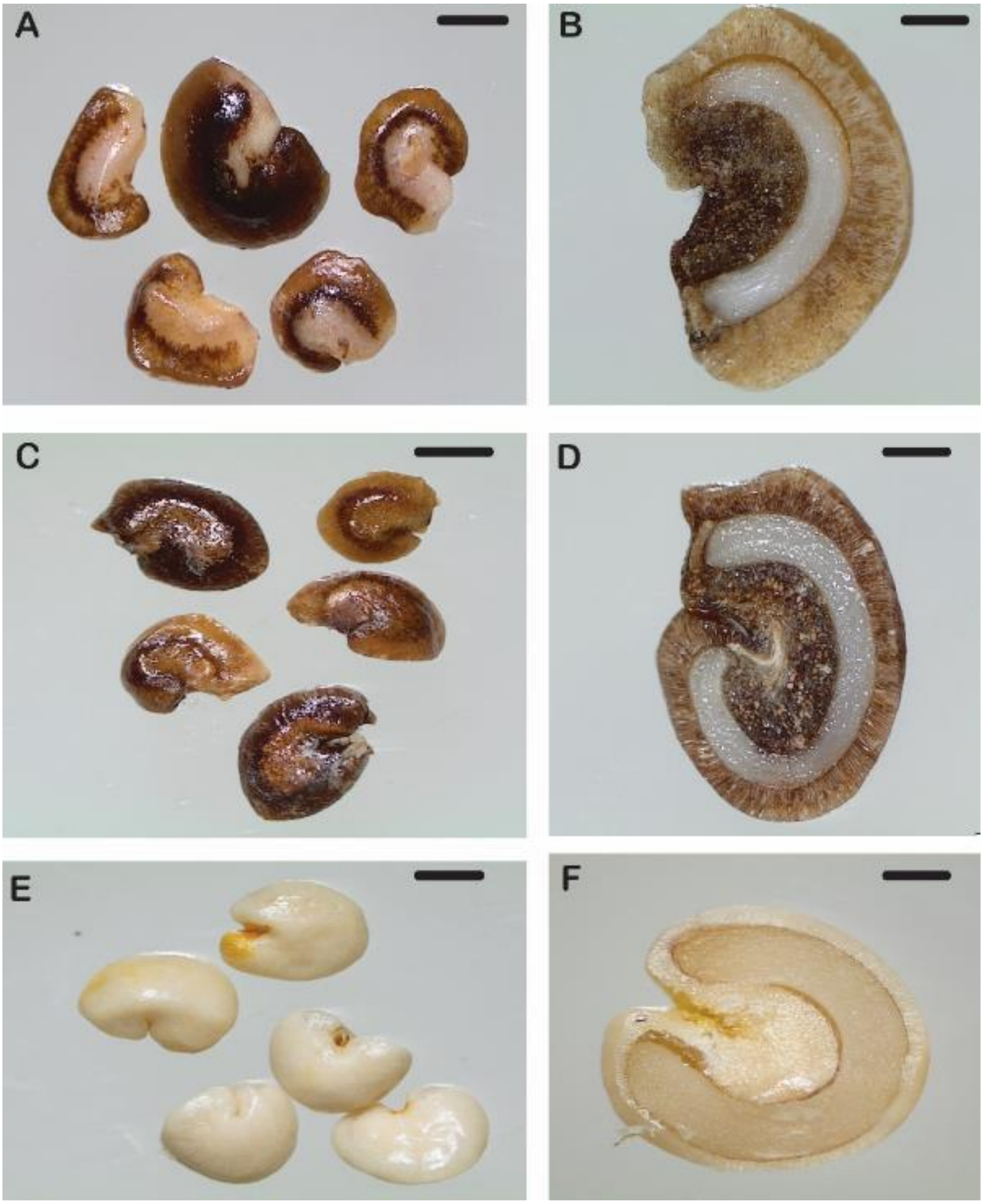


Figure 3.3: Whole and dissected seed illustrating embryo size and structure in *L. bullata* (A & B), *L. obcordata* (C & D) and *Neomyrtus pedunculata* (E & F). Black scale bars are equivalent to 1 mm.

Table 3.4: Fruit weight, number of seed and seed characteristics of *Lophomyrtus bullata*, *L. obcordata* and *Neomyrtus pedunculata*. Letters in each column indicate significant difference at $P < 0.05$ based on Fisher's LSD test

Species	Fruit		Seed		
	Weight (g)	**Number seed**	**Weight (mg)**	**Diameter (mm)**	**Moisture Content (g H_2O/ g DW)**
L. bullata	0.46 ± 0.07	14.1 ± 4.8[a]	9.3 ± 1.0[a]	4.06 ± 0.68[a]	0.52 ± 0.04[a]
L. obcordata	0.007 ± 0.002	3.1 ± 4.8[b]	7.1 ± 1.0[b]	1.47 ± 0.23[b]	0.35 ± 0.02[b]
N. pedunculata	n/a	n/a	3.2 ± 1.0[c]	0.77 ± 0.05[c]	0.15 ± 0.001[c]

3.3.2 Biochemical composition of Lophomyrtus seed

Fresh *L. bullata* seed consisted of more total solids with higher fat and crude protein content compared to *L. obcordata* (Fig. 3.4). Starch and sugar accounted for less than 1% of the seed composition in both species. The fatty acid profile showed that both *Lophomyrtus* species' seed comprised mostly of polyunsaturated fatty acids (PUFA), specifically linoleic acid (Table 3.5). *Lophomyrtus bullata* seed stored for 15 months at 5 and -196°C showed c. 0.8 and 1.0% decrease in saturated fatty acid (SAFA); 0.3 and 1.3% decrease in monounsaturated fatty acids (MUFA); whilst showing an increase of 1.2 and 2.3% in polyunsaturated fatty acid PUFA respectively (Table 3.5). *Lophomyrtus obcordata* contained small amounts of undecamolic acid (C11) which was not present in *L. bullata*.

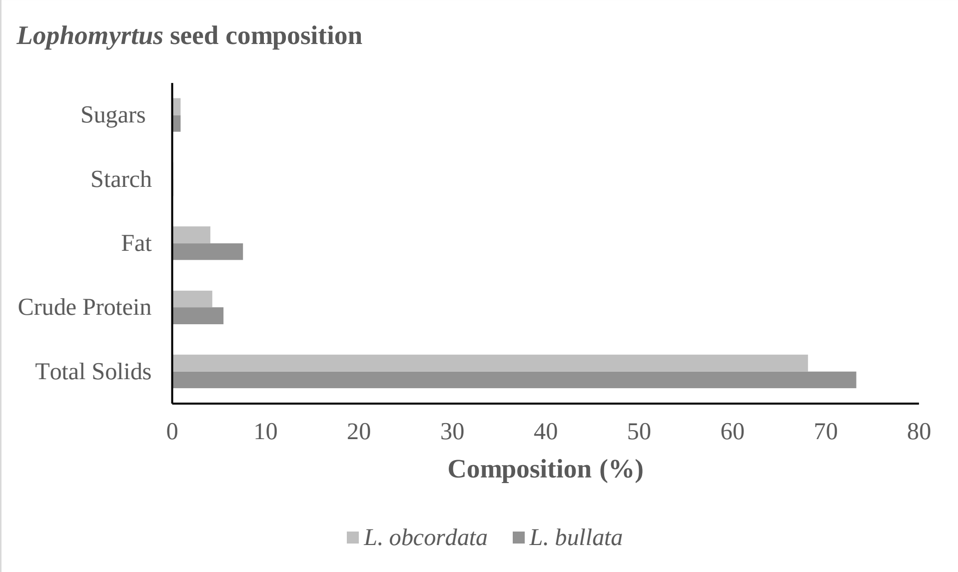


Figure 3.4: Biochemical composition of fresh *Lophomyrtus bullata* and *L. obcordata* seeds

Table 3.5: Fatty acid as a percentage of total composition of fresh *Lophomyrtus obcordata* and *Lophomyrtus bullata* seed as well as desiccated (~15% eRH) *L. bullata* seed stored for 15 months at 5°C and -196°C

Lipid	***L. obcordata***	***L. bullata***		
	Fresh seed (%)	Fresh Seed (%)	Desiccated & stored for 15 months at 5°C (%)	Desiccated & stored for 15 months at -196°C (%)
Caproic (C6:0)	0.22	0.13	0.09	0.12
Undecanoic (C11:0)	0.22	0	0	0
Myristic (C14:0)	0.22	0.13	0	0
Palmitic (C16:0)	6.51	7.71	7.55	7.4
Margaric (C17:0)	0.22	0.13	0.06	0.07
Stearic (C18:0)	2.6	3.27	3.12	3.03
Arachidic (C20:0)	0.43	0.53	0.48	0.43
Heneicosanoic (C21:0)	0.22	0	0	0
Behenic (C22:0)	0.22	0.13	0.07	0.08
Tricosanoic (C23:0)	0.22	0.13	0	0
Lignoceric (C24:0)	0.22	0.13	0.06	0.12
Total SAFA	**11.78**	**12.29**	**11.46**	**11.27**
Palmitoleic (C16:1)	0.22	0	0	0
Oleic (C18:1)	11.06	11.63	11.35	10.42
Vaccenic (C18:1)	0.43	0.39	0.38	0.36
Eicosenoic (C20:1)	0.22	0.13	0.06	0.06
Total MUFA	**11.9**	**12.1**	**11.8**	**10.8**
Linoleic (C18:2)	75.9	74.9	76.1	77.3

Lipid	***L. obcordata***	***L. bullata***		
	Fresh seed (%)	Fresh Seed (%)	Desiccated & stored for 15 months at 5°C (%)	Desiccated & stored for 15 months at -196°C (%)
Alpha linolenic (C18:3)	0.7	0.7	0.6	0.6
Eicosadienoic (C20:2)	0.2	0	0	0
Total PUFA	**76.8**	**75.6**	**76.8**	**77.9**

SAFA = Saturated fatty acids
MUFA = Monounsaturated fatty acids
PUFA = Polyunsaturated fatty acids

3.3.3 Tetrazolium chloride viability staining tests

Both *Lophomyrtus* species had similar staining patterns. TZ staining identified three categories of seed viability; a) non-viable seed in which embryos did not stain, b) intermediate staining which included partial or light pink staining and c) viable embryos with a consistent dark red stain (Fig. 3.5). Similar staining patterns were observed in *N. pedunculata* with disintegrated embryos also frequently encountered (Fig. 3.6). There was a strong positive correlation (R^2 = 0.996) between germination and embryos stained dark red in the TZ test for *L. bullata, L. obcordata* and *L. bullata x L. obcordata* (Fig. 3.7). Inclusion of seed which displayed intermediate staining patterns resulted in a weak positive correlation (R^2 = 0.09) (data not shown).

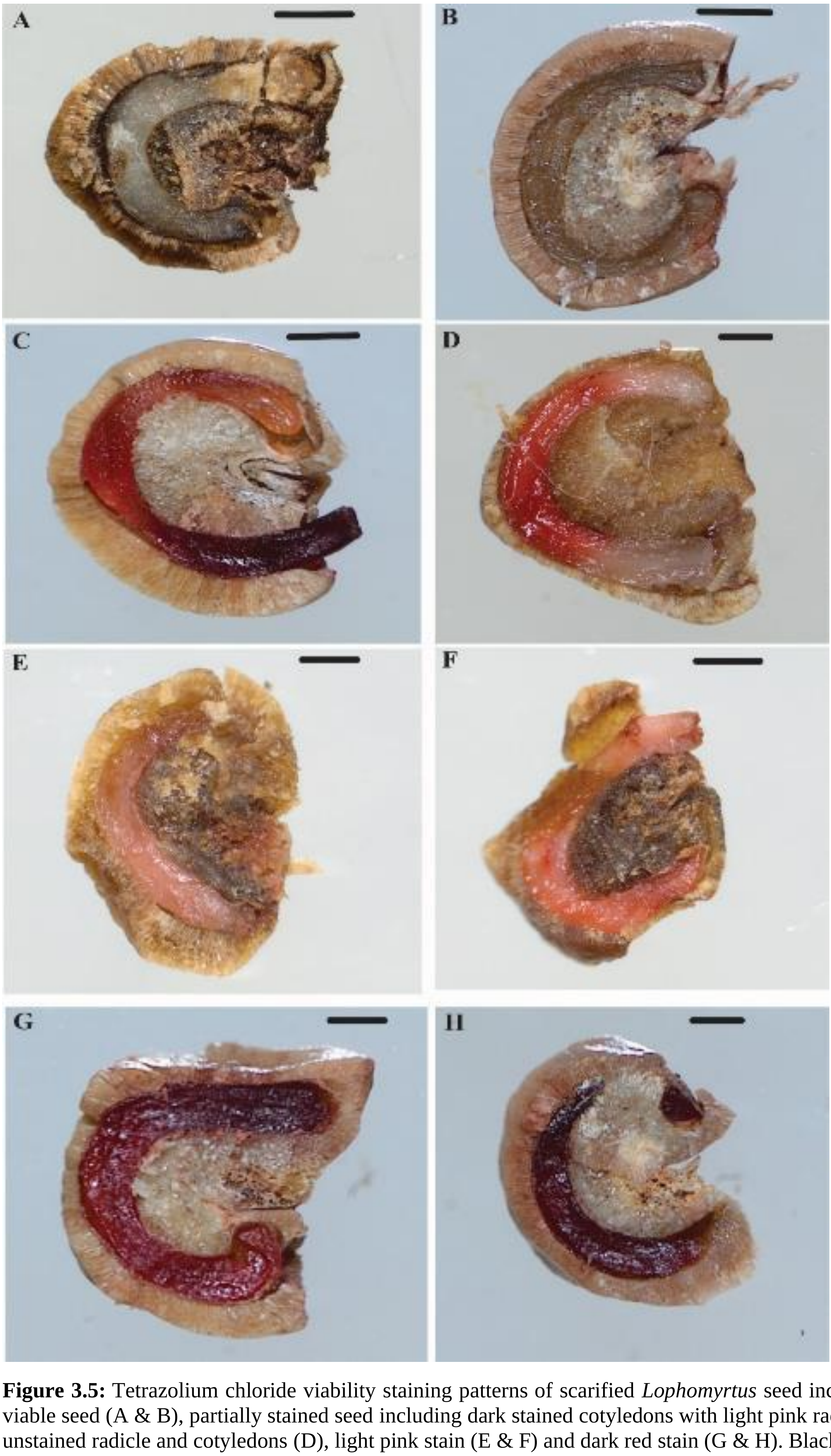


Figure 3.5: Tetrazolium chloride viability staining patterns of scarified *Lophomyrtus* seed included non-viable seed (A & B), partially stained seed including dark stained cotyledons with light pink radicle (C) or unstained radicle and cotyledons (D), light pink stain (E & F) and dark red stain (G & H). Black scale bars are equivalent to 1 mm.

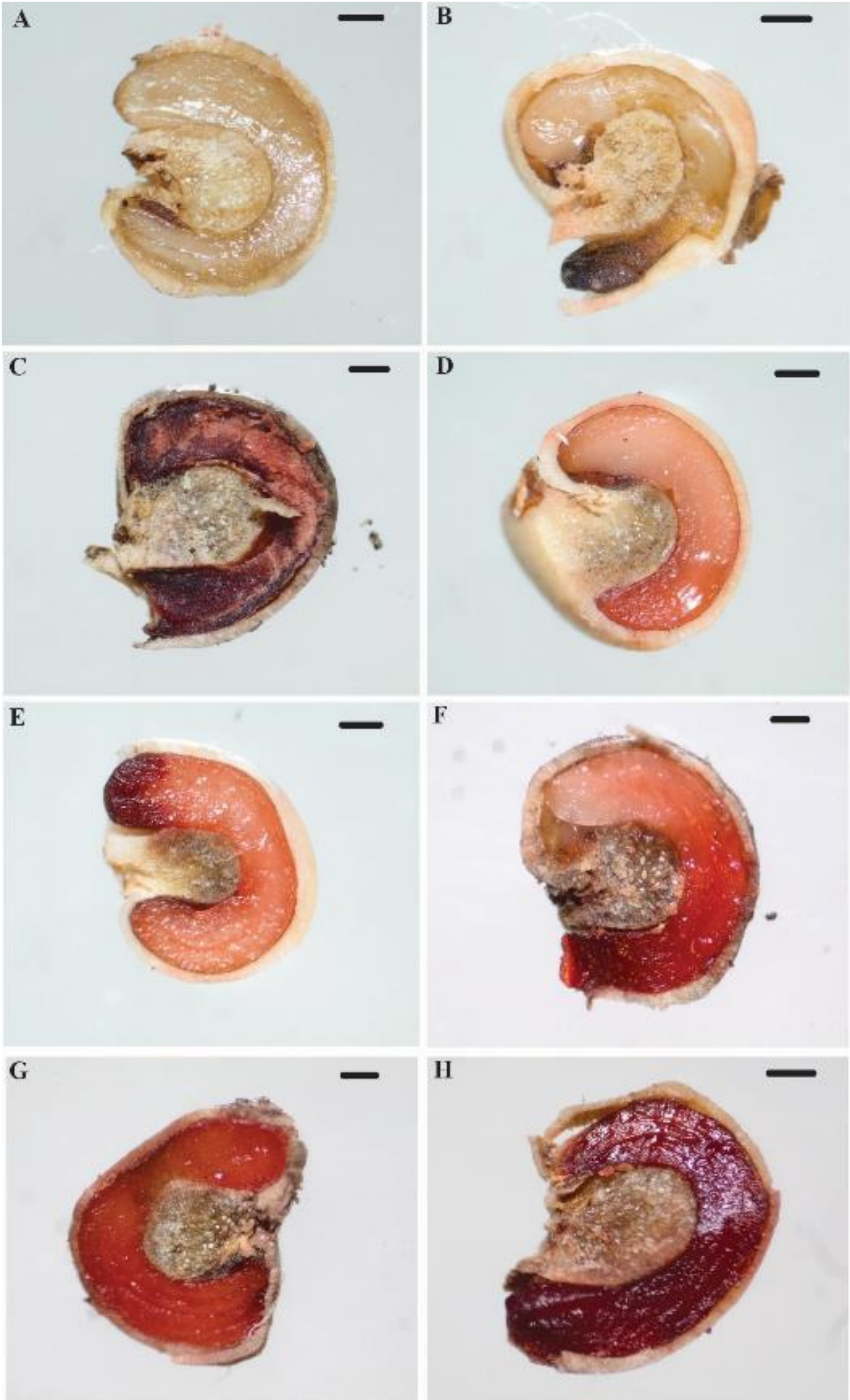


Figure 3.6: Tetrazolium chloride viability staining patters for *Neomyrtus pedunculata* seed included unstained (A & B), disintegrated (C), light pink (D & E), partial staining of the cotyledons with radicle light pink or unstained (F), light red (G) and dark red (H). Black scale bars are equivalent to 1 mm.

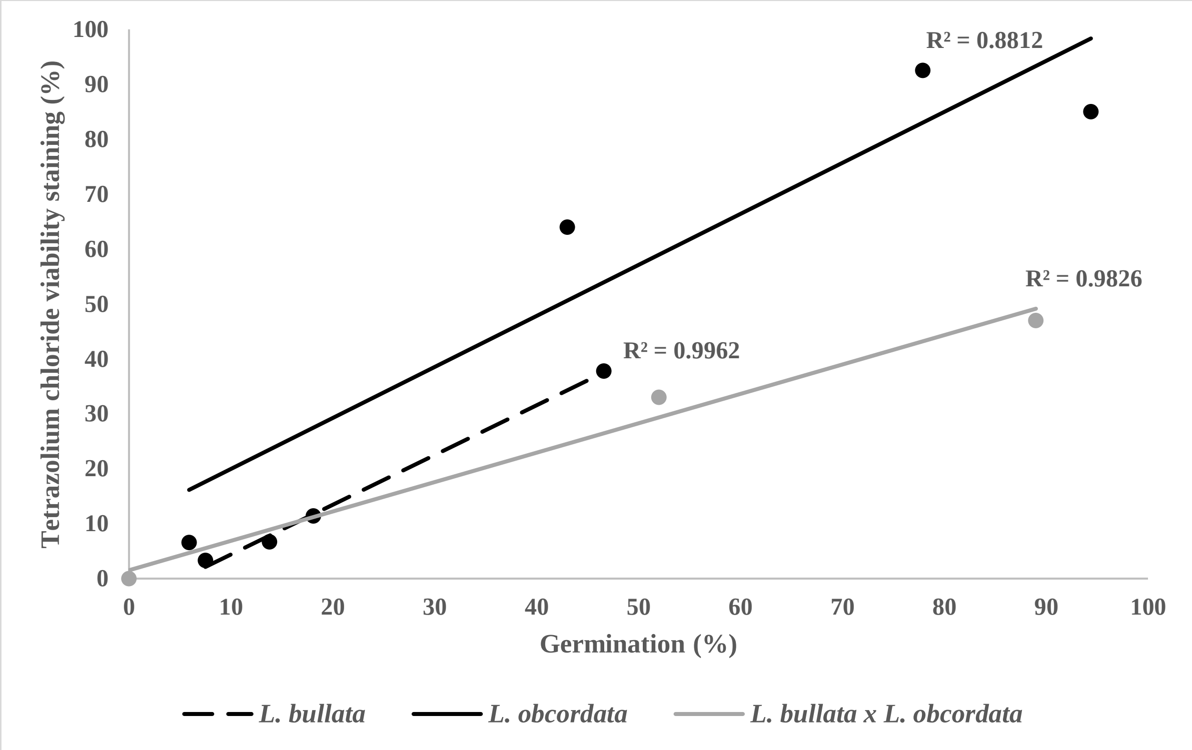


Figure 3.7: There was a strong positive correlation between germination and Tetrazolium Chloride (TZ) viability staining results for *Lophomyrtus* seed

3.3.4 Seed germination test and seedling vigour assessment

Lophomyrtus bullata seed collected in April or May failed to germinate within six weeks but TZ staining revealed high viability (100%). Radicle emergence was recorded within 10 days of seed collecting during June, and maximum germination was reached within six weeks. Radicle emergence was significantly higher in fresh non-stratified seed (100%) compared to seed stratified dry for four weeks (74%). Germination and seedling height were similar in non-stratified (46.9% and 47.1 mm) and stratified (45.9% and 45.3 mm) seed (Table 3.6).

Stratification did not impact radicle emergence or germination in *L. obcordata* with final germination of 85% and 82.5% for non-stratified and stratified seed respectively. Seedling height ranged from 12.3 to 35 mm with the mean seedling height after eight weeks 35.7 mm and 34.5 mm for non-stratified and stratified seed respectively (Table 3.6).

Seed collected from natural *L. bullata x L. obcordata* hybrids did not have dormancy with radicle emergence similar in non-stratified (c. 82%) and stratified (78%) seed.

Germination was lower than radicle emergence for stratified and non-stratified seed (c. 71% and 66% respectively) (Table 3.6). Seedling vigour was not recorded in *L. bullata x L. obcordata*.

Stratification time (4 vs 20 weeks) and method (dry vs wet) influenced radicle emergence in *N. pedunculata* seed. Dry stratification failed to stimulate radicle emergence irrespective of the stratification period (Table 3.7). Wet stratification for 4 weeks stimulated radicle emergence (c. 38%) with significantly higher radicle emergence after 20 weeks of wet stratification (c. 72%; P = 0.014; Table 3.7). All the seeds which produced a radicle completed the germination process (Table 3.7). Seeds exposed to wet stratification for up to 20 weeks also started to germinate during stratification (Fig. 3.8A), while those exposed to dry stratification showed no visual signs of germination during the stratification period (Fig. 3.8B). Wet stratification time (4 vs 20 weeks) did not have a significant impact on seedling height, although seedlings regenerated from seed stratified wet for 20 weeks were slightly taller (c. 20 mm) compared to those stratified for 4 weeks (c. 15 mm).

Table 3.6: Germination, tetrazolium viability staining results and seedling vigour of fresh seed from *L. bullata, L. obcordata and L. bullata x L. obcordata*. Parameter values (radicle emergence, germination and seedling vigour) within each species followed by the same letter do not differ significantly (Tukey's HSD; $P \leq 0.05$; N = 10)

Species	Radicle Emergence (% mean ± SD)		Germination (% mean ± SD)		Seedling vigour			
					Seedling Height (mm) (mean ± SD)		# of leaves (mean ± SD)	
	NS	DS	NS	DS	NS	DS	NS	DS
L. bullata	100 ± 0[a]	74 ± 17[b]	66.7 ± 5.8[a]	45.9 ± 26.8[a]	47.1 ± 11.3[a]	45.3 ± 10.2[a]	1.9 ± 1.3[a]	1.6 ± 1.3[a]
L. obcordata	85.0 ± 5.8[a]	82.5 ± 9.6[a]	85.0 ± 5.8[a]	82.5 ± 9.6[a]	35.7 ± 11.5[a]	34.5 ± 9.5[a]	1.9 ± 0.7[a]	1.7 ± 0.5[a]
L. bullata x L. obcordata	81.6 ± 7.1[a]	78.2 ± 5.6[a]	71.2 ± 6.5[a]	66.3 ± 8.6[a]	NT	NT	NT	NT

NS = Non-stratified
DS = Dry Stratified
NT = Not Tested

Table 3.7: Tetrazolium staining results, radicle emergence, germination, and seedling vigour for *Neomyrtus pedunculata* seed exposed to wet and dry stratification for 4 and 20 weeks. Parameter values (radicle emergence, germination and seedling vigour) followed by the same letter do not differ significantly (Tukey's HSD; $P \leq 0.05$; $N = 10$).

Stratification Time (weeks)	**Radicle Emergence (% mean ± SD)**		**Germination (% mean ± SD)**		**Seedling Vigour**			
					Seedling Height (mm) (mean ± SD)		**# of leaves (mean ± SD)**	
	WS	**DS**	**WS**	**DS**	**WS**	**DS**	**WS**	**DS**
4	37.5 ± 13.5[a]	0[c]	37.5 ± 13.5[a]	0[c]	15.8 ± 3.6[a]	NG	1.1 ± 0.3	NG
20	71.9 ± 21.9[b]	0[c]	71.9 ± 21.9[b]	0[c]	20.8 ± 8.5[a]	NG	1.1 ± 0.3	NG

DS = Dry stratified
WS = Wet Stratified
NG = No Germination

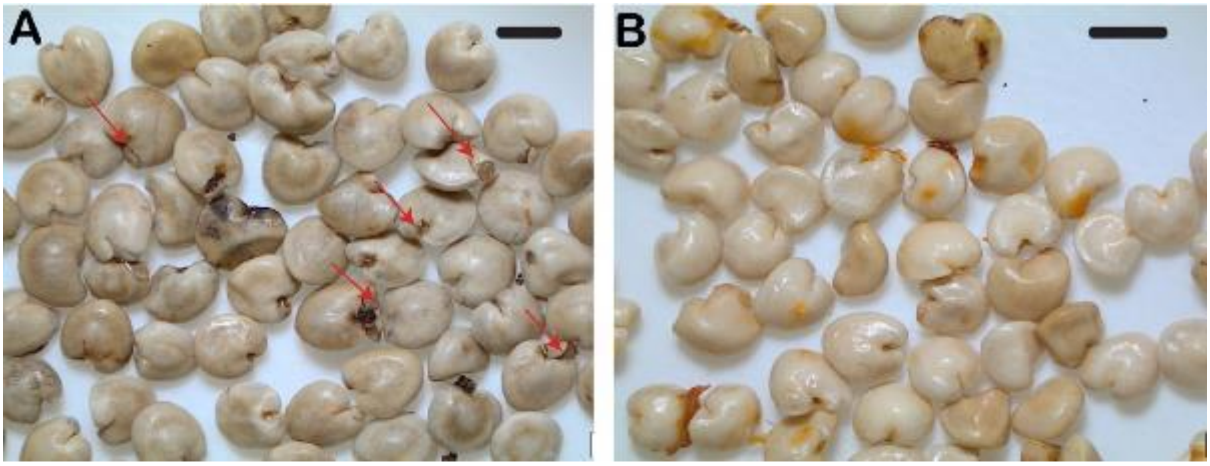


Figure 3.8: Radicle emergence was recorded in *Neomyrtus pedunculata* seeds during wet stratification (A) (red arrows indicate emerging radicles). Seed stratified in dry conditions did not display any radicle emergence during the stratification period (B). Black scale bars are equivalent to 1 mm

3.3.5 Seed desiccation trial

Lophomyrtus bullata seeds displayed desiccation sensitivity with a decrease in radicle emergence and TZ staining corresponding to a decline in MC (Table 3.8). Desiccation to 30% eRH resulted in a decrease in viability, although this was not significant. Further desiccation to 15% eRH decreased radicle emergence and germination to c. 48% and 39% respectively with seedling height decreasing from c. 47 mm in fresh seed to c. 31 mm following desiccation (Table 3.8). Although germination declined and seedling vigour was reduced when MC was lowered, this difference was not statistically significant.

Desiccation, irrespective of the eRH, had no impact of *L. obcordata* seed viability or seedling vigour. Radicle emergence in *L. bullata x L. obcordata* was reduced from c. 82% in non-desiccated seed to 55% in seed desiccated to 15% eRH with all seed showing radicle emergence completing the germination process (Table 3.8). *Neomyrtus pedunculata* seed desiccated 15% eRH had low (c. 4%) radicle emergence and germination compared to c. 72% in non-desiccated seeds. This is in agreement with the TZ test result which showed viability declined from 75% in non-desiccated seed to 25% in desiccated seeds (Table 3.8). The seedlings of desiccated seed also only reached c. 50% of the height recorded by seedlings from non-desiccated seeds (Table 3.8).

Table 3.8: Desiccation treatment, moisture content, viability (TZ stain, radicle emergence and germination) and seedling vigour (seedling height and number of leaves) for desiccated *L. bullata, L. obcordata, L. bullata x L. obcordata* and *N. pedunculata* seed. Fresh seed is represented by non-stratified seed in *L. bullata, L. obcordata* and *L. bullata x L. obcordata* with seed stratified wet for 20 weeks representing fresh seed for *N. pedunculata*. Parameter values (radicle emergence, germination and seedling vigour) for each species followed by the same letter do not differ significantly (Tukey's HSD; $P \leq 0.05$; N = 10).

Species	Desiccation Treatment	Moisture Content (g/g) (Mean ± SD)	TZ Stain (% Mean ± SD)	Radicle Emergence (% Mean ± SD)	Germination (% Mean ± SD)	Seedling vigour	
						Height (mm) (Mean ± SD)	# of leaves (Mean ± SD)
L. bullata	Fresh	0.52 ± 0.04	100 ± 0[a]	100 ± 0[a]	66.7 ± 5.8[a]	47.1 ± 11.3[a]	1.9 ± 1.3[a]
	30% eRH	0.42 ± 0.03	82.2 ± 9.6[b]	75.0 ± 17.3[ab]	70.0 ± 24.5[a]	33.2 ± 8.8[a]	1.6 ± 0.5[a]
	15% eRH	0.095 ± 0.001	52.1 ± 12.2[c]	47.5 ± 20.6[b]	42.5 ± 17.1[a]	31.1 ± 8.4[a]	1.8 ± 0.5[a]
L. obcordata	Fresh	0.35 ± 0.02	94.4 ± 11[a]	85 ± 5.8[a]	85 ± 5.8[a]	37.1 ± 9.5[a]	1.9 ± 0.7[a]
	30% eRH	0.2 ± 0.004	90.2 ± 8.9[a]	87.1 ± 6.3[a]	87.1 ± 6.3[a]	35.7 ± 11.5[a]	1.9 ± 0.4[a]
	15% eRH	0.094 ± 0.01	92.3 ± 2.2[a]	85 ± 5.8[a]	85 ± 5.8[a]	43.1 ± 11.0[a]	2.0 ± 0.7[a]
	Fresh	0.23 ± 0.003	NT	81.6 ± 7.1[a]	71.2 ± 6.5[a]	NT	NT

Species	Desiccation Treatment	Moisture Content (g/g) (Mean ± SD)	TZ Stain (% Mean ± SD)	Radicle Emergence (% Mean ± SD)	Germination (% Mean ± SD)	Seedling vigour	
						Height (mm) (Mean ± SD)	# of leaves (Mean ± SD)
L. bullata x L. obcordata	30% eRH	NT	NT	NT	NT	NT	NT
	15% eRH	0.094 ± 0.004	NT	54.6 ± 17[b]	54.6 ± 17[a]	NT	NT
N. pedunculata	Fresh	0.15 ± 0.001	75 ± 9.6[a]	71.9 ± 21.9[a]	71.9 ± 21.9[a]	20.8 ± 8.5[a]	1.1 ± 0.3[a]
	30% eRH	0.12 ± 0.006	NT	NT	NT	NT	NT
	15% eRH	0.06 ± 0.001	25.3 ± 12[b]	4.0 ± 8.0[b]	4.0 ± 8.0[b]	10.5 ± 0[b]	0[b]

NT = Not Tested

3.3.6 Combination of desiccation and freezing sensitivity assessment

Lophomyrtus bullata displayed freezing sensitivity at -18°C and -196°C with a declining trend in viability (TZ staining), radicle emergence, germination and seedling vigour associated with a decrease in storage temperature. This trend was accelerated with lowering the MC. Interestingly for seed desiccated 15% eRH, germination and seedling vigour remained constant with similar results for the non-stored seeds for both 5°C and -18°C. However, germination and seedling vigour declined significantly in seed exposed to -196°C (Table 3.9). *Lophomyrtus obcordata* showed an overall declining trend in radicle emergence, germination and seedling vigour following freezing at -18°C and -196°C, although the decline in most cases was not statistically significant (Table 3.9). The exception was seed desiccated to 15% eRH and exposed to -196°C which retained radicle emergence and germination (c. 84% and 74% respectively) which was comparable to fresh seed (c. 85%). Similarly, there was an overall reduction in seedling vigour (height and number of leaves) following freezing (Table 3.9) except for seed desiccated to 30% eRH and exposed to -18°C. *Lophomyrtus bullata x L. obcordata* displayed sensitivity to storage at -18°C with a non-significant decline in radicle emergence (c. 33%) and a significant decrease in germination (c. 29%) compared to seed exposed to 5°C and -196°C

(Table 3.9). Seedling vigour was not recorded in *L. bullata x L. obcordata*. For *L. bullata, L. obcordata* and *L. bullata x L. obcordata,* the interactive effect between desiccation and temperature on radicle emergence was not significant. Desiccation was found to be the most influential explanatory value. *Neomyrtus pedunculata* showed a decline in viability (based on TZ testing) following exposure to -18°C (Table 3.9). Since seed germination was limited due to dormancy, no seedling vigour assessment was carried out.

3.3.7 Seed storage trial

Lophomyrtus bullata seed stored for 24 to 36 months showed extreme sensitivity to freezing at -18°C and -196°C with a significant decline confirmed in TZ viability staining, radicle emergence and germination. Desiccation was however essential to retain viability in seed stored at 5°C (Table 3.10). Time in storage, seed lot or the combined effect of time and seed lot, did not have a significant influence on the viability of seed in storage, with temperature found to be most influential variable. Overall seedling vigour was not impacted by storage temperature, except for the single seedling obtained from seed stored at -18°C which was significantly taller (c. 40 mm) and produced more leaves (c. 2.0) compared to other treatments (Table 3.10). The *L. obcordata* seed storage trial was represented by three seed lots: Ōtari 19, Matai and Old Mill. Two of the seed lots, Ōtari 19 and Matai, showed freezing sensitivity illustrated in the decline in viability (TZ staining), radicle emergence, germination, and seedling height (Table 3.10). Response to storage at -18°C was also influenced by seed lot, with seed from Old Mill having significantly higher radicle emergence, germination and seedling height compared to Ōtari and Matai. The interactive effect of time in storage, temperature and seed lot was not significant. Seed from *L. bullata x L. obcordata* also continued to decline in viability after 12 months at sub-zero temperature storage (Table 3.10). Interestingly, although radicle emergence was lower in seed stored at -18°C (42%) and -196°C (33%) compared to seed stored at 5°C (55%), the difference was not significant (P = 0.170). In contrast to radicle emergence, germination was significantly higher in seed stored at 5°C (P = 0.001; 42.5%) compared to germination in seed stored for 12–36 months at -18°C (19.3%) and -196°C (22%) (Table 3.10). Seedling vigour was similar irrespective of the storage temperatures.

Table 3.9: The combined effect of desiccation (15% and 30% eRH) and temperature exposure (5°C, -18°C and -196°C) for 14 days on the seed viability (TZ stain and radicle emergence), germination and seedling vigour of *Lophomyrtus bullata*, *L. obcordata*, *L. bullata x L. obcordata* and *Neomyrtus pedunculata*. Values followed by the same letter for various parameters (TZ stain, radicle emergence, germination, seedling size and number of leaves) within each species do not differ significantly (Tukey HSD, $P \leq 0.05$, N = 10)

Species	Desiccation Treatment	Moisture Content (g/g) (Mean ± SD)	Temperature (°C)	TZ Stain (% Mean ± SD)	Radicle Emergence (% Mean ± SD)	Germination (% Mean ± SD)	Seedling vigour	
							Size (mm) (Mean ± SD)	# of leaves (Mean ± SD)
L. bullata	Fresh	0.52 ± 0.04	NA	82.3 ± 10.3^{a}	100 ± 0^{a}	66.7 ± 5.8^{ab}	47.1 ± 11.3^{a}	1.9 ± 1.3^{a}
	30% eRH	0.42 ± 0.03	NA	82.2 ± 9.6^{a}	75.0 ± 17.3^{ab}	70.0 ± 24.5^{a}	33.2 ± 8.8^{b}	1.6 ± 0.5^{ab}
			5	77.3 ± 10.1^{a}	76.7 ± 5.8^{ab}	60.0 ± 10^{ab}	33.2 ± 8.8^{b}	1.8 ± 0.5^{ab}
			-18	60.1 ± 5.6^{bc}	47.5 ± 12.6^{bc}	35.0 ± 19.1^{ab}	28.6 ± 7.6^{bc}	1.3 ± 0.5^{ab}
			-196	49.0 ± 11.1^{bc}	44.4 ± 13.3^{bc}	31.9 ± 9.0^{ab}	24.8 ± 10^{bc}	0.8 ± 0.5^{ab}
	15% eRH	0.095 ± 0.001	NA	52.1 ± 12.2^{bc}	47.5 ± 20.6^{bc}	42.5 ± 17.1^{a}	31.1 ± 8.4^{bc}	1.8 ± 0.5^{a}
			5	52.0 ± 6.3^{bc}	47.6 ± 20.5^{bc}	41.0 ± 10.1^{ab}	31.1 ± 8.4^{bc}	1.8 ± 0.5^{a}
			-18	57.2 ± 7.5^{bc}	57.5 ± 6.6^{bc}	49.1 ± 20.7^{ab}	26.1 ± 7.3^{bc}	1.3 ± 0.4^{ab}
			-196	39.3 ± 12.3^{c}	35.6 ± 9.9^{c}	23.6 ± 10.5^{b}	20.8 ± 8.1^{c}	0.8 ± 0.5^{b}
L. obcordata	Fresh	0.35 ± 0.02	NA	94.4 ± 11^{a}	85.0 ± 5.8^{a}	85.0 ± 5.8^{a}	37.1 ± 9.5^{a}	1.9 ± 0.7^{a}

Species	Desiccation Treatment	Moisture Content (g/g) (Mean ± SD)	Temperature (°C)	TZ Stain (% Mean ± SD)	Radicle Emergence (% Mean ± SD)	Germination (% Mean ± SD)	Seedling vigour	
							Size (mm) (Mean ± SD)	# of leaves (Mean ± SD)
	30% eRH	0.2 ± 0.004	NA	90.2 ± 8.9[a]	87.1 ± 6.3[a]	87.1 ± 6.3[a]	35.7 ± 11.5[a]	1.9 ± 0.4[a]
			5	91.2 ± 9.6[a]	84.6 ± 12.6[a]	78.8 ± 7.4[a]	33.7 ± 9.5	1.9 ± 0.7[a]
			-18	75.2 ± 3.0[b]	68.8 ± 1.9[a]	58.3 ± 0[b]	36.7 ± 10.3[a]	1.9 ± 0.7[a]
			-196	91.1 ± 5.3[a]	72.2 ± 6.9[a]	68.9 ± 1.9[ab]	28.7 ± 11.3[b]	1.7 ± 0.8[b]
	15% eRH	0.094 ± 0.01	NA	92.3 ± 2.2[a]	85 ± 5.8[a]	85 ± 5.8[ab]	43.1 ± 11.0[a]	2.0 ± 0.7[a]
			5	NT	84.6 ± 12.6[a]	71.6 ± 15.2[ab]	43.0 ± 11.0[a]	2.0 ± 0.7[a]
			-18	NT	68.6 ± 8.0[a]	65 ± 4.9[ab]	28.2 ± 9.2[b]	1.6 ± 0.7[b]
			-196	NT	84.1 ± 11.4[a]	73.7 ± 25.6[a]	25.6 ± 7.6[b]	1.6 ± 0.7[b]
L. bullata x L. obcordata	Fresh	0.23 ± 0.003	NA	NT	81.6 ± 7.1[a]	71.2 ± 6.5[a]	NT	NT
	30% eRH		NT	NT	NT	NT	NT	NT
	15% eRH	0.094 ± 0.004	NA	NT	54.6 ± 17[b]	54.6 ± 17[b]	NT	NT
			5	NT	59.0 ± 12.5[b]	50.8 ± 10.5[b]	NT	NT
			-18	NT	33.3 ± 2.8[b]	29.3 ± 2.8[c]	NT	NT
			-196	NT	50.3 ± 15.6[b]	44.2 ± 12.3[b]	NT	NT
N. pedunculata	Fresh	0.15 ± 0.001	NA	75 ± 9.6	71.9 ± 21.9	71.9 ± 21.9	20.8 ± 8.5	1.1 ± 0.3
	30% eRH	0.12 ± 0.006	NT	NT	NT	NT	NT	NT
	15% eRH	0.06 ± 0.001	NA	25.3 ± 12[a]	4.0 ± 8.0	4.0 ± 8.0	10.5 ± 0	0

Species	Desiccation Treatment	Moisture Content (g/g) (Mean ± SD)	Temperature (°C)	TZ Stain (% Mean ± SD)	Radicle Emergence (% Mean ± SD)	Germination (% Mean ± SD)	Seedling vigour	
							Size (mm) (Mean ± SD)	# of leaves (Mean ± SD)
			-18	19.9 ± 15.2^{a}	0	0	0	0

NA = Not applicable
NT = Not Tested

Table 3.10: Seed storage trial for non-desiccated seed and seed desiccated to 15% eRH for *Lophomyrtus bullata, L. obcordata and L. bullata x L. obcordata*. Seed was stored at 5°C, -18°C and -196°C for 12 to 36 months. Values followed by the same letter for various parameters (TZ stain, radicle emergence, germination, seedling size and number of leaves) within each species do not differ significantly (Tukey HSD, P ≤ 0.05, N = 10)

Species	Seed Lot	Desiccation Treatment	MC (g/g)	Time in storage (months)	Storage Temperature (°C)	TZ Stain	Radicle Emergence	Germination	Seedling vigour	
									Seedling size (mm)	# of Leaves
L. bullata	Kap 19	ND	0.52 ± 0.04	24	5	0^{b}	0^{b}	0^{b}	n/a	n/a
		15% eRH	0.087 ± 0.02		5	46.7 ± 15.6^{a}	37.8 ± 21.7^{a}	22.5 ± 17.1^{a}	25.0 ± 8.4^{a}	0.6 ± 0.8^{a}
					-18	7.5 ± 9.5^{b}	3.3 ± 3.8^{b}	3.3 ± 3.8^{b}	*40.2 ± 0^{b}	*2.0 ± 0^{b}

Species	**Seed Lot**	**Desiccation Treatment**	**MC (g/g)**	**Time in storage (months)**	**Storage Temperature (°C)**	**TZ Stain**	**Radicle Emergence**	**Germination**	**Seedling vigour**	
									Seedling size (mm)	# of Leaves
					-196	18.1 ± 8.9^{b}	11.4 ± 13.3^{b}	0^{b}	n/a	n/a
	Butchers	15% eRH	0.083 ± 0.03	36	-18	NT	9.2 ± 4.3^{b}	9.2 ± 4.3^{b}	20.5 ± 0^{a}	0.3 ± 0.6^{a}
	Wrights	15% eRH	0.09 ± 0.02	36	-18	13.8 ± 4.5^{b}	6.9 ± 5.4^{b}	6.9 ± 5.4^{b}	*20.9 ± 0^{a}	*1.0 ± 0^{a}
L. obcordata	Ōtari 19	ND	0.35 ± 0.02	24	5	0	0^{c}	0^{c}	n/a	n/a
		15% eRH	0.08 ± 0.002		5	77.5 ± 9.6^{a}	92.5 ± 9.6^{a}	80 ± 14.1^{a}	30.6 ± 9.7^{a}	1.1 ± 0.3^{a}
					-18	5.9 ± 7.4^{b}	6.6 ± 9.4^{c}	3.3 ± 3.8^{c}	15.3 ± 5.3^{b}	*1.0 ± 0^{a}
	Matai	15% eRH	0.08 ± 0.002	36	-18	NT	12.3 ± 8.9^{c}	7 ± 5.8^{c}	18.0 ± 2.5^{ab}	*1.0 ± 0^{a}
	Old Mill		0.08 ± 0.002	36	-18	NT	64.8 ± 9.8^{b}	41.4 ± 9.8^{b}	29.6 ± 9.2^{a}	1.4 ± 0.6^{a}
	Skyline 20	15% eRH	0.08 ± 0.004	12	5	NT	55.0 ± 17.3^{a}	42.5 ± 5^{a}	29.2 ± 14.4^{a}	1.6 ± 0.5^{a}

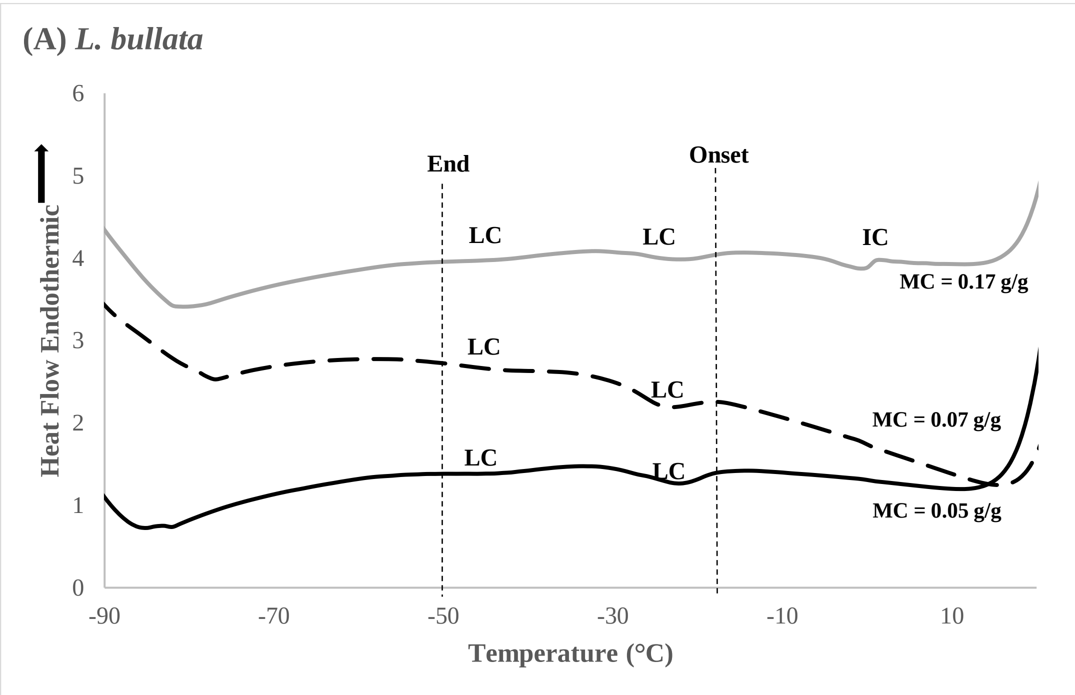
Species	Seed Lot	Desiccation Treatment	MC (g/g)	Time in storage (months)	Storage Temperature (°C)	TZ Stain	Radicle Emergence	Germination	Seedling vigour	
									Seedling size (mm)	# of Leaves
L. bullata x L. obcordata					-18	NT	41.6 ± 5.9[a]	19.3 ± 7.1[b]	31.7 ± 11.6[a]	1.3 ± 0.5[a]
					-196	NT	33.3 ± 18.0[a]	21.7 ± 6.4[b]	22.9 ± 9.5[a]	1.2 ± 0.6[a]

ND = Non-Desiccated
NT = Not Tested
* Based on a single seedling

3.3.8 Differential Scanning Calorimetry

The DSC thermograms provided measurements for critical parameters including enthalpy of phase transitions that occur between -90°C and 20°C. For the cooling cycle, all three species showed two lipid crystallization events with onset and end temperatures spread from -18°C to -50°C (Figure 3.9; Table 3.11). The onset temperature for lipid crystallization for *L. bullata* is slightly higher i.e. -18°C compared to -23°C and -25°C in *L. obcordata* and *N. pedunculata* respectively (Fig. 3.9). There was a small ice crystallization event noted for *L. bullata* seeds equilibrated to 30% eRH with seed moisture content c. 0.17 g/g (Fig. 3.9A).

Upon warming, all three species displayed three lipid melting events spread from -77.4 to 5.7°C (Table 3.11). *Lophomyrtus bullata* seed equilibrated to 30% eRH with seed moisture content c. 0.17 g/g displayed the fourth peak which is related to ice melt (Fig. 3.10A). The lipid melting events for *L. bullata* seeds ranged from -19.7 to 4.8°C, whereas for *L. obcordata* it ranged from -24.3 to 3.7°C and -25.3 to 1.2°C for *N. pedunculata*. Melting events spanned similar temperature ranges (24 to 28°C) in all three species. The average of total melt enthalpy (cumulative value of all three melting events) was 3.1 J/g for *L. bullata*, 4.3 J/g for *L. obcordata* with the highest enthalpy recorded in *N. pedunculata* at 6.6 J/g dw (Table 3.11).



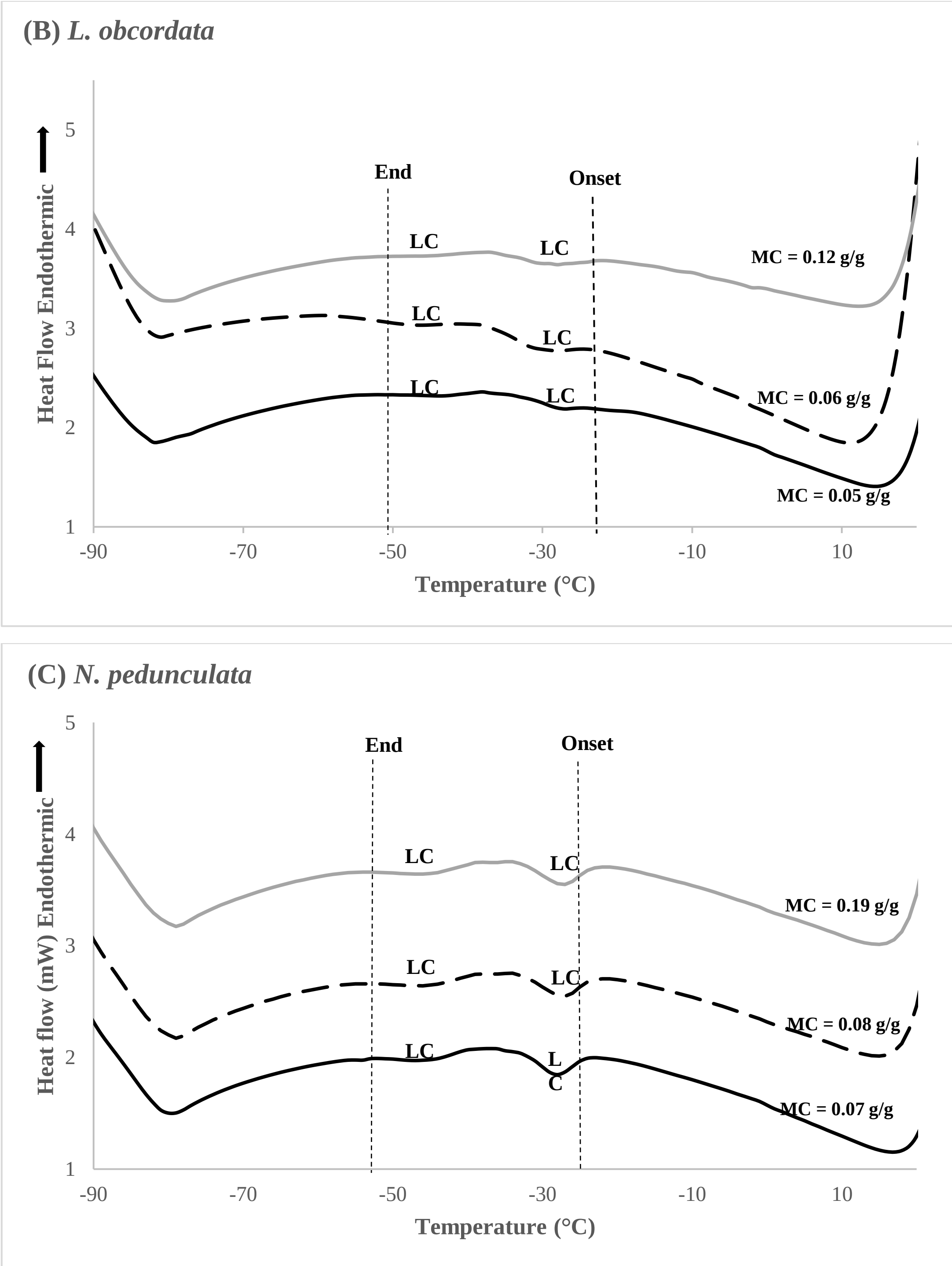
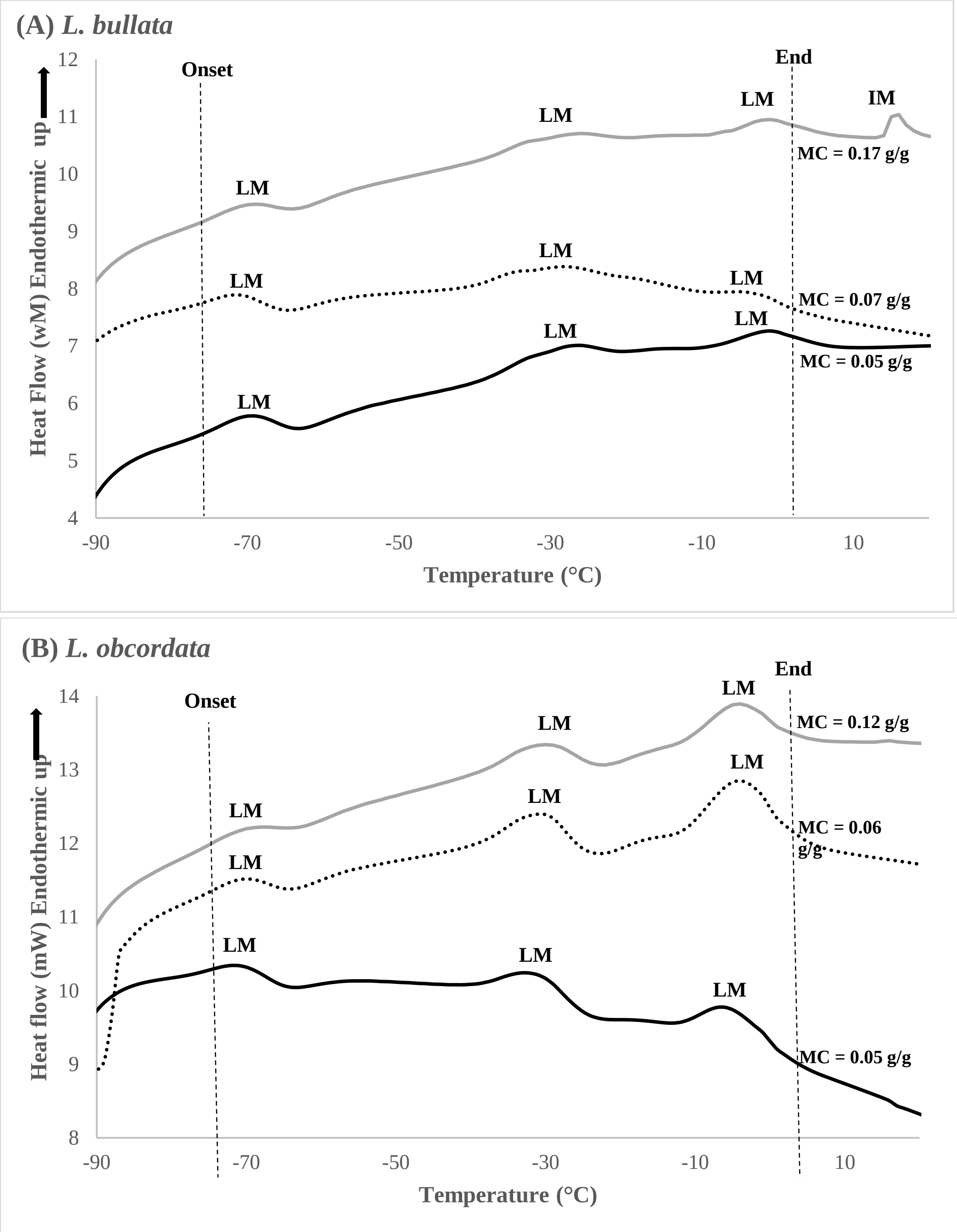


Figure 3.9 DSC cooling thermograms for *Lophomyrtus bullata* (A), *L. obcordata* (B), and *Neomyrtus pedunculata* (C) seed equilibrated to 5% (black line), 15% (broken line) and 30% eRH (grey line). Ice crystallization (IC), lipid crystallization (LC) and temperature range from onset to end of crystallization are indicated. Samples were scanned at 10 min^{-1} from 25°C to -100°C. Each sample consisted of three replicates with three seeds in each replicate.


(A) L. bullata
Heat Flow (wM) Endothermic up
Temperature (°C)
Onset
End
LM
IM
MC = 0.17 g/g
MC = 0.07 g/g
MC = 0.05 g/g
12
11
10
9
8
7
6
5
4
-90
-70
-50
-30
-10
10
(B) L. obcordata
Heat flow (mW) Endothermic up
Temperature (°C)
Onset
End
LM
MC = 0.12 g/g
MC = 0.06 g/g
MC = 0.05 g/g
14
13
12
11
10
9
8
-90
-70
-50
-30
-10
10

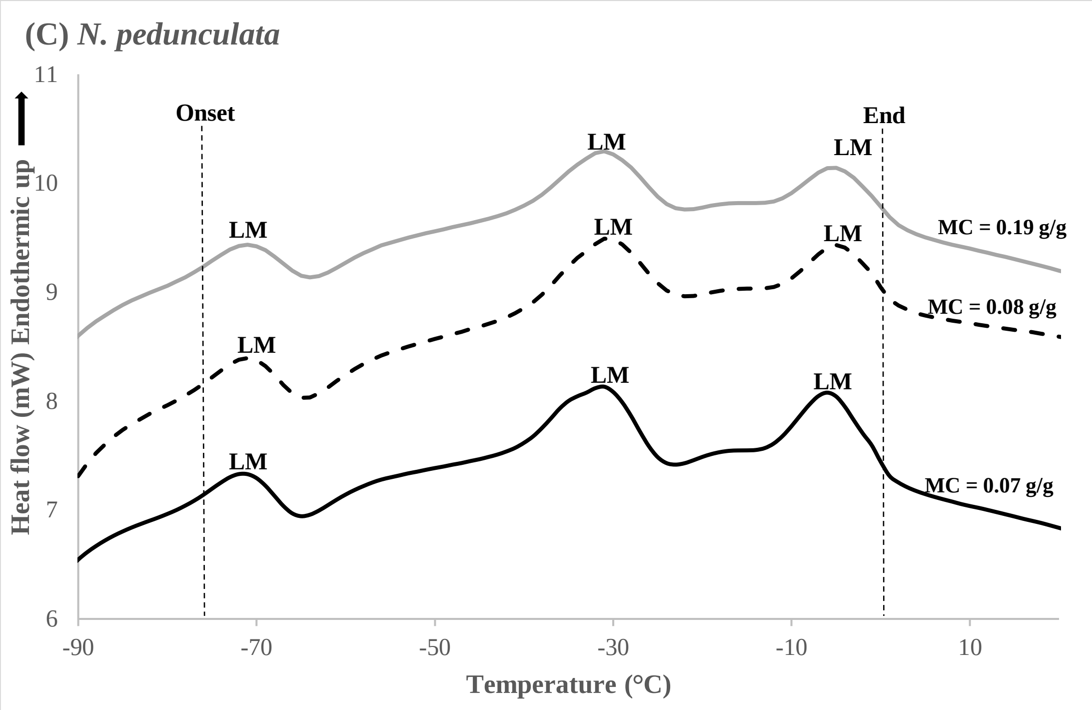


Figure 3.10: DSC warming thermograms for *Lophomyrtus bullata* (A), *L. obcordata* (B), and *Neomyrtus pedunculata* (C) seed equilibrated to 5% (black line), 15% (broken line) and 30% eRH (grey line). Lipid melt transitions are identified (LM) and ice melt as (IM). Samples were scanned at 10 min^{-1} from -100°C to 25°C. Each sample consisted of three replicates with three seeds in each replicate.

Table 3.11: Cooling and warming thermodynamic properties of *Lophomyrtus bullata, L. obcordata* and *Neomyrtus pedunculata* seed following desiccation to various moisture contents. Superscript of different letters within a column indicates significantly difference at p < 0.05 based on a one-way ANOVA analysis (Tukey HSD test). Data are presented as mean ± SD (P < 0.05) for three different treatments.

Species	**Moisture Content (g/g)**	**Sample Weight (mg)**	**Cooling**				**Warming**			
			***Onset Temperature of Crystallization (°C)**	****End Temperature of Crystallization (°C)**	*****Area of crystallization (mJ)**	*****Enthalpy of Melt (J/g)**	***Onset Temperature of Melt (°C)**	****End Temperature of Melt (°C)**	*****Area of Melt (mJ)**	*****Enthalpy of Melt (J/g)**
L. bullata	0.053 ± 0.001	17 ± 3.9	-18.4 ± 1.4^{a}	-49.5 ± 2.8^{a}	7.3 ± 1.8^{a}	0.4 ± 0.9^{a}	-76.5 ± 0.8^{ab}	5.7 ± 0.8^{a}	48.9 ± 14.5^{a}	2.9 ± 0.6^{a}
	0.067 ± 0.001	16.4 ± 5.5	-19.7 ± 0.7^{ab}	-49.3 ± 1.6^{a}	7.3 ± 3.8^{a}	0.4 ± 0.1^{a}	-77.6 ± 0.4^{d}	3.0 ± 1.6^{abc}	68.7 ± 38.2^{a}	4.1 ± 2.1^{a}
	0.17 ±0.002	14.1 ± 3.3	-19.2 ± 2.0^{ab}	-47.0 ± 2.6^{a}	3.5 ± 1.2^{a}	0.3 ± 0.06^{a}	-76.4 ± 0.7^{ab}	4.8 ± 1.5^{ab}	37.2 ± 12.7^{a}	2.2 ± 0.5^{a}
L. obcordata	0.048 ± 0.001	13.1 ± 3.1	-23.2 ± 3.6^{abc}	-50.8 ± 4.2^{b}	3.9 ± 2.8^{a}	0.3 ± 0.2^{a}	-77.1 ± 0.6^{abcd}	2.1 ± 2.5^{abc}	51.6 ± 8.8^{a}	4.2 ± 1.6^{a}
	0.06 ±0.001	19.3 ± 4.7	-23.7 ± 2.6^{abc}	-50.3 ± 0.4^{b}	6.3 ± 0.9^{a}	1.0 ± 0.9^{a}	-76.8 ± 0.4^{abc}	3.7 ± 0.4^{abc}	88.8 ± 48.5^{a}	4.6 ± 1.7^{a}
	0.12 ± 0.006	14.7 ± 2.6	-24.3 ± 2.3^{bc}	-47.5 ± 5.9^{b}	4.8 ± 3.1^{a}	0.3 ± 0.1^{a}	-76.3 ± 0.6^{a}	2.2 ± 2.7^{abc}	32.3 ± 31.5^{a}	4.1 ± 2.0^{a}
N. pedunculata	0.069 ± 0.002	10.4 ± 0.6	-25.3 ± 0.5^{c}	-52.9 ± 1.0^{b}	7.2 ± 1.8^{a}	0.6 ± 0.1^{a}	-76.9 ± 0.7^{abcd}	0.15 ± 0.3^{c}	80.9 ± 49.7^{a}	7.6 ± 4.2^{a}
	0.084 ± 0.003	11.9 ± 0.4	-24.3 ± 0.6^{bc}	-51.3 ± 1.5^{b}	8.7 ± 2.1^{a}	0.7 ± 0.1^{a}	-77.6 ± 0.3^{cd}	1.2 ± 0.5^{bc}	72.1 ± 11.9^{a}	5.9 ± 0.9^{a}

Species	Moisture Content (g/g)	Sample Weight (mg)	Cooling				Warming			
			*Onset Temperature of Crystallization (°C)	**End Temperature of Crystallization (°C)	***Area of crystallization (mJ)	***Enthalpy of Melt (J/g)	*Onset Temperature of Melt (°C)	**End Temperature of Melt (°C)	***Area of Melt (mJ)	***Enthalpy of Melt (J/g)
	0.17 ± 0.004	10 ± 0.6	-25.4 ± 1.4[c]	-52.6 ± 3.6[b]	6.5 ± 1.9[a]	0.6 ± 0.2[a]	-77.3 ± 0.4[bcd]	-0.3 ± 1.6[c]	63.3 ± 11.9[a]	6.2 ± 3.0[a]
P value			*= 0.026*	*< 0.001*	*= 0.307*	*= 0.37*	*= 0.03*	*< 0.02*	*= 0.326*	*= 0.625*

* Onset temperature of the first peak that appeared during cooling or warming programmes
** End temperature of the last peak that appeared during cooling or warming programmes
*** Cumulative area / enthalpy of all the peaks that appeared during cooling or warming programmes

3.4 Discussion

This study investigated the seed biology and storage physiology of *Lophomyrtus* and *Neomyrtus* two threatened genera of Myrtaceae endemic to New Zealand in order to recommend optimum storage conditions for long-term conservation of these species.

Fruit and seed morphology have been used to increase foundational knowledge into seed responses to storage processes such as desiccation (Hamilton *et al.*, 2013). The fruit and seed morphology of *L. bullata* was studied in fruit from two developmental stages distinguished by the colour of the fruit pulp (green and red). Seed desiccation tolerance is normally acquired at the time when seeds reach complete maturity and thus reached their maximum dry weight (Ellis, 2019; Whitehouse *et al.*, 2020). This study found that the MC of *L. bullata* seed collected from green fruit was higher than the MC of seed collected from red fruit. Similarly, seed from red fruit collected earlier in the season (May) had higher MC compared to seed from fruit collected a month later (June). A decrease in MC generally indicates that seeds have reached maturity and the vascular connection with the maternal plant has been terminated allowing the seeds to equilibrate to the ambient conditions in preparation for dispersal (Ellis, 2019; Hay *et al.*, 1995). It is postulated that the decrease in MC observed in *L. bullata* seed in this study indicated that seed had reached maturity and was at the optimal stage for collection. Mature *L. bullata* seed had a significantly higher MC (0.52 g/g) compared to *L. obcordata* (0.35 g/g) (Table 3.4). Desiccation sensitive seeds from rainforest species in Australia and Africa all had moisture contents at maturity of greater than 0.5 g/g (~35% fresh weight) (Hamilton *et al.*, 2013; Pritchard, 2004). Hong *et al.* (1996) indicated that seed moisture content of < 25% could be used as an indicative of desiccation tolerance.

Another important seed characteristic for seed storage is lipid content, particularly the physical phase of the lipid at storage temperatures (Crane *et al.*, 2003; Hamilton *et al.*, 2013). Seed maturity is often associated with the accumulation of specific biochemical compounds related to the seed’s ability to tolerate desiccation and survive prolonged periods of unsuitable conditions for germination (Ballesteros *et al.*, 2020). Loss of germination in stored seed is accompanied by a decrease in embryo viability and deterioration of storage nutrients (Oenel *et al.*, 2017). Since the degradation of storage lipids can have important physiological and ecological consequences for seed, an understanding of the seed’s fatty acid composition can aid in the optimisation of seed storage protocols. During storage, fatty acids are vulnerable to lipid degradation through oxidation or enzymatic hydrolysis (Hulbert, 2005). Hamilton *et al.* (2013) found that several characteristics including seed lipid content, could be used to flag Australian rainforest species which have unknown seed storage behaviour. The total lipid content of *L. bullata* and *L. obcordata* seed in this study was 7.6% and 4.1% of the total seed weight respectively (Fig. 3.4). Interestingly, linoleic acid (C18:2) made up 75% of the fatty acids in both species. Linoleic acid is abundant in many species including *Vitis vinifera* (Vitaceae; grape), *Nicotiana tabacum* (Solanaceae; tobacco) and *Ribes nigrum* (Grossulariaceae; blackcurrant) (see Diantina *et al.*, 2021 and references within). Polyunsaturated fatty acids, which includes linoleic acid, are particularly prone to lipid oxidation during the seed aging process (Oenel *et al.*, 2017). This study found that there was a slight increase in linoleic acid from c. 75% in fresh *L. bullata* seed to c. 77% and 78% in desiccated *L. bullata* seed stored for 15 months at 5°C and -196°C respectively (Table 3.5). Most studies report a decrease in linoleic acid in aged seed, for example, almond seed (Zacheo *et al.*, 1998), various rice species (Yasumatsu & Moritaka, 1964), tomatoes (Pichardo-González *et al.*, 2014) and orchids (Diantina *et al.*, 2021). It is thus expected that lipid content and the amount of total fatty acids in ageing seeds are likely to decrease due to lipid oxidation (Oenel *et al.*, 2017). The changes in fatty acid composition reported in this study could indicate that the molecular structure of the crystals are reorganising into denser and lower energy forms as suggested by (Mira *et al.*, 2019). However, since these changes are relatively small, it is postulated that in *L. bullata* seed, particularly the increase in linoleic acid, are likely due to intraspecific variation in the composition of the seed collected at different development stages and various genotypes.

Seed germination tests are considered the ultimate method for assessing seed viability. This however requires knowledge of seed dormancy and the identification of optimum germination conditions (Whitehouse *et al.*, 2020). Staining tests using tetrazolium chloride is another method often used to evaluate seed viability. One of the main disadvantages of TZ staining tests is the difficulty in interpreting the staining patterns and thus possibly leading in subjective assessments (Quain *et al.*, 2009; Whitehouse *et al.*, 2020). However, once staining protocols have been developed, TZ testing is considered one of the most reliable and quick tests to evaluate seed quality (Custódio *et al.*, 2016). TZ staining tests in this study revealed clear categories for viable (dark red) and non-viable (unstained/white) in both *Lophomyrtus* species and *N. pedunculata*. Seed viability, defined as dark red uniformly stained embryos in the TZ test, strongly correlated with the germination tests. The staining protocol described here can therefore the used as a reliable method to evaluate the physiological quality of the seed, particularly for *N. pedunculata* which displayed dormancy.

Though seed germination test is regarded as the best method to assess seed viability, the outcome of a germination test can be affected by seed dormancy. Seed dormancy is defined as the suspension of germination under conditions that are unfavourable for embryo emergence (Nonogaki, 2019). One of the challenges in seed germination is defining the environmental conditions that are required to release a seed from dormancy (Baskin & Baskin, 2004). In addition, seed can cycle through various dormancies during development and into maturation (Baskin *et al.*, 2004). For example, *Brassica napus* (Brassicaceae) seed is dormant during seed development but lack dormancy once seed reached maturity (Huang *et al.*, 2016). In this study, *L. bullata* seed collected during April or early May failed to germinate despite high viability as confirmed through TZ staining. When seed from the same population (Kap 19) was collected at the end of June, radicle emergence was observed within seven days. Minimum temperatures for Paekakariki, the closest town to the Kapiti population, during April ranged from 2.2 to 7.5°C while minimum temperatures in June drops to around -1.3°C (Chappell, 2014). Seed collected in June has therefore have been exposed to lower temperatures which likely served as a cue to break the dormancy associated with early season collection. There was no dormancy recorded for *L. obcordata* irrespective of the population or time of collection while *N. pedunculata* seeds required moist stratification (5°C) for up to 20 weeks to stimulate germination.

Species occurring in climates with seasonal variation (summer and winter) often have seed which becomes non dormant over winter resulting in germination during spring (Baskin *et al.*, 2014). Winter rainfall in these areas furthermore increases the likelihood that wet cold stratification will be required to break dormancy. *Neomyrtus pedunculata* seed in this study was collected from Taranaki which is located in central North Island. It is postulated that high winter rainfall coupled with low minimum temperatures during winter breaks dormancy and facilitates germination in spring. Dormancy which requires a longer cold stratification period is likely a strategy to prevent early emergence of seedlings during the cold winter months (Phartyal *et al.*, 2003). Dormancy can furthermore vary between populations and genotypes. For example, Andersson and Milberg (1998) found that seed dormancy varied between mother plants, populations and years in *Silene noctiflora* (Caryophyllaceae), *Sinapis arvensis* (Brassicaceae) and *Spergula arvensis* (Caryophyllaceae). Similarly, dormancy breaking requirements varied between three *Actinidia* (Actinidiaceae) species, although all the species showed increased germination after five weeks cold stratification (Maghdouri *et al.*, 2021). It is recommended that *N. pedunculata* seed is collected from different populations and tested to establish if dormancy is present.

Conventional seed banking requires desiccation to about 15% eRH and storage at -18°C (FAO, 2014). Desiccation tolerance in *L. bullata* and *L. obcordata* was investigated by drying the seeds to 30% and 15% eRH. Neither species suffered a significant decrease in radicle emergence or germination following desiccation to 30% eRH. Further desiccation to 15% eRH did not have an impact on *L. obcordata*, while a significant decrease in radicle emergence and germination was recorded in *L. bullata* (Table 3.8). The desiccation sensitivity in *L. bullata* in this study contrasted with (Nadarajan *et al.*, 2021) where *L. bullata* seed was found to be desiccation tolerant. The standard deviation observed for radicle emergence and germination in *L. bullata* in this study was high (c. 21 and 17% respectively). This could indicate that there is intraspecific diversity towards desiccation tolerance (15% eRH) (Dussert *et al.*, 2004). Intraspecific desiccation tolerance can be variable and influenced by factors such as seed development at time of harvesting, parental plant characteristics, post-harvest seed handling, and climatic conditions between years (Hay *et al.*, 1995, 2013). It was interesting to note that *L. bullata x L. obcordata* appears to share desiccation characteristics from both parental plants with seed slightly less sensitive to desiccation compared to *L. bullata* (Table 3.8). This has also been found in cultivated species of *Coffea arabica* (Rubiaceae) which showed a level of desiccation tolerance intermediate between its two parental species (Dussert *et al.*, 2004). The results from this study found that seed from *L. bullata*, *L. obcordata* and *L. bullata x L. obcordata* falls along a continuum of desiccation tolerance, supporting the hypothesis that non-orthodox species displays very high variation in desiccation sensitivity (Dussert *et al.*, 2004; Pammenter & Berjak, 2007).

Seed from the intermediate storage physiology can display tolerance to desiccation but sensitivity to freezing (Merritt *et al.*, 2021; Sommerville *et al.*, 2021). To test for freezing sensitivity, *L. bullata* and *L. obcordata* seed desiccated to 30% eRH and 15% eRH were exposed to 5°C, -18°C and -196°C for 14 days. Although *L. bullata* seed tolerated desiccation to 30% eRH, freezing seed at this MC resulted in a decrease in seed viability (TZ test and radicle emergence) and germination. This is likely due to the presence of freezable water which resulted in the formation of ice crystals. Although there was an initial decrease in viability following desiccation to 15% eRH, viability remained similar when seed was stored at 5°C and -18°C but a further decrease in viability was associated with storage at -196°C. *Lophomyrtus bullata* seed stored for 24 to 36 months at -18°C suffered significant viability loss. Seed from the same seed lot stored for 24 months at 5°C did not lose significant viability compared to desiccated seed without storage. Since the seed for the same seed lot which was stored at 5°C had greater viability than seeds stored at -18°C, it can be concluded that *L. bullata* seeds do not tolerate freezing temperature storage.

Lophomyrtus obcordata seed was not sensitive to desiccation and although there was a decrease in seed viability following exposure to -18°C for 14 days, it was not significant. However, when desiccated (15% eRH) seed was stored for 24 to 36 months at -18°C, two collections (Matai and Ōtari), lost significant viability while seed collected from Old Mill Road retained 63% radicle emergence (Table 3.6). Loss of seed viability during storage can be seed lot specific (Godefroid *et al.*, 2011; Hay *et al.*, 2013) with some accessions rapidly losing viability in storage while freeze induced viability loss can take multiple years in others (Chau *et al.*, 2019). Intraspecific variability in seed physiology has been reported in species such as *Aesculus hippocastanum* (Sapindaceae), *Silene noctiflora* (Carophyllaceae), *Sinapis arvensis* (Brassicaceae), *Spergula arvensis* (Carophyllaceae) and *Thlaspi arvense* (Brassicaceae) (Andersson *et al*., 1998; Daws *et al*., 2004). Although not fully understood, reasons for this intraspecific variability include genetic heterogeneity, climatic conditions, seed development stage and post-harvest treatments (Hay *et al.*, 1995, 2013; Walters *et al.*, 2005). The *L. obcordata* seed from Old Mill Road and Matai was collected at the same time and from the same general area (Marlborough). It is therefore postulated that the difference in seed storage physiology reported here is likely due to genetic variation.

DSC was used to characterise the phase behaviour of lipids. The lack of large endothermic peaks suggest that no ice nucleation was present, except for *L. bullata* seeds which had been dried to 0.17 g/g (~30% eRH). Transitions observed in the cooling and warming thermograms are therefore due to lipids. The crystallization and melt fingerprints of *L. bullata* seed spanned 87°C [-71°C (onset) to 16°C (end)]. Mira *et al.* (2019) found that oily seeds which stored relatively poorly at -20°C tended to have lipids with crystallization and melting temperatures that spread over a wide range and spanned the storage temperature. The study furthermore highlighted that when lipids were in a mixture of solid and liquid phase (metastable) at the storage temperature, the Brassicaceae seeds aged more rapidly. The DSC thermograms indicated lipid crystallization *L. bullata* occurred between -18 and -28°C, thus overlapping the conventional seed banking temperature of -18°C. Long-term storage of this seed at temperatures close to or within the lipid phase transitions should be avoided at this could lead to rapid seed deterioration due to unforeseen consequences to the seed cell structure (Hamilton *et al.*, 2013; Walters *et al.*, 2001). For *L. obcordata* and *N. pedunculata* the onset temperature of crystallization is below -23°C. Although slightly outside of the storage temperature, it is likely that the lipids are in a metastable state and could accelerate seed aging. In addition, it has been suggested that long-term storage of seed at specific temperatures could affect the lipid phase behaviour, especially onset and end of melt temperatures, enthalpies, and crystallization events (Mira *et al.*, 2019; Vertucci, 1992). Interestingly, seed viability in *L. bullata*, *L. obcordata* and *L. bullata x L. obcordata* was also not retained when desiccated seed was stored at -196°C. At least two possibilities can be proposed to explain the low survival rate following exposure to LN: (1) seeds were already aging prior to storage at -196°C; (2) the cooling and thawing conditions employed in this study were sub-optimal. The first possibility in which seeds continued to age has been highlighted by Walters (1998) indicating that aging occurs in all seed whether in soil seed banks, conventional seed banks or in cryostorage. However, in this study, seed viability remained constant in *L. bullata* and *L. obcordata* after storage for 24 months at 5°C while significant viability was lost when the same seed lots were stored at -18°C. This was similar for *L. bullata x L. obcordata* seed stored for 12 months at 5°C, -18°C and -196°C. This indicates that over the course of the study period, freeze sensitivity, and not seed aging, caused viability loss in *L. bullata, L. obcordata* and *L. bullata x L. obcordata*.

The second possibility, cooling and warming rates employed in the study was sub-optimal is derived from studies by (Dussert *et al.*, 2001; Vertucci, 1989). Sensitivity to rapid cooling of 100°C to 200°C min^{-1} has been reported in dry seed of various *Coffea* spp. (Dussert *et al.*, 2001). Slow cooling at 1°C min^{-1} to -50°C resulted in 70% germination in *Coffea arabica* while direct submersion in LN was fatal (Dussert *et al.*, 1997). Similarly, slow cooling of 3°C $hour^{-1}$ was required for cryopreservation of lettuce seed (Jaganathan & Liu, 2014). This study was limited to rapid cooling through direct submersion of seed into LN and slow cooling rates to minimize lipid crystallization should be investigated.

3.5 Conclusion

The study revealed that seed of *L. bullata, L. obcordata*, *L. bullata x L. obcordata* and *N. pedunculata* should be considered as intermediate in storage physiology. *Lophomyrtus bullata* was sensitive to desiccation to MCs suitable for conventional seed banking (~15% eRH). Although seed viability and seedling vigour were improved following desiccation to 30% eRH, DSC analysis revealed ice nucleation due to the presence of freezable water. In addition, *L. bullata* is also freezing sensitive, with seed stored at -18°C impacted by lipid crystallization. *Lophomyrtus obcordata* seed was not desiccation sensitive but lost significant viability during sub-zero temperature storage. One seed lot was found to retain viability in seed stored at -18°C which could indicate intraspecific variation in storage physiology. Lipid thermal fingerprints of *L. obcordata* indicate that lipids are in a metastable state at conventional storage temperatures and likely to continue deteriorating. No long-term data was available for *N. pedunculata* although TZ staining revealed the species is desiccation sensitive and likely to be freezing sensitive with similar lipid thermal fingerprints to *L. obcordata.* It is recommended that seed of all three species are stored outside of lipid transition temperatures and cooling rates are optimised. In addition, cryopreservation protocols using cryoprotectants should be investigated for *L. bullata* which is desiccation sensitive.

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Chapter 4.

Phenology, fruit morphology and seed characteristics of *Syzygium maire* (Myrtaceae), a threatened endemic New Zealand tree

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Abstract

Efforts to conserve the world's most threatened plant species are hindered by gaps in fundamental knowledge regarding their seed biology. *Syzygium maire* (A.Gunn.) Sykes et Garn.-Jones is a highly threatened endemic tree in New Zealand. In this study, we investigated fruit and seed development on *S. maire* trees at different sites and between seasons and used short-term seed storage protocols to investigate the storage potential of harvested seeds. Fruit development took 8 to11 months with three of the four identified stages occurring during the warmer spring and summer months. Embryos were found to be viable from an early stage, but seedlings from final stage embryos were more robust. The moisture content of the fruit pulp varied between populations and within populations among seasons, but embryo moisture content remained more constant. *Syzygium maire* seeds have high germinability when removed from the fruit pulp, and removal from the pulp is essential for germination. Seeds in fruits stored hydrated at 5 °C remained viable for up to 12 weeks, although some germination was observed from 8 weeks. From this study we have defined developmental stages for *S. maire* seeds, which could be used to inform optimal times for seed collection. The seed germination and short-term storage information provided by this study, will be useful for further studies that investigate long-term storage strategies for *S. maire* seeds. This paper highlights the importance of developing the fundamental knowledge needed to inform overall conservation of this critically endangered tree.

Keywords: embryo, endangered, germination, myrtle rust, Pacific, phenology, seed banking

4.1 Introduction

Syzygium Gaertn. is the largest genus in the Myrtaceae family and with an estimated 1200–1800 species and is one of the ten largest genera of flowering plants (Ahmad *et al.*, 2016; Nair, 2017). The Pacific region is considered the centre of diversity for *Syzygium* with 250 species in regions such as Borneo and New Guinea. Although the New Caledonia region, which includes Lord Howe Island and New Zealand, has 75 known species (Ahmad *et al.*, 2016), *S. maire* (A.Gunn.) Sykes et Garn.-Jones (swamp maire, maire tawake, waiwaka) is the only native *Syzygium* species in New Zealand (Dawson *et al.*, 2011). *Syzygium maire*, previously known as *Eugenia maire* (Sykes & Garnockjones, 1979), is a glabrous tree up to 16 m high with smooth, grey-brown bark often flaking in irregular shards (Fig. 4.1A). Pneumatophores, lateral roots adapted for oxygen uptake in plants growing in swamps, mangroves, or muddy environments, are common on *S. maire* (Fig 4.1B). The evergreen leaves are opposite and often bear small galls and leaf blisters. The creamy white flowers that are borne in clusters of up to 35 in number on terminal racemes are approximately 90 to100 mm in diameter and appear between February and April (Fig. 4.1C). The fruits are fleshy berries developing over 8 to 11 months and are initially green in colour turning bright red during the final 3 to 4 weeks of development (Fig. 4.1D & E). Fruit matures between November and April depending on the locality. Mature fruit and flowers can be observed on the same plant at the same time especially in late flowering populations.

Figure 4.1: *Syzygium maire* (A) in habitat with pneumatophores (B) common in muddy environments. Flowers (C) are creamy white and present between February and April with (D) green berries turning (E) red in autumn.

Syzygium maire is endemic to New Zealand and found mostly in coastal or lowland riparian forest in waterlogged ground or on the margins of streams. Although more common in lower lying areas, it is also found in some montane forest and cloud forest (de Lange, 2021), such as the wetland vegetation in Egmont Ecological District, where *S. maire* is a major component of the forest canopy (McEwen, 1987). Distribution is largely confined to the North Island, although small populations occur in the northern corner of the South Island around Blenheim and Nelson. Except for an in-depth study by (Clarkson, 2014) on *S. maire* in Taranaki, the decline of *S. maire* has not been well documented in New Zealand. Accounts from pioneer farmers in the Taranaki region describe farms of 76 ha consisting of 74 ha of swamp land (Clarkson, 2014). The decline of *S. maire* during recent times in Taranaki, and likely throughout its distribution range, is the result of clearing and draining of large tracts of forest and wetlands for settlements and pasture. According to the International Union for the Conservation of Nature (IUCN) only 133 *Syzygium* species have been assessed for their conservation status globally. Of the assessed species 12.8% are Critically Endangered, 20.3% are Endangered, 22.6% Vulnerable, 3% Near Threatened and 36.1% considered to be of Least Concern (IUCN, 2021). Despite significant loss of habitat, *S. maire* was not considered to be threatened based on assessments conducted between 2004 and 2012 (de Lange *et al.*, 2013). The arrival of *Austropuccinia psidii* (Myrtle Rust) in 2017, combined with the slow terminal decline of remnant populations on partially drained farmland, resulted in a revision of the conservation status to Nationally Critical (de Lange, 2018).

Within the Myrtaceae family the two genera *Eugenia* and *Syzygium* are valued for food and medicine. Medicinal properties have been formally studied for at least 24 *Syzygium* species, while there is anecdotal evidence of medicinal or traditional use for many more species in Africa, Australia, and India (Goyal & Ayeleso, 2019). Although the fruits are more commonly used, dried flowers of *S. aromaticum* (clove) is a universal spice and well known for antioxidant and antimicrobial activities, while leaf extracts of *S. australe* and *S. leuhmannii* also have antimicrobial activities (Cock, 2012). The fruit of *S. aqueum, S. cordatum, S. cumini, S. jambos, S. malaccense* and *S. samarangense* are widely used for food and medicine (Hardman, 2017; Shü *et al.*, 2011; Van Wyk *et al.*, 1997). *Syzygium cumini* berries have exceptionally high antioxidant capacity (Nair, 2017) and similarly *S. maire* berries have 18 times the concentration of antioxidants of blueberries or 29.8 ascorbic acid (vitamin C) equivalents on a dry weight basis (Gould *et al.*, 2006). Although *S. maire* was unlikely to be an important food supply, the berries were used to make waiwaka puddings as a treat for children (Clarkson, 2014; Gould *et al.*, 2006). *S. maire* was, however, valued by both Māori and European settlers for its strong and durable timber.

Conservation efforts of the world's most threatened plant species are hindered by gaps in fundamental biological information (Cavender *et al.*, 2015) and their seed biology (Pritchard *et al.*, 2014). These gaps include knowledge about tree taxonomy and phylogeny, reproductive ecology, seed biology and distribution information (Cavender *et al.*, 2015). Of these factors, reproductive ecology is often considered the most important aspect for conserving rare and threatened species (Emery & Offord, 2019). In contrast to *S. maire,* for which the ecology and reproductive biology are largely unknown, species such as *S. cormiflorum* (Crome & Irvine, 1986), *S. cumini* (Anandalakshmi *et al.*, 2005; Coelho de Araújo *et al.*, 2008; Sivasubramaniam & Selvarani, 2012), *S. paniculatum* (Blando *et al.*, 2013; Thurlby *et al.*, 2012) and *S. samarangense* (Shü *et al.*, 2011) have been extensively studied. As with other genera within the Myrtaceae family, *Syzygium* flower morphology suggests a generalist pollination syndrome. This is supported by observations that up to 45 animal species including butterflies, hawk moths, honeybees, various birds and bats associated with the pollination of *S. cormiflorum, S. paniculatum* and *S. tierneyanum* (Crome *et al.*, 1986; Hopper, 1980; Ramírez & Kallarackal, 2019).

Limited information has been published on the pollination of *S. maire* but as with other *Syzygium* species, a range of pollinators including honey bees and birds, such as tūī (*Prosthemadera novaeseelandiae),* stitchbird/hihi (*Notiomystis cincta*) and bellbird (*Anthornis melanura*), have been observed competing for access to the nectar of *S. maire* in Zealandia ecosanctuary in Wellington (Clarkson, 2014). Little is known about the fruit dispersal of *Syzygium* spp. and knowledge of dispersal agents is mainly based on deductions from fruit morphology (Lughadha & C, 1996). Some of these deductions include suggestions that the Australian rainforest species *S. cormiflorum* develops berries on the main trunk and large branches to enable larger marsupials to eat the fruits (Beardsell, 1993). However, despite the palatable fleshy fruit of *S. paniculatum* there was no evidence of birds or small mammals consuming these within its native range in Australia (Thurlby *et al.*, 2012) or as a cultivated species in Columbia (Ramírez *et al.*, 2019). It is speculated that the New Zealand pigeon/kererū (*Hemiphaga novaeseelandiae*) aids in distribution of *S. maire* based on photographs of these pigeons feeding on mature berries in Ngā Manu Reserve (Clarkson, 2014). Although there are no official records, it is also possible that other native species such as North Island kōkako (*Callaeas wilsoni*), tūī (*Prosthemadera novaeseelandiae*) and bellbird or korimako (*Anthornis melanura*) consume the berries, but most of these species are no longer common in the wild.

Syzygium fruits are typically a fleshy single-seeded berry that are orange, red or black in colour (Lughadha *et al.*, 1996). Fruit development, from anthesis to maturity, in *Syzygium* species ranges from less than 50 days in *S. samarangense*, 54–73 days in *S. jambos*, 89 days for *S. pycnanthum*, to 82–112 days for *S. creaghii* (Khandaker & Boyce, 2016; Mudiana, 2010). Blando *et al.* (2013) reported a fruit development period of approximately 60 days for *S. paniculatum* in natural populations in Australia while Ramírez *et al.* (2019) found the same species to take in excess of 135 days for plants in cultivation in Columbia. In contrast to the more tropical species, *S. maire* has a fruit development period of 240–330 days (Clarkson, 2014). Fruit size varies significantly within the genus ranging from 4 mm diameter in *S. badescens* from Northern Borneo (Ashton, 2006) to 60 mm in *S. samarangense* from Taiwan, Indonesia and Malaysia (Shü *et al.*, 2011). Polyembryony i.e. multiple embryos arising within the embryo sac of the zygotic proembryo, has been observed in many species including *S. cumini* (Sivasubramaniam *et al.*, 2012), *S. malaccense* (Shü *et al.*, 2011)*, S. mundagom* (Jose *et* *al.*, 2009) and *S. paniculatum* (Thurlby *et al.*, 2012) but although recorded frequently it is not considered the norm for this genus (Lughadha *et al.*, 1996). Most *Syzygium* seeds are shed at high moisture contents ranging from 44% to 60% and have 89–100% viability (Abbas *et al.*, 2003; Lughadha *et al.*, 1996; Nagendra *et al.*, 2019). *Syzygium* seeds are highly hydrated and metabolically active and can therefore not tolerate desiccation resulting in a post-harvest lifespan of days to months (Berjak & Pammenter, 2008; Nagendra *et al.*, 2019). *Syzygium cumini* seeds stored between 12°C and 20°C lasted for 4 to 6 months with a 30% loss in viability (Anandalakshmi *et al.*, 2005; Coelho de Araújo *et al.*, 2008). Both *S. cumini* and *S. samarangense* seeds or fruit suffered from chilling injury and significant seed viability loss when stored below 10°C (Abbas *et al.*, 2003; Shü *et al.*, 2011). However, Horng (1988) as referenced by Shü *et al.* (2011), however, found that the chilling injury in *S. samarangense* fruit varied between seasons and cultivars with fruits produced in summer more susceptible to chilling injury.

The seed biology of *S. maire* remains largely unstudied, hampering its effective conservation. The aims of this study were to: (1) compare seed biology among sites and seasons, (2) examine the fruit development stages to inform the optimal time for fruit collection, (3) determine a seed germination protocol, and (4) establish a seed storage methodology to maximize seed longevity. The findings from this study can be used as a model to optimize seed collection and maximize the short-term storage of *S. maire* seeds.

4.2 Materials and Methods

4.2.1 Study sites

Fruit morphology and seed biology for *S. maire* were based on fruit collected from four sites (Fig. 4.2; Table 4.2) over three consecutive summers: 2018, 2019 and 2020. Study sites were selected based on the presence of flowering and fruiting individuals, habitat type, range of climatic conditions, accessibility, and landowner consent or a combination of these factors. Three of the sites were in the central to lower North Island, while a single planted specimen was located at the Queens Gardens in Nelson at the top of South Island. The tree in Queens Gardens is the only tree in this study not part of an established population and was included due to fruit availability in 2019 and 2020. The original provenance of the tree is unknown.

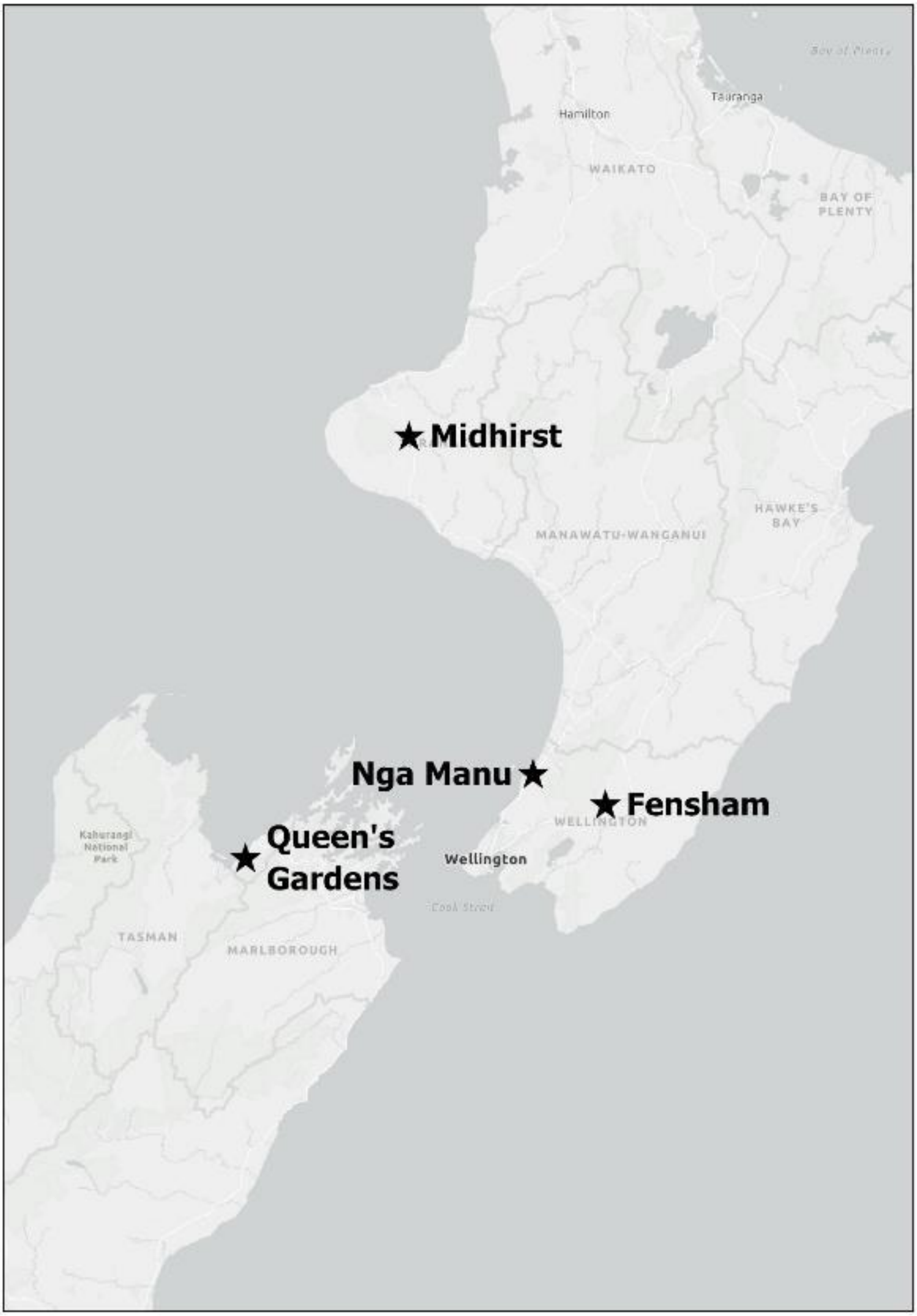

Figure 4.2: Locality of the four sites where *Syzygium maire* seed material was collected

Table 4.1: Locality information for the four sites where *Syzygium maire* fruit was collected

Population/ Locality	Region	GPS Coordinates (DMS)	Altitude (meters above sea level)	Approximate size of population (adult trees)	Land use	Mean monthly rainfall (mm)	Mean maximum temperature (°C)	Mean minimum temperature (°C)
Fensham R eserve	Carterton, Wairarapa, North Island	40°59’41.59” S 175°30’17.89 E	121	50	Forest and Bird Reserve	66.4 ± 46.5	24.4 ± 4.9	-0.04 ± 3.2
Midhist	Taranaki, North Island	39°17’55.02” S 174°11’16.56 E	574	100+	Large population on private farm on poorly drained volcanic ring plain between Midhirst and Egmont National Park	84.1 ± 38.3	20.8 ± 3.6	2.9 ± 3
Ngā Manu Reserve	Waikanae, Kapiti Co ast, North Island	40°51’45.59” S 175°03’33.01 E	10	35	Coastal swamp forest between SH1 and Waikanae	72.6 ± 36.5	22.9 ± 1	2.5 ± 3.4
Queen’s Gardens	Nelson, South Island	41°16’25.07” S 173°17’28.02 E	2	1	Planted specimen in Queen’s Gardens, Nelson.	69.5 ± 42.5	22.5 ± 4.6	3.7 ± 4.0

4.2.2 *Environmental factors*

Automated Weather Stations (AWS) closest to each site were used to obtain mean monthly temperatures (minimum and maximum) and rainfall (Meteorological Service of New Zealand Ltd). Environmental records used in this study covered the period from January 2018 to March 2020 (27 months). These records were used to investigate phenological patterns and compare seed biology among localities and seasons.

4.2.3 *Plant material*

Fruit was collected from as many individuals at each site as possible. Fruits were harvested from trees with the use of pole loppers and arborists. Only fruit collected directly from trees were used for this study. Fruits were placed in polystyrene boxes and transported to the research facility within 24 hours of collection. Fruits were mixed with two parts medium grade (1 to 4 mm granule size) vermiculite (Ausperl, Australia) and stored in airtight containers at 5°C until use.

4.2.4 *Fruit development stages*

Fruit development stages for *S. maire* were based on information collected between August 2018 and January 2019 in Fensham Reserve and verified in Taranaki between January 2019 and March 2020. Fruit was collected in November and December 2018 to determine weight, moisture content and viability for early development stages with subsequent collections in January 2019. Moisture content and germination was conducted as per methodology described below.

4.2.5 *Fruit morphology and embryo biochemical properties*

Fruits with no visible insect damage or disease symptoms were randomly selected for morphological characterization. Fruit length and maximum diameter were taken to the nearest 1.0 mm with a digital calliper, while fruit weights were taken to the nearest 0.01 g with a precision scale (Model: AUW120D, Shimadzu, Japan). Fruits were then manually separated into the cotyledons, embryo and pulp and the anatomical parts were weighed separately.

To determine the biochemical properties of the cotyledons and embryos, the fruit pulp and the seed coat were removed, and the EA separated from the cotyledons. The plant material was freeze dried and then pulverized using a grinding machine. Standard methods of analysis prescribed by AOAC International were used for the determination of total solids, crude protein, fat, starch and sugars (AOAC, 2021). Dry matter was measured by drying samples at 135°C for two hours. Biochemical analysis was conducted by the Manawatū Nutrition Lab Massey University, Palmerston North.

4.2.6 Moisture content

Moisture content was determined for cotyledons, embryo and pulp and whole fruits. Pulp was manually removed before cotyledons and embryos were separated using a scalpel. The components were placed in aluminium boats and weighed with a 0.01 mg precision scale before drying for 17 hours at 103°C. Moisture content (%) was based on the fresh weight basis according to the Rules of the International Seed Testing Association (ISTA) using the following formula (ISTA, 2018):

$$\% \text{ Moisture Content} = \frac{\text{Weight of fresh seeds} - \text{Weight of dry seeds}}{\text{Weight of fresh seeds}} \times 100\%$$

Moisture content of the various fruit components was determined for development stages as well as collections from each site over three years (2018, 2019 and 2020).

4.2.7 Seed germination

Fruits were surface sterilized inside a laminar flow cabinet by submersion in 50% commercial bleach, Janola® (4% sodium hypochlorite) for 5 min, rinsed three times with sterile water and air dried on sterile paper towels. Treatments included seed with pulp (intact fruit), seed de-pulped by hand and seed de-pulped by pigeons, collected from Fensham Reserve, as well as seed collected from various development stages. Germination medium consisted of 7% bacteriology grade agar (Lab Supply A0949) in 500 ml plastic tubs. Two temperature settings; 15/25°C and 20/30°C both with a 16/8-hour light cycle were used for germination experiments. Germination was scored once a week and a seed was considered germinated when the radicle protruded a minimum of 2 mm. Germination experiments were terminated after two months. Viability of ungerminated seeds was assessed with a cut test. Seed germination trials were conducted in incubators at Massey University and The New Zealand Institute for Plant and Food Research Limited, Palmerston North, as well as at the Lions Ōtari Plant Conservation Laboratory in Wellington.

4.2.8 Seed storage

The effect of storage temperature, storage substrate and de-pulping on seed longevity was investigated. To determine the role of storage temperature, freshly collected seeds were mixed with two parts medium grade (1–4 mm granule size) vermiculite (Ausperl, Australia) and placed in airtight containers stored at either 5°C or room temperature. Based on the results from the storage temperature experiment all further seed storage was done at 5°C. The interactive effect of substrate and de-pulping was investigated by storing de-pulped seed and seed with pulp (intact fruit) in two substrates: a) vermiculite and b) submerged in water. Seed viability was determined through a germination test as described above, unless seed was observed to have germinated in storage or have become soft and decomposed. All treatments consisted of four replicates containing a minimum of 25 seeds. Samples for viability tests were randomly selected from each treatment at 4-week intervals.

4.2.9 Statistical analysis

Variables between sites and across seasons, as well as germination results, were compared using ANOVA (for normally distributed data) or Kruskal Wallis test (data not normally distributed). Arcsine transformation was used to normalize percentage or proportion data where needed. All statistical analysis was conducted using XLStat Software version 1.3 (2020).

4.3 Results

4.3.1 Environmental factors

Temperatures below freezing are common in Fensham Reserve between April and October. With a mean annual minimum temperature of -0.04 ± 3.16°C, it is significantly colder than Ngā Manu (2.49 ± 3.4°C), Queens Gardens (3.71 ± 3.92) or Taranaki (2.88 ± 3.01) ($P < 0.001$; Fig. 4.3A). Although mean monthly maximum temperatures were similar over the course of a year (data not shown), the mean monthly maximum temperatures during spring and summer were significantly higher in Fensham Reserve ($P = 0.006$; Fig. 4.3).

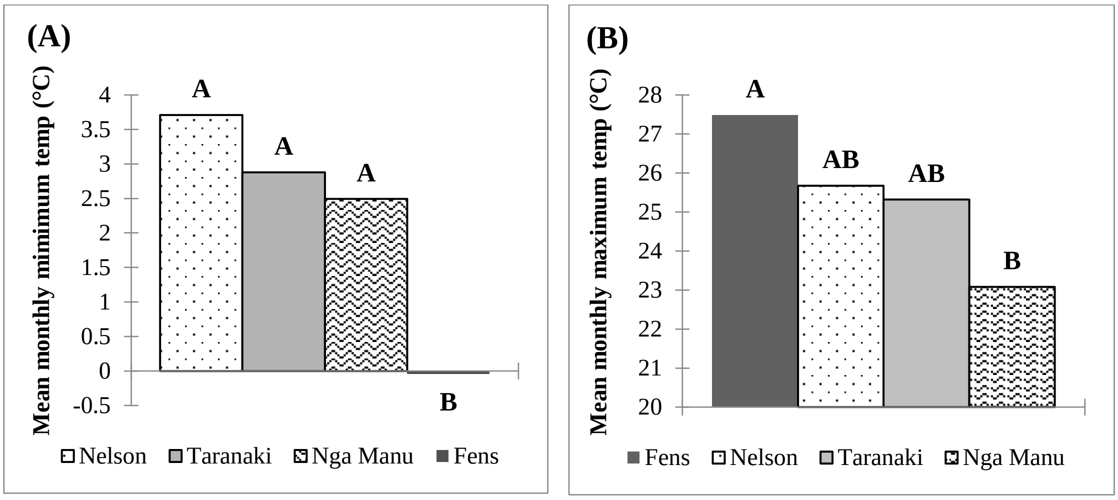

Figure 4.3: The (A) mean monthly minimum temperature and, (B) mean monthly maximum temperatures over spring and summer between 2018 and 2020 at Fensham Reserve, Nelson, Ngā Manu and Midhirst. Values in each graph with different letters are significantly different at $P < 0.05$ based on Kruskal Wallis test**.**

4.3.2 Fruit development stages

Syzygium maire fruits start to develop in mid-spring (October) in lower lying coastal sites such as Fensham, Ngā Manu and Queens Gardens and early summer (December/January) in the elevated areas of Taranaki. Although for the purpose of this study fruit development is divided into four stages, development occurs along a continuum and an individual fruit often has characteristics of more than one stage. During stage 1 fruit showed no tissue specialisation and although included in the description of development stages here, fruit was not harvested for morphological characterization. Stage 2 is characterized by swelling of the fruit as the cotyledons and embryo start to develop. The pulp expands early in stage 3, with a slight change in colour from green to light pink noticeable until fruit reach full maturity, characterized by bright red fleshy berries at stage 4 (Fig. 4.4A–D). Stages 2 – 4 can be present on the same tree, especially during the early part of the fruiting season. At Fensham Stage 1 and 2 occur around October and November, while stages 3 and 4 can be found from mid-December to January. Fruit development in Taranaki starts approximately 6 – 8 weeks later, with stages 1 and 2 from mid-December to January, while stages 3 and 4 occur from late March to April. Fruit weight differed significantly ($P < 0.001$) between the final three stages of development, which is most likely due to the increase in moisture content of the pulp as there was no significant difference in the moisture content of cotyledons or embryos between stages 2 to 4 ($P = 0.115$; Table 4.2).

Table 4.2: Weight, pulp colour and moisture content for four fruit development stages in *Syzygium maire* at different times after flowering. N = 10.

Development Stage	Months after Flowering	Weight (g)	Colour	Moisture Content (%)		
				Cotyledon	Embryo	Pulp
1	7–8	n/a	Green	n/a	n/a	n/a
2	8–9	5.8±0.4[a]	Green	66.2±2.9[a]	69.7±2.7[a]	85.7±0.9[a]
3	9+	9.2±0.6[b]	Pink	61.1±6.3[a]	68.7±1.7[a]	88.7±0.2[b]
4	9+	13.1±1.4[c]	Red	58.3±0.6[a]	65.9±1.7[a]	90.1±0.3[c]

4.3.3 Fruit morphology

Mature *S. maire* berries are approximately 10 to 15 mm in diameter, 8 to 12 mm long and have a mean weight of 1.11 ± 0.26 g (n = 40). The fruit consists of thin seed coat, fleshy pulp, cotyledons, and embryo. Biochemical analysis revealed that the cotyledons were high in starch with embryos consisting of 1.4% fat (Table 4.3).

Table 4.3: Biochemical properties of *Syzygium maire* cotyledons and embryos

Sample	Total Solids (%)	Crude Protein (%)	Fat (%)	Starch (%)	Sugars (g/100g)
Cotyledons	42.0	3.5	0.9	13.5	1.9
Embryo	26.4	3.2	1.4	< 0.1	1.1

4.3.4 Moisture content

Embryo moisture content was similar for all sites in 2018 and 2019, although embryos from the tree at Queens Gardens in Nelson had significantly lower moisture content in 2020 than those from the other sites ($P < 0.001$; Fig. 4.5). When embryo moisture content was compared for the same site across two or three seasons it was found that Ngā Manu embryos had significantly lower embryo moisture content ($P = 0.026$) in the summer of 2018 with no difference among any of the seasons for Fensham and Taranaki (Fig. 4.5). There was significant variation in pulp moisture content among seasons at the same sites except for Taranaki where the pulp moisture content was similar in 2019 and 2020. Ngā Manu was the only site in which there was a difference in cotyledon moisture content among seasons ($P < 0.001$; Fig. 4.5). Due to limited seed availability, the moisture content of cotyledons and pulp was not determined for the Queens Garden tree.

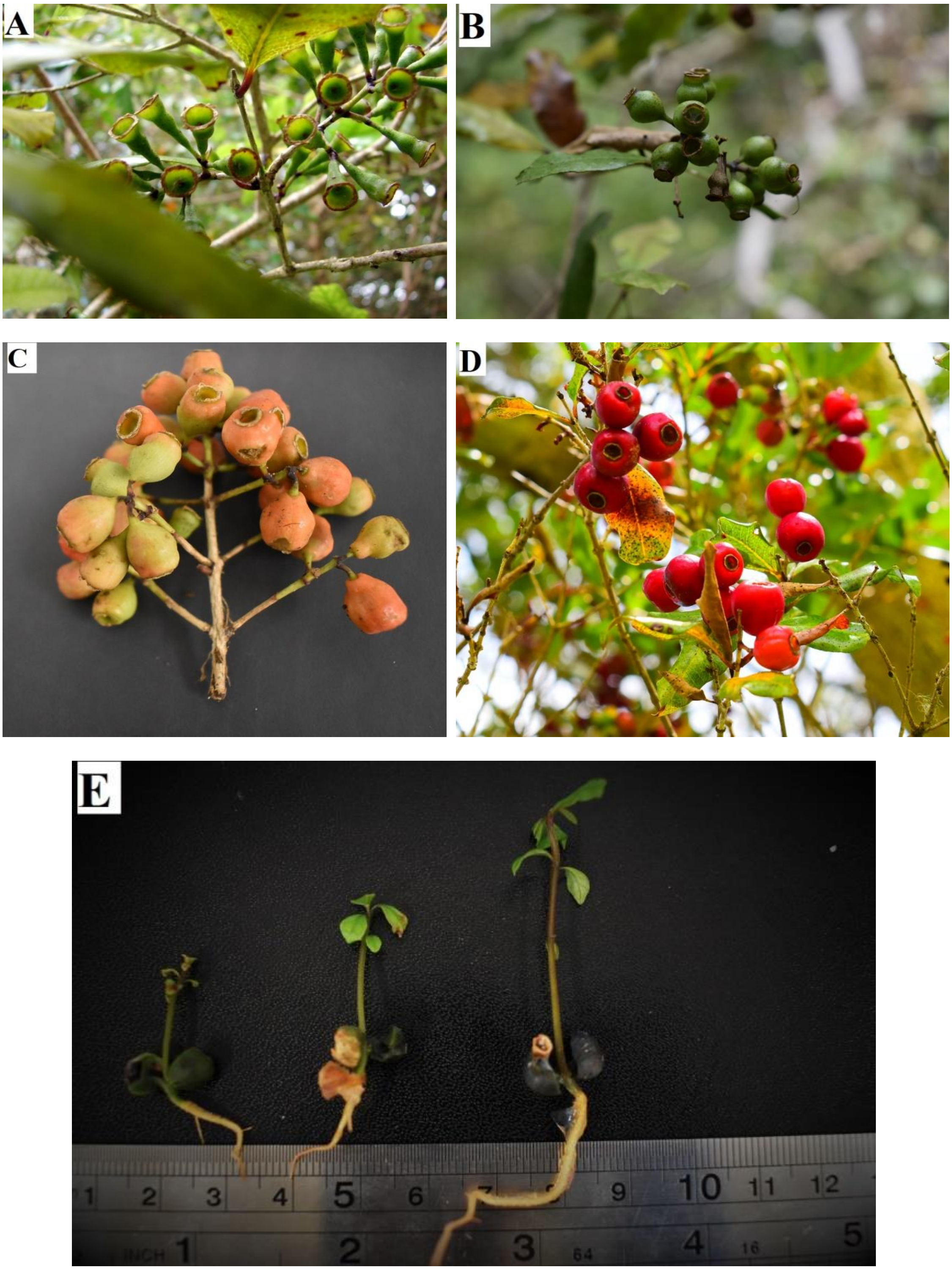

Figure 4.4: *Syzygium maire* fruit developmental stages 1-4 (A-D) and (E) size differences between 2-month-old seedlings from seed collected during stage 2 (left), 3 (middle) and 4 (right).

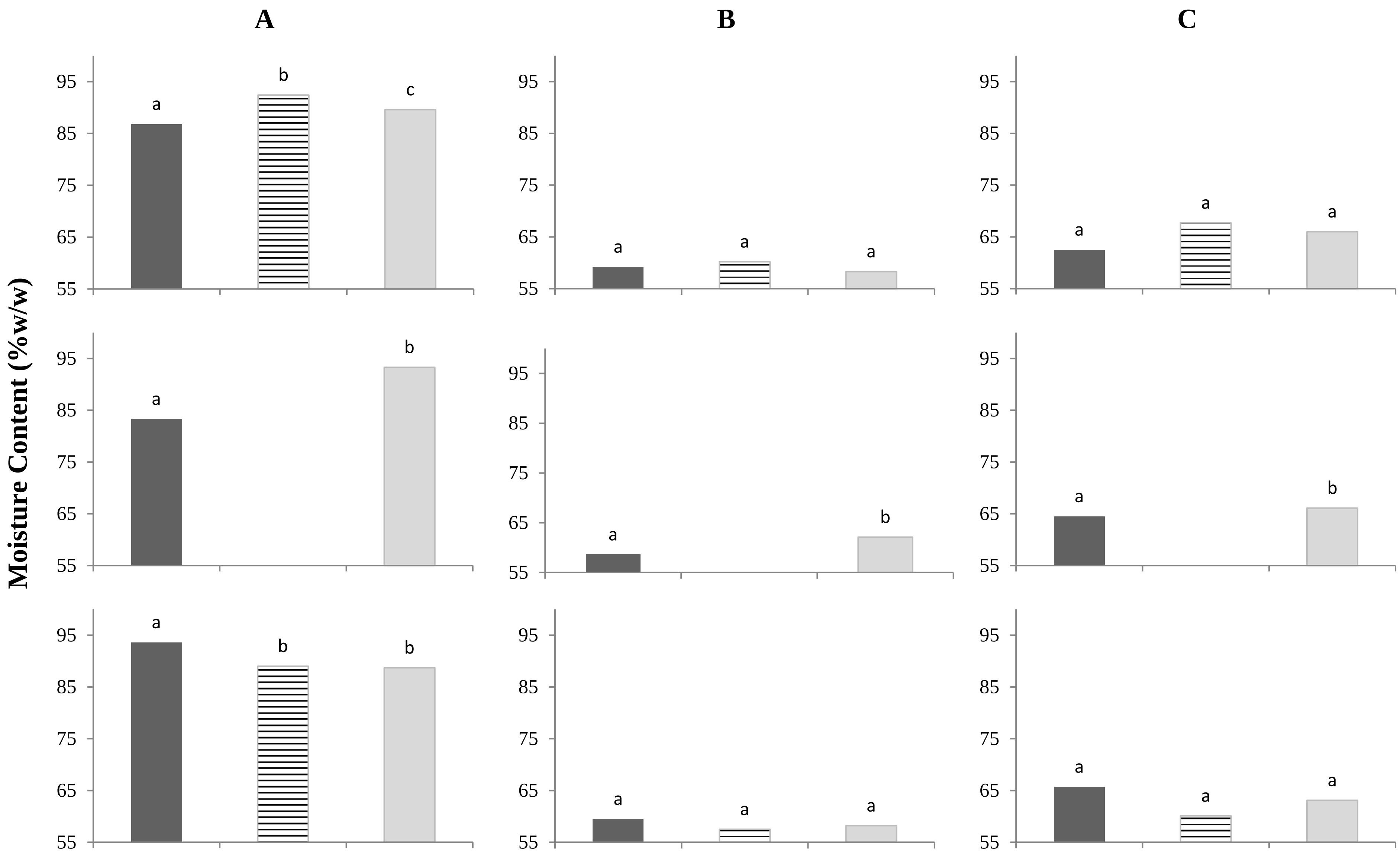

Figure 4.5: Moisture content for (A) pulp, (B) cotyledons and (C) embryos for 2018 (solid black bars), 2019 (broken bars) and 2020 (grey bars) taken from fruit collected in Fensham Reserve (top row), Ngā Manu (middle row) and Midhurst (bottom row). Bars in each graph with different letters are significantly different at P < 0.05 based on Kruskal Wallis test

4.3.5 Seed germination

Removal of pulp had a significant impact on seed germination with uncleaned seed showing final germination of ~10% (Fig. 4.6). High germination was achieved irrespective of the method of cleaning (hand vs kererū). Both temperature settings tested resulted in high final germination in cleaned seed with the lower temperature of 15/25°C resulting in slightly higher final germination (Fig. 4.6). Seed was viable from stage 2 onwards and although there was no significant difference in final germination percentage of seed from stage 2 (96.7% ± 5.2), 3 (100% ± 0.0) or 4 (98% ± 4.2), seedlings from the seeds of stage 4 fruit developed faster and were found to be more vigorous after 60 days (P = 0.037; Fig. 4.4E).

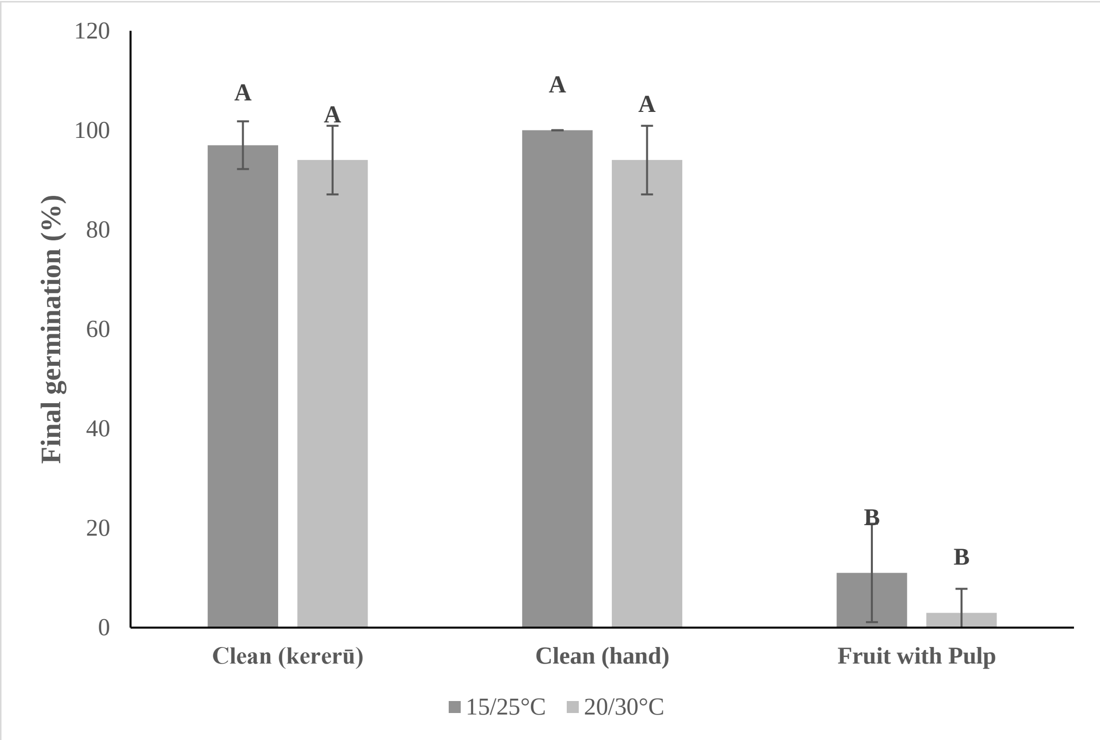

Figure 4.6: Final germination (%) of *Syzygium maire* seed cleaned by kererū, hand cleaned and seed with pulp still attached. Seed was germinated at 15/25°C or 20/30°C. Letters above bars and line graphs for each desiccation treatment indicate significant difference at P < 0.05 based on Tukey's HSD. Error bars represent SD of the mean.

4.3.6 Seed storage

Maximum seed longevity was obtained by mixing fruits with vermiculite in airtight containers stored at 5°C (Table 4.4). However, despite the low temperature seeds started to germinate in storage after 8 weeks, with 55% percent germination recorded by week 12. Viability loss at 12 weeks was 9%. Seed which was de-pulped and stored in vermiculite was the only other treatment that supported seed longevity for more than 4 weeks. Seed submerged in water lost all viability in less than four weeks.

Table 4.4: Percentage viable seed for capsules collected in 2018 and 2020. Results are displayed as mean ± SD, letters in each column indicate significant difference at P < 0.05 based on Fishers LSD test.

	4 Week Viability (%)	**8 Week Viability (%)**	**12 Week Viability (%)**	**16 Week Viability (%)**
Vermiculite, pulp on seed	100	100	91% (55% germinated in storage)	n/a
Vermiculite, depulped	91% (80% germinated in storage)	0	0	n/a
Submerged in water, pulp on seed	0	0	0	0
Submerged in water, depulped	0	0	0	0

n/a = Not available

4.4 Discussion

The study investigated fruit collected from four sites, three from North Island and one from the top end of South Island. All four study sites are located along the western portion of New Zealand and therefore are exposed to similar weather patterns. Mean annual rainfall ranges from 767 mm for Fensham Reserve to 2000 mm for Taranaki with highest percentile of the annual rainfall during the winter months for all sites (Chappell, 2014a, 2014b). Two of the sites, Fensham and Taranaki, receive frost on 11 to 18 days per annum (Chappell, 2014a; Macara, 2016), but frost events are associated with the months during which no reproduction occurs (April to September). Taranaki is the only site where snow might be a factor, although at 554 meters above sea level the *S. maire* population is located below the frequent snow line (1000 m). Despite this, snow fall has been recorded down to 300 m, although these events are unusual (Chappell, 2014a; pers. comm. Bill Clarkson, 2018). Based on the frequent occurrence of sub-zero temperatures, it is clear that *S. maire*, although not dormant, can withstand frost during non-reproductive months, a characteristic shared by many trees from temperate climates (Vitasse *et al.*, 2014). Temperature is one of the key environmental factors in plant reproductive systems affecting floral and fruit development (Distefano *et al.*, 2018) and to escape freezing temperatures trees will often adjust to spring and summer phenology (Vitasse *et al.*, 2014). *Syzygium maire* flowers from the end of summer (Fensham, Ngā Manu and Queens Gardens) to autumn (Taranaki) leaving pollinated flowers to overwinter in a state of no development until the daily maximum temperatures start to exceed 20°C, after which fruit development is initiated.

To effectively conserve seeds, it is important to not only understand the role of ambient temperature and the moisture content of seeds (Daws *et al.*, 2006), but also optimize the development stage at which the fruit or seeds are harvested (Hay & Probert, 2013). It is also likely that due to variability in flowering time for wild species, individual plants in the same population contain seeds at different development stages (Hay *et al.*, 2013). Our study found that *S. maire* seeds can germinate from stage 2 and although the embryo is sufficiently developed and moisture content remains constant from stages 2 to 4, the seedlings from stage 2 are not robust and therefore unlikely to survive in natural conditions.

The immature fruits of most *Syzygium* species are green, becoming fleshy and brightly coloured ranging from purple in *S. cumini* and *S. pycnanthum* (Deden & Esti Endah, 2010; Patel & Rao, 2014), red in *S. samarangense* (Chang *et al.*, 2003) to deep magenta in *S. paniculatum* (Juniper & Britton, 2010; Thurlby *et al.*, 2012) at maturity. Mature *Syzygium* fruits have high nutritional value including minerals, water-soluble vitamins and carbohydrates (Coelho *et al.*, 2016). Despite the visually attractive and highly nutritional berries of many *Syzygium* species, records of animals consuming the berries are limited (Beardsell, 1993; Lack & Kevan, 1984; Lughadha *et al.*, 1996). During this study the New Zealand pigeon/kererū (*Hemiphaga novaeseelandiae*) was observed consuming large quantities of *S. maire* berries and excreting the de-pulped seeds within the same population or in adjacent bush clumps. Gut passage of seed through an animal's digestive system aid in removing fruit pulp and scarify the seed coat and thereby increasing the ability of seed to germinate (Chimera & Drake, 2010; Robertson *et al.*, 2006). This study found that the removal of the pulp significantly increased the germination of *S. maire* seeds. There was no difference in the final germination percentage or germination rates between fruits de-pulped by pigeons and fruits de-pulped by hand (Fig. 4.6). This is supported by earlier studies on fleshy Myrtaceae, which found that seed germination is dependent upon the removal of the pericarp by weathering or ingestion by animals (Beardsell, 1993).

Mature *S. maire* seeds start to germinate within 7 days when incubated at 15/25°C with a 16/8-hour light cycle with the first set of true leaves fully developed within 21 days. Seed germination in *Syzygium* species is generally rapid (12 to 14 days), although some Australian species have been found to germinate over a period of 300 days with considerable variation within and between species (Hyland, 1983). *Syzygium maire* fruits mature from late summer to mid-autumn with seedling emergence in late autumn and winter when ambient temperatures are often below freezing. Studies have found that trees from temperate climates, coordinate seedling emergence time with environmental cues to maximize the growing season even if the risk of frost during the initial germination is high (Bianchi *et al.*, 2019; Verdú & Traveset, 2005). Since in New Zealand the highest percentile rainfall is recorded in winter, the swamps in which *S. maire* grows are likely to be inundated during seed germination and seedling establishment, providing the essential moist substrate required for germination. Furthermore, it is likely that despite frequent frost days at some of the *S. maire* sites, seedlings are protected from frost damage by the dense forest canopy.

Seeds of *Syzygium* species studied to date have been found to be recalcitrant (intolerant to drying) and suffer from chilling injury when stored at temperatures below 10°C (Abbas *et al.*, 2003; Berjak *et al.*, 2008; Nagendra *et al.*, 2019; Shü *et al.*, 2011). This study found that *S. maire* seeds can be stored for 6 to 8 weeks in a fully hydrated state at 5°C with limited viability loss. However, since *S. maire* is adapted to a temperate environment, seeds can germinate at low temperatures, and it was found that after 12 weeks more than 50% of the seeds in storage had germinated. Within the natural habitat of *S. maire*, fruits and seeds (pulp removed) are often dropped into the swamps or wetlands below the mother trees, possibly delaying germination until the pulp has been removed or decomposed. It was found that storing the fruits submerged in water (with or without pulp) was not an effective storage method since all seeds lost viability in less than 4 weeks.

4.5 Conclusion

This study defined the developmental stages of *S. maire* fruit, which can be used to inform optimal seed collection time which range from January (for populations at lower altitudes) to March (for populations at higher altitude). Information on seed germination and the short-term storage potential of *S. maire* seeds provides useful information that could be further expanded to investigate strategies for long-term storage of *S. maire* seeds. The influence of different geographical sites on phenology, seed biology and fruit morphology presented in this study, contributes to making informed-decision on suitable conservation strategies for this critically endangered species including:

- To ensure robust seedlings, seed should be collected when the fruit pulp turns red and become fleshy.
- Fruit can be stored for up to six weeks by mixing freshly collected fruit containing pulp with equal amounts of vermiculite and storing it in an airtight container at 5°C (fridge).
- Seed germination requires the removal of fruit pulp and seed will readily germinate over temperatures ranging from 15-30°C.

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Chapter 5.

Oxidative stress, ultrastructure modification and physiological impacts of rapid desiccation on *Syzygium maire* zygotic embryos

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Publication: Van der Walt, K., Burritt, D.J., & Nadarajan, J. (2022). Impacts of rapid desiccation on oxidative status, ultrastructure and physiological functions of *Syzygium maire* (Myrtaceae) zygotic embryos in preparation for cryopreservation. *Plants* 11(8). Doi: 10.3390/plants11081056.

Abstract

Syzygium maire is a highly threatened tree endemic to New Zealand. Due to its recalcitrant seed and embryos, cryopreservation is the only viable long-term *ex situ* conservation option for this species. This study investigated oxidative stress, thermal properties, and ultrastructure of zygotic embryo axes (EAs) desiccated to various moisture contents (MC) and evaluated viability following desiccation and cryopreservation. Fresh EAs had a MC of c. 1.9 g/g with 100% viability but rapid desiccation to MC to < 0.3 g/g significantly impacted viability and decreased the activities of the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPOX), with a 7-fold increase in the production of protein carbonyls and lipid peroxides. This indicated that the antioxidant system was overwhelmed and could no longer effectively scavenge free radicals. Exogenous application of ascorbic acid (AsA) increased antioxidant activity in desiccated EAs but not by a significant amount. Differential Scanning Calorimetry (DSC) analysis showed large thermal transitions in fresh EAs while endothermic peaks of EAs desiccated to MCs of < 0.3 g/g were significantly smaller. There was no thermal events in EAs desiccated to MC of < 0.2 g/g, indicating that all freezable water has been removed, but this was lethal to both EAs and enzymatic antioxidants. The ultrastructure of desiccated EAs showed signs of plasmolysis while fully hydrated EAs exposed to cryogenic temperature had ultrastructural disintegration and membrane damage. As no EA survived cryopreservation, this study aimed at explaining the effects of rapid desiccation on various indicators of oxidative stress as a function of MC, viability, and thermal transitions. The decline in enzymatic antioxidant activities and the increase in lipid peroxidation suggests that *S. maire* EA viability loss is due to oxidative stress rather than structural impacts. It is postulated that faster cooling and warming rates might overcome ice nucleation phenomenon when desiccated EAs are exposed to cryogenic temperatures.

Keywords: Critically Endangered, DSC, free radicals, recalcitrant, ROS, TEM, thermal transitions, recalcitrant seeds, threatened, ultrastructure.

5.1 Introduction

Strategies for the long-term storage of desiccation sensitive germplasm remains a challenge for many commercially important crop and endangered wild plant species (Wesley-Smith *et al.* 2001). Cryopreservation, the storage of biological material at ultralow temperatures, is the only viable strategy currently available for the conservation of such species (Engelmann, 2004). The cryopreservation process comprises several steps as described in Fig. 5.1 which needs to be optimised for each species or type of material used.

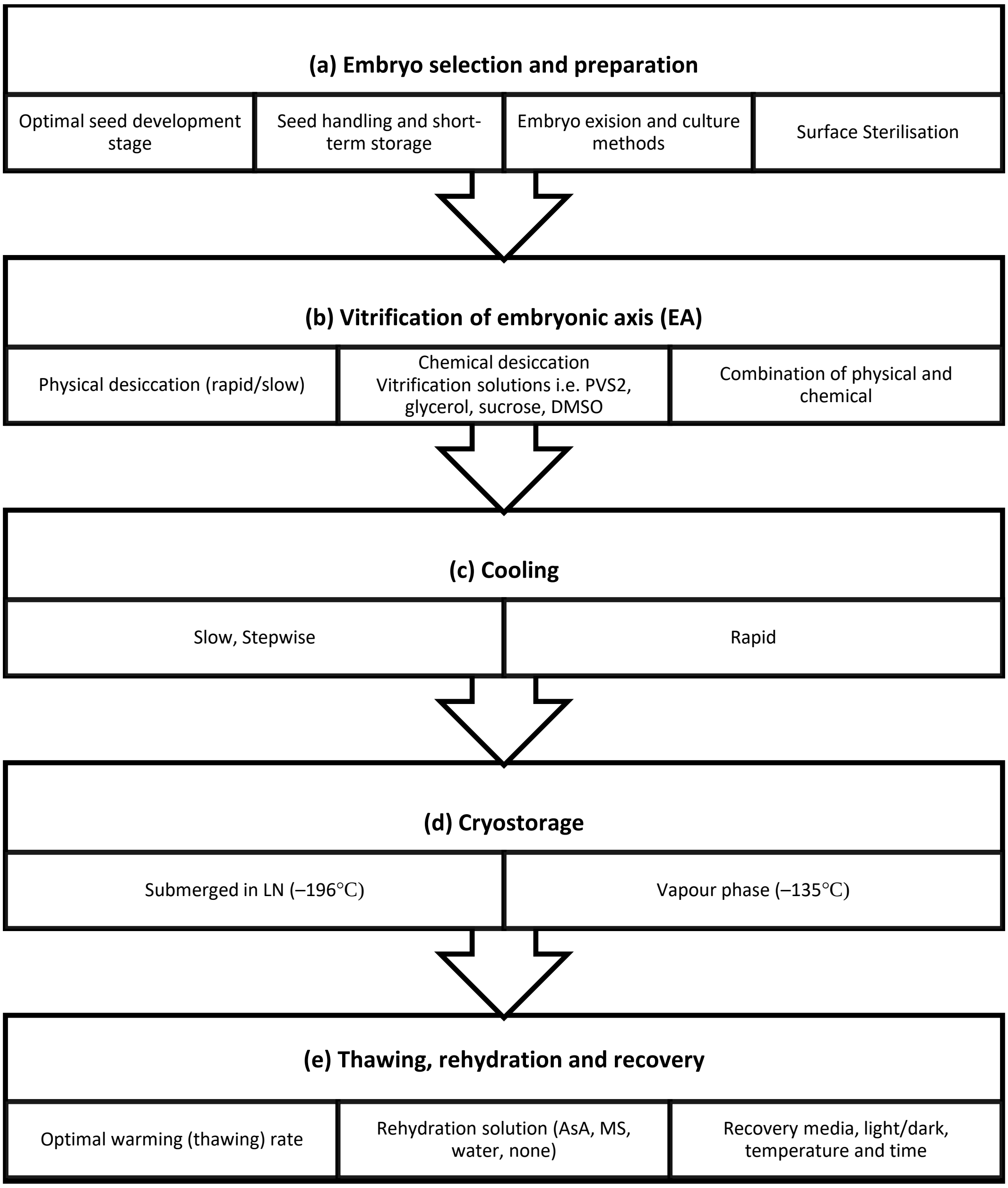


Figure 5.1: Generalised cryopreservation protocol for zygotic embryos of desiccation sensitive species.

Cryopreservation provides a viable strategy for the long-term conservation of desiccation sensitive species, but its application is challenged by the fact that recalcitrant seeds are generally large, shed at high moisture contents and are metabolically active (Berjak & Pammenter, 2008). Large seed poses a significant challenge to cryopreservation since desiccation and cooling rates are too slow to outrun the kinetics of ice nucleation (Fahy & Wowk, 2014). Furthermore, due to their larger size and tissue heterogeneity, a uniform dehydration of whole seed is impossible to achieve (Nadarajan & Pritchard, 2014). One way to decrease the size of the material used is through the surgical removal of zygotic embryos from recalcitrant seed. Excised zygotic embryos for use in cryopreservation is widely applied to crop species for example *Citrus* (Sakai *et al.*, 1990), *Cocos nucifera* (Arecaceae) (N'Nan *et al.*, 2012), *Coffea* (Rubiaceae) (de Freitas *et al.*, 2016) and *Havea brasiliensis* (Euphorbiaceae) (Normah *et al.*, 1986). Although less often used, cryopreservation of zygotic embryos from wild recalcitrant species has been applied to a number of trees including *Acer saccharinum* (Sapindaceae) (Wesley-Smith *et al.*, 2014), *Ekebergia capensis* (Meliaceae) (Bharuth & Naidoo, 2020; Pammenter *et al.*, 1998) and *Quercus* (Fagaceae) species (Ballesteros & Pritchard, 2020). Despite the advantages of using embryonic axis (EAs) for cryopreservation, tolerance to desiccation can vary within and among recalcitrant species. Intra-species variation can be influenced not only by provenance and season (Ellis *et al.*, 1991) but also by the stage of seed development (Daws *et al.*, 2006) and rate of desiccation (Pammenter *et al.*, 1998). Isolated axes appear to tolerate desiccation to lower moisture content levels when desiccated rapidly, not because rapid drying elicits desiccation tolerance, but rapid drying does not allow sufficient time for aqueous-based deleterious reactions to progress (Pammenter *et al.*, 1998).

In order for cryopreservation to be successful, it is essential that most of the freezable water from the plant tissue is removed to prevent the formation of lethal ice-crystals (Walters *et al.*, 2008). Classical plant cryopreservation technique which involves slow cooling step (Benson, 2008b) has largely been replaced by vitrification-based procedures (Sakai, 2004). The simplest approach to vitrification is desiccating germplasm through osmotic dehydration or evaporative desiccation (Benson, 2008a). However, since recalcitrant species are sensitive to the removal of moisture, vitrification is usually achieved through a combination of cryoprotective measures (Reed, 2008; Sakai, 2004). These measures include (modified from Benson, 2008a):

- Desiccation: Evaporative desiccation through air (rapid and slow), chemicals (saturated salts) and desiccants (silica gel).
- Osmotic dehydration (sucrose enriched media).
- Encapsulation-dehydration: Osmotic dehydration combined with encapsulation in alginate beads and evaporative desiccation (encapsulation-dehydration).
- Cryoprotection: Highly concentrated cryoprotective agents such as Plant Vitrification Solution (PVS2) are used to remove free water.
- Encapsulation-vitrification: Cryoprotectants combined with encapsulated beads.
- Droplet-vitrification: Plant material is covered with micro-droplets of chemical vitrification solution (i.e., PVS2) and cooled at ultra-rapid rates.

Following desiccation or attaining a vitrified state, optimisation of cooling rates is the next critical step in cryopreservation protocol development. Cooling rate can influence the formation of ice crystals especially in desiccation sensitive germplasm where the critical water content might still include free water (Vertucci, 1989; Walters *et al.*, 2008). Cooling rates can be described as slow, intermediate, stepwise or rapid (Naidoo, 2010). During slow freezing, material is cooled between 0.5°C and 2°C min^{-1} by using programmable freezing devices (Sakai, 2004). Intermediate cooling rates of 3 to 10°C s^{-1} is achieved by placing plant material in cryovials before direct submersion in liquid nitrogen (LN) (Panta *et al.*, 2015). Fast cooling rates of > 10 to 300°C generally involves droplet freezing, aluminium foil strips/envelopes, super-cooled liquid nitrogen, or a combination of these methods (Wesley-Smith *et al.*, 1995). Post cryo-survival of zygotic embryos of various Amaryllid species (Sershen *et al.*, 2008), *Poncirus trifoliata* (Rutaceae) (Wesley-Smith et al., 2004) and *Quercus robur* (Fagaceae) (Ntuli et al., 2011) was achieved through rapid cooling. However, rapid cooling rates have been detrimental to the survival of *Landlophia kirkii* (Apocynaceae) embryos (Kistnasamy *et al.*, 2011) emphasising the importance of optimising cooling rates for each species.

The overall purpose of cryopreservation is to maintain quality, integrity, and functionality of material stored in LN (at -196°C) or in the vapour phase (between -140°C and -180°C) (Bajerski *et al.*, 2021; Panis *et al.*, 2020). The temperature at which plant material is cryopreserved depends on the type of material (vegetative or seed), water content, stability of the glass, risk of contamination and operational risks (storage in vapour phase minimises risks associated with handling or samples in LN). It is generally recommended that seed, including embryonic axes, are stored in LN to prevent devitrification (Walters *et al.*, 2001).

The prevention of injury during retrieval from cryostorage is another critical step which requires optimisation of the thawing and rehydration process for each species (Naidoo, 2010). For desiccated explants, thawing and subsequent rehydration can be damaging due to ice crystal formation during warming and imbibitional injury caused by direct contact with liquid (Dussert *et al.*, 2003; Wesley-Smith *et al.*, 2004). Rehydration can be done rapidly by direct submersion into the solution or through osmotic methods. Although many studies have found direct rehydration critical for embryo survival (i.e. Berjak & Mycock, 2004; Peran *et al.*, 2004), species such as *Poncirus trifoliata* (Rutaceae) and *Coffea arabica* (Rubiaceae) were dependant on slow or osmotic rehydration for survival (Pinto *et al*., 2016; Wesley-Smith *et al.*, 2004). Another aspect that requires optimisation is the type of solution used for rapid rehydration. Although rehydration of isolated axes is normally done through direct plunging into distilled water (Wesley-Smith *et al.*, 2001), the addition of Ca^{2+} and Mg^{2+} cations to the rehydration medium has been found to increase survival in Amaryllid species (Sershen *et al.*, 2008) and improved regeneration in *Quercus robur* (Fagaceae) and *Trichilia dregeana* (Meliaceae) (Berjak *et al.*, 2004). Rehydration solutions containing antioxidants such as ascorbic acid has also been found to increase survival in various species (Reed, 2014). Like all the steps in the cryopreservation process, rehydration technique and solution also need to be optimised for every species.

The effects of partial dehydration on inducing oxidative stress in recalcitrant axes and zygotic embryos has been identified as a main source of cryoinjury in recalcitrant species (Benson *et al.*, 1992; Sershen, Varghese, *et al.*, 2012; Walters *et al.*, 2008). When the metabolically active cells of recalcitrant species are dehydrated to intermediate MC levels, the cells continue to respire but can no longer effectively scavenge toxic metabolic by-products also known as Reactive Oxygen Species (ROS) (Walters *et al.*, 2001). ROS are implicated in normal metabolism, however, the stresses associated with various steps in the cryopreservation process elevate ROS production which can overwhelm the endogenous antioxidant capacity of EAs (Berjak *et al.*, 2011; Whitaker *et al.*, 2010).

Superoxide ($\bullet O_2$) are often implicated as the main ROS associated with desiccation damage in recalcitrant material (Berjak *et al.*, 2008). Under normal circumstances $\bullet O_2$ would be effectively removed by superoxide dismutase (SOD). Plant cells contain a range of enzymatic antioxidants such as SOD, catalases (CAT), glutathione peroxidase (GPOX) and several other peroxidases (Berjak *et al.*, 2011; Walters *et al.*, 2008; Whitaker *et al.*, 2010). Protection can also be provided by non-enzymatic antioxidants such as vitamin E, ascorbate, and glutathione as well as metabolically inert carbohydrates and proteins (Roach *et al.*, 2008). The high toxicity of free radicals and ROS is further enhanced by secondary oxidative reactions when these radicals attack lipid membranes and causes the formation of lipid peroxides (Benson *et al.*, 1992). Cell membranes have been identified as main areas of oxidative injury during metabolism disruption in recalcitrant seed (Berjak *et al.*, 2008) and therefore the accumulation of lipid peroxidation is widely used as a biomarker of oxidative stress during desiccation of these species (Ntuli *et al.*, 2011; Parkhey *et al.*, 2012). In addition to desiccation, the cooling, rewarming and recovery time after cryopreservation has also been identified as critical stages during which there is a significant increase in oxidative stress (Funnekotter *et al.*, 2017; Whitaker *et al.*, 2010). Exogenous application of antioxidants, such as ascorbic acid (AsA) and glutathione (GSH), has been successful in strengthening the ROS scavenging capacity of plants under abiotic stress conditions (Xu & Huang, 2017) and to increase survival and regeneration following cryopreservation (Ren *et al.*, 2015; Wang & Deng, 2004).

Lowering MC in recalcitrant tissues not only causes oxidative stress but the physical structure of their cells can also be compromised during water removal (Pammenter *et al.*, 1998). Ultrastructural studies can be used to compare subcellular organisation at various steps of the desiccation and freezing process to ensure cell integrity is maintained (Coelho *et al.*, 2020; Sershen, Berjak, *et al.*, 2012b; Wen *et al.*, 2012). The alteration of cellular substructure has been identified as a main source of cryoinjury in embryos of recalcitrant species such as *Amaryllis belladonna* (Amaryllidaceae) (Sershen, Berjak, *et al.*, 2012a), *Livistona chinensis* (Arecaceae) (Wen *et al.*, 2012), *Podocarpus henkelii* (Podocarpaceae) (Woodenberg *et al.*, 2018) and *Pisum sativum* (Myrtaceae) (Wesley-Smith *et al.*, 1995). By comparing subcellular organisation between fresh, desiccated, and desiccated/frozen EAs, fatal changes in subcellular organisation can be identified and thereby provide essential information for the development of species-specific cryopreservation protocols.

Despite the low, or lack of, tolerance to desiccation in recalcitrant species, it is essential that excess moisture is removed from cells to prevent the formation of lethal ice crystals during freezing. Ice nucleation will impact the structural and osmotic integrity of cells resulting in physical raptures and mechanical injury, while colligative damage will result from excessive solute concentrations (Benson, 2008b). Vitrification, when liquids are solidified without crystallization, has been achieved through desiccation of plant material (Benson, 2008b; Sakai, 2004). During vitrification the system is amorphous and comprises a glassy state which lacks organized structure but has the mechanical and physical properties of a solid (Taylor & Brockbank, 2004). To evaluate the efficiency of desiccation to achieve a glassy state, thermal analysis using differential scanning calorimetry (DSC) can be used. DSC analyses thermal properties of plant material to demonstrate the relationship between presence of water freezing or melting transitions and damage at sub-freezing temperatures (Volk & Walters, 2006). Plant material that survives sub-freezing temperatures generally have MC of 0.25–0.4 g H_2O g dm^{-1} (dm = dry mass) (Ballesteros *et al.*, 2014). Since the motional restrictions are too great for the formation of ice crystals when the MC is less than 0.25 g/g, water contents vitrify either at room temperature or during cooling (Volk & Walters, 2006). If small exothermic events are still present after initial desiccation, it can be limited by increasing cooling/warming rates, or decreasing moisture content through increased desiccation or by incubation in cryoprotectant solutions (Volk& Walters, 2006).

This study aimed to quantify desiccation stress by assessing the degree of physical and biochemical damage and metabolic disruption inducted during desiccation of *S. maire* zygotic embryos. *Syzygium* Gaertn. is the largest genus in the Myrtaceae family and with an estimated 1 136species it is one of the ten largest genera of flowering plants (Ahmad *et al.*, 2016; Nair, 2017). The Pacific region is considered the centre of diversity for *Syzygium* with 250 species in regions such as Borneo and New Guinea. Although the New Caledonia region, which includes Lord Howe Island and New Zealand, has 75 known species (Ahmad *et al.*, 2016), *S. maire* (A.Gunn.) Sykes et Garn.-Jones (swamp maire, maire tawake, waiwaka) is the only native *Syzygium* species in New Zealand (Dawson *et al.*, 2011). Significant loss of habitat, coupled with a threat from the fungal pathogen myrtle rust (*Austropuccinia psidii*), has resulted in the species listed as Nationally Critical on the New Zealand Threat Classification System (de Lange, 2018). *Syzygium maire* berries are 10–15 mm in diameter and have a mean weight of 1.11 ± 0.3 g while the embryos are 4–5 mm in size (Chapter 4; Van der Walt *et al.*, 2021b). Seeds and embryos are recalcitrant and do not tolerate desiccation to MC below 20% (Nadarajan *et al.*, 2021; Van der Walt *et al.*, 2021b). To date, there has been no studies published on the successful cryopreservation of zygotic embryos of any *Syzygium* species, and while desiccation and the use of cryoprotectants have been tested for the cryopreservation of *S. maire* zygotic embryos, neither method was successful (Chapter 6; Van der Walt *et al.*, 2021a).

The specific objectives of the study were to: 1) Determine the impact of desiccation on embryo viability and plantlet development, 2) Determine the suitability of evaporative desiccation in the cryopreservation of *S. maire*, 3) Use antioxidant enzymes (SOD, CAT and GPOX) and oxidative damage markers (lipid peroxide and protein carbonyls) to quantify the oxidative stress induced during desiccation, 4) Measure thermal transitions using DSC in desiccated zygotic embryos to assess ice nucleation for various desiccation treatments, and 5) Investigate subcellular impacts of desiccation and freezing on zygotic embryos.

5.2 Materials and Methods

5.2.1 Plant material

. Fruits used to determine embryo desiccation sensitivity were collected from three sites in North Island (Fensham - Wairarapa, Parkwood Village – Kapiti Coast, and Taranaki) as well as one site in South Island (Nelson) in 2020 and 2021. Assessment of oxidative stress, thermal properties, and ultrastructure observations used seed collected from Taranaki in 2021. After collection fruits were transported to the Lions Ōtari Plant Conservation Laboratory in Wellington where they were mixed with two parts medium grade vermiculite (Ausperl, Australia) and stored in airtight containers at 5°C until use. Fruits were not surface cleaned before storage. Prior to embryo excision, fruits were surface sterilized by immersion in 50% commercial bleach (4% sodium hypochlorite) for 5 min and then rinsed three times with sterile distilled water. The fruit pulp and seed coat were removed aseptically to expose the radicle tip, enabling excision of the embryonic axis.

5.2.2 Embryonic axis excision and in vitro *germination for viability assessment*

The embryonic axes (EAs) were excised by cutting through the cotyledon, leaving 1 mm of cotyledon still attached to the embryos. EAs were 4–5 mm in size. The EAs were sterilised by immersion in 5 g/L sodium dichloroisocyanurate (NaDCC) for 10 min and rinsed three times using sterile distilled water. EAs were cultured on half strength Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and 0.2 g^{-1} ascorbic acid (AsA), hereafter referred to as growth media (GM). Cryopreserved EAs were kept in the dark for 1 week prior to exposure to light. All incubations were done at 15/25°C with a 16 h light/8 h dark photoperiod provided by fluorescent tubes (PFD of 30–50 $\mu mol/m^2/s$). EA survival was assessed using the criterion of normal seedling development, namely emergence of the radicle geotropic grown of more than 5 mm (survival) and opening of the cotyledonary leaves (plantlet development). Embryo survival and plantlet development were assessed every 7 days. Survival was expressed as the percentage of final radicle emergence, while mean germination over time was used to assess number of EAs which showed survival each week.

5.2.3 Embryo desiccation

EAs were excised in batches of 10. Prior to the desiccation treatment, EAs were submerged in 0.2 g^{-1} AsA kept in the dark for a maximum of 15 min, blotted dry and then desiccated. After the desiccation treatment, EAs were rehydrated for 30 min in the dark in one of four solutions: (a) sterile distilled water (DW), (b) 0.2 g^{-1} AsA, (c) calcium/magnesium solution (1µM $CaCl_2$ + 1 mM $MgCl_2$) or (d) liquid MS. Rehydration was followed by sterilisation of the EAs as described above. MC for all treatments were determined gravimetrically after drying at 103°C ± 1°C for 17 h. Values represent the mean MC of ten axes replicated four times and expressed on a g H_2O per g dry matter (g/g).

5.2.3.1 Rapid desiccation

Rapid desiccation was conducted in a modified chamber originally described by Wesley-Smith *et al.* (2001). Plastic columns of 250 mm x 130 mm (height x diameter) were created using a ANet A8 3D printer. A computer cooling fan (12V) was mounted in the middle of the column with airflow directed upwards towards the gauze which supported the EAs (Fig. 5.2A & C). Two columns were placed in a 27 L airtight storage container containing 1 kg of activated silica gel (Fig. 5.2B). The air inlet into the pipe was buried in the silica gel to ensure uptake of desiccated air. EAs were desiccated in the chamber for 0, 60, 120, 150, 180 and 210 min.

5.2.3.2 *Slow desiccation*

EAs were placed on sterile filter paper and kept under laminar air flow for 0, 1, 2, 3, 4, 5 or 6 h.

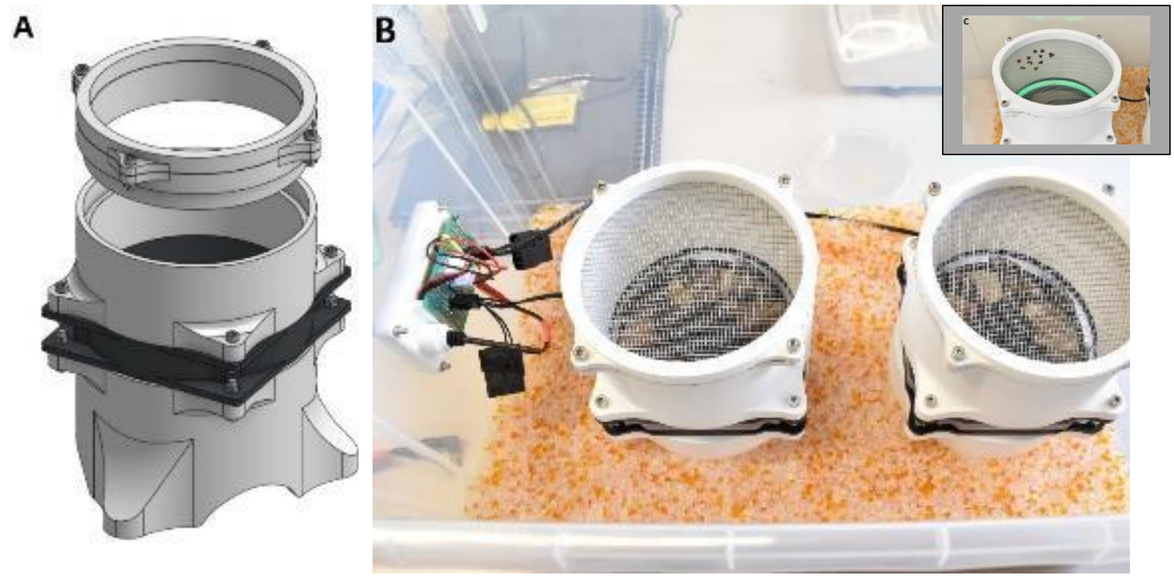


Figure 5.2: Rapid desiccation chamber showing location of gauze (A) in the 3D printed plastic column with two columns (B) located in an airtight container with silica gel. Insert (C) illustrates *Syzygium maire* EAs on the gauze.

5.2.4 *Cryopreservation of desiccated EAs*

Following slow and rapid desiccation treatments described above, EAs were transferred to 1.8 mL sterile cryovials and plunged into LN. After a minimum of 1 h in LN, EAs were rapidly thawed by stirring the cryovials in a water bath at 40 ± 2°C for 2–3 min before rehydration in sterile 0.2 g^{-1} AsA for 30 min in the dark. Sterilized EAs were placed on GM and kept in the dark for at least 7 days. Survival and plantlet development were determined as described above.

5.2.5 *Oxidative stress*

EAs were excised and rapidly desiccated for 0, 60, 120, 150, 180 and 210 min. After the desiccation treatment, EAs were rehydrated in 0.2 g^{-1} AsA or DW in the dark for 30 min. To reflect accumulated metabolic damage, EAs were recovered in the dark for 48 h *in vitro* and then stored in LN until further processing.

5.2.5.1 *Extraction of total proteins and semi-purification of protein extracts*

Due to high levels phenolics and polyphenolics in the EAs, these molecules were removed by adding polyvinylpolypyrrolidone (PVPP) to the extraction buffer and the semi-purification of crude protein extracts by repeated ultrafiltration was essential to preserve and assay enzyme activities. Frozen, pre-weighed EAs were placed in 2 mL screw-capped tubes with 900 μM ice-cold extraction buffer (EB; 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM Na_2 EDTA, 3% PVPP, and 1mM phenylmethylsulfonyl fluoride (PMSF). To homogenize the EAs, five 1.4 mm ceramic beads were added to each tube and the tubes were place on ice. A mini-bead beater was used to homogenize the EAs for 10 sec at a time, repeated 4 times. Once tissue was sufficiently homogenised, the tubes were centrifuged (Eppendorf Centrifuge 5424R, Germany) at 4°C for 30 min at 15 000 rpm to obtain a pellet of cell debris and a supernatant containing the proteins (enzymes). The supernatant was then semi-purified to remove small interfering molecules by ultrafiltration using 0.5 mL Amicon® Ultra centrifugal filters (10 kD cut-off). The supernatant (500 µl) was placed in the ultrafilter and centrifuged at 4°C for 30 min at 10 000 rpm. The concentrate was then made up to 500 µl 100 mM potassium phosphate buffer (pH 7.0) and ultrafiltration process was repeated three times by reconstituting the supernatant with phosphate buffer and transfer the protein sample to a fresh ultrafilter filter. After the third ultrafiltration, the reconstituted protein sample was transferred to a 1.5 mL centrifuge tube and centrifuged for 5 min at 15,000 rpm. The semi-purified protein extracts were then store at –80°C until assayed, as detailed below. The protein contents of the semi purified extracts were determined using a Lowry protein assay as per Fryer *et al.* (1986) with a bovine serum albumin (BSA) standard. The pellet of cell debris was retained and used for the lipid extraction as detailed below.

5.2.5.2 *Extraction of lipids*

The pellet retained from the protein extraction was homogenized in 0.6 mL methanol:chloroform (2:1 v/v) for 1 min before adding 0.4 mL chloroform and 0.4 mL deionised water and centrifuging for 30 sec at 13 300 rpm. The phases were allowed to separate before transferring the chloroform phase to new tubes. All samples were stored at -80°C until analysed.

5.2.5.3 *Antioxidant enzyme assays*

Superoxide dismutase (SOD) activity was assayed using the semi-purified protein extracts following the microplate assay protocol of Ewing and Janero (1995) with minor modifications. The activity in each of the extracts were calculated with reference to a standard (prepared from bovine liver SOD (Sigma–Aldrich, St. Louis, MO, U.S.A.) and expressed as units of SOD mg^{-1} of total protein. One unit of SOD corresponds to the amount of enzyme that inhibits the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25°C.

Catalase (CAT) was assayed using methodology developed by Summermatter *et al.* (1995) and adapted by Gillespie *et al.* (2011) for 96-well microplates. The activity in each of the extracts were calculated with reference to a standard (purified bovine liver CAT; Sigma–Aldrich, St. Louis, MO, U.S.A.) and expressed as μM of H_2O_2 consumed min^{-1} mg^{-1} of total protein.

The activity of glutathione peroxidase (GPOX) was measured using the spectrophotometric method described by Paglia and Valentine (1967), with modifications for use with a microplate reader. GPOX activity was calculated with reference to a standard (GPOX purified from bovine erythrocytes; Sigma–Aldrich, St. Louis, MO, U.S.A.) and expressed as nmoles min^{-1} mg^{-1} of total protein.

5.2.5.4 *Protein carbonyls*

Protein carbonyl (PC) levels were determined using the semi-purified protein extracts via reaction with 2.4-dinitrophenylhydrazine (DNPH) (Reznick & Packer, 1994), adapted for measurement in a microplate reader. Protein carbonyl contents (nmol) were determined using the extinction coefficient of DNPH at 370 nm (0.022 μM^{-1} cm^{-1}), corrected for the calculated pathlength of the solution (0.6 cm).

5.2.5.5 *Lipid peroxide assay*

The lipid peroxide (LPOx) content was determined using the ferric thiocyanate (Mihalievic *et al.*, 1996) method adapted for measurement in a microplate reader, using glass microplates. A calibration curve with t-butyl hydroperoxide was used and lipid peroxide content calculated as nmol lipid peroxide g^{-1} FW.

5.2.6 *Differential Scanning Calorimetry (DSC)*

Thermal behaviour in EAs for each desiccation period (0, 60, 120, 150, 180 and 210 min) were determined using a Perkin-Elmer differential scanning calorimeter (DSC 8500) (Shelton, USA), calibrated for temperature and heat flow with zinc (melting point 419.5 °C) and indium (melting point 156.6 °C). One EA per treatment was hermetically sealed into a large volume (60 µL) stainless steel capsule (sample pan) using an O-ring with the aid of a Perkin-Elmer Universal Crimper (Shelton, USA). Sample weight was measured using a micro-balance (Model: XPR6UD5; Mettler-Toledo, Switzerland), and subjected to calorimetric assessment within 5 min of sample preparation. Samples were cooled from 25°C to -100°C and held for 1 min before rewarming to 25°C at a rate of 10°C min^{-1}. Exothermic- and endothermic-heat changes were derived from the crystallization and melt endotherm during the cooling and warming cycles. The onset temperature of the transition was determined as the temperature at which the tangent of the sharpest portion of the first peak intersected the baseline. The onset, peak and end temperatures were calculated using PYRIS software. The areas of melt and crystallization peaks were calculated from the area above the baseline and expressed as millijoule (mJ). The enthalpies for these transitions are presented as joule per gram of sample weight (J/g). Calorimetric data were collected from three replicates per treatment. After calculating the dry weight of each sample (as described above), the frozen and unfrozen water proportions were calculated. Since 1 g of water releases 334.5 joules of heat energy during the conversion to and from ice, the osmotically active water content of the sample was calculated from the endothermic heat changes derived from the melt endotherm (Block, 2003; Nadarajan *et al.*, 2008).

5.2.7 *Ultrastructural observations - Transmission Electron Microscopy (TEM)*

The effects of desiccation stress are unlikely to be instantaneous, thus freshly excised EAs and those subjected to desiccation with and without freezing, were processed for TEM after rehydration and a subsequent 48 h *in vitro* recovery period. To study the effect of dehydration and freezing on the ultrastructure of meristematic cells in EAs, photomicrographs of desiccated root meristems were compared between fresh, desiccated and desiccation plus frozen samples. Immediately after the desiccation treatment (0, 60, 120, 150 or 180 min), EAs were placed in 2 ml cryovials and stored in LN for 24 h. After 2 min thawing at 40°C, EAs were rehydrated in 0.2 g^{-1} AsA for 30 min. Non-frozen samples were rehydrated after the desiccation treatment. EAs were then recovered on GM for 48 h in the dark before small blocks containing the root meristems were fixed for TEM. Samples were fixed in 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH 7.2) for 24 h at 4°C. After several rinses with phosphate buffer, specimens were post-fixed in 0.5% aqueous osmium tetroxide for 1 h at room temperature and rinsed three times with phosphate buffer. Specimens were then dehydrated in a graded acetone series (30%, 50%, 75% for 5 min), followed by 100% acetone for 10 min. This was followed by infiltration and embedding in low-viscosity resin and polymerisation for 8 h at 70°C. Specimens were sectioned using Ultracut E ultramicrotome (Leica, Austria). Ultra-thin sections of root meristem showing copper/gold interference colours were collected on 600 mesh copper grids and contrasted for electron microscopy using standard double-staining procedure saturated (2.5%) uranyl acetate followed by lead citrate. TEM was carried out at the Manawatū Microscopy and Imaging Centre, Massey University, Palmerston North.

5.2.8 Statistical analysis

All germination experiments involved 10 EAs for each treatment, repeated four times. Survival and plantlet assessment results as well as MC between populations were compared using ANOVA, followed by Fisher's protected least significant difference test for significantly different means (at $P < 0.05$). Inter-treatment differences in lipid peroxidation, protein carbonyls and enzyme activity were tested using ANOVA followed by Tukey's HSD test for pairwise comparisons. Correlations between viability and antioxidant enzyme activity were tested using Pearson correlation analysis. For correlation analysis, percentages were transformed (arcsin) to conform to parametric test assumptions. ANOVA followed by Tukey's HSD test was used to investigate differences in onset of melt temperature, freeze temperature and enthalpy of the melt. All statistical analysis were performed at 0.05 level of significance and results are expressed as mean ± SD. Statistical analysis was conducted using XLStat Software version 1.3 (2021) and SAS/STAT version 14.2.

5.3 Results

5.3.1 Embryo response to desiccation and cryopreservation

MC of fresh EAs was similar across years and provenances ranging from 1.66 ± 0.18 g/g to 1.93 ± 0.19 g/g. Overall rapid desiccation reduced MC to approximately 0.44 g/g within 120 min while it took 150 min to achieve the same MC during slow drying (Fig. 5.3A). Moisture loss during the first 60 min was rapid in both methods, but significantly more moisture was removed when using rapid desiccation (0.75 ± 0.13 g; $P < 0.001$) compared to slow desiccation (1.02 ± 0.25 g). Based on this, rapid desiccation was applied to all further desiccation experiments. MC levels for EAs from different seed lots were significantly different after 120 min and 150 min of rapid desiccation but after 180 min, MCs were similar across all seed lots (Fig. 5.3B). Rehydration with AsA significantly improved embryo survival following 120- and 150-min desiccation while improved survival was also achieved after rehydration with AsA and DW following 180 min desiccation (Fig. 5.3C). Desiccation delayed onset of radicle emergence by at least 7 days while final embryo survival was also significantly impacted by desiccation time (Fig. 5.3D). At MC levels below 0.28 g/g, the formation of cotyledons and development of plantlets were significantly reduced with less than 50% of germinated EAs producing leaves (Fig. 5.4A & B). After four months *in vitro* all surviving plantlets from non-desiccated and desiccated EAs were similar in size (14.1 ± 3.1 mm and 18.7 ± 7.6 mm respectively; Fig. 5.4C) although out of all the treatments, three plantlets produced shoots with no roots (Fig. 5.4D). No survival was recorded after cryopreservation, irrespective of the desiccation method or moisture content (Table 5.1).

Table 5.1: Comparison of embryo moisture content and survival (%) before (-LN) and after (+LN) cryopreservation for embryonic axes (EAs) desiccated slowly (0–6 h) or rapidly (0–210 min). Results are displayed as mean ± SD. Survival values followed by the same letter in each column do not differ significantly (Fisher's, $P \leq 0.05$; N = 10).

Slow desiccation				**Rapid desiccation**			
Desiccation time (min)	**Embryo MC (g/g)**	**Survival (%)**		**Desiccation time (min)**	**Embryo MC (g/g)**	**Survival (%)**	
		-LN	**+LN**			**-LN**	**+LN**
0	1.860 ± 0.51	100 ± 0.0^{a}	0	0	1.94 ± 0.93	100 ± 0^{a}	0
60	1.019 ± 0.25	100 ± 0.0^{a}	0	60	0.75 ± 0.06	78.7 ± 30.8^{ab}	0
120	0.731 ± 0.25	100 ± 0.0^{a}	0	120	0.44 ± 0.15	56.5 ± 24.6^{b}	0
180	0.457 ± 0.18	86.6 ± 15.3^{ab}	0	150	0.35 ± 0.06	50.7 ± 20.1^{bc}	0
240	0.643 ± 0.31	40 ± 17.3^{def}	0	180	0.28 ± 0.05	28.5 ± 30.2^{cd}	0

300	0.478 ± 0.06	16.7 ± 11.5^{fg}	0	210	0.2 ± 0.02	0^{d}	0
360	0.407 ± 0.18	0^{g}	0				0

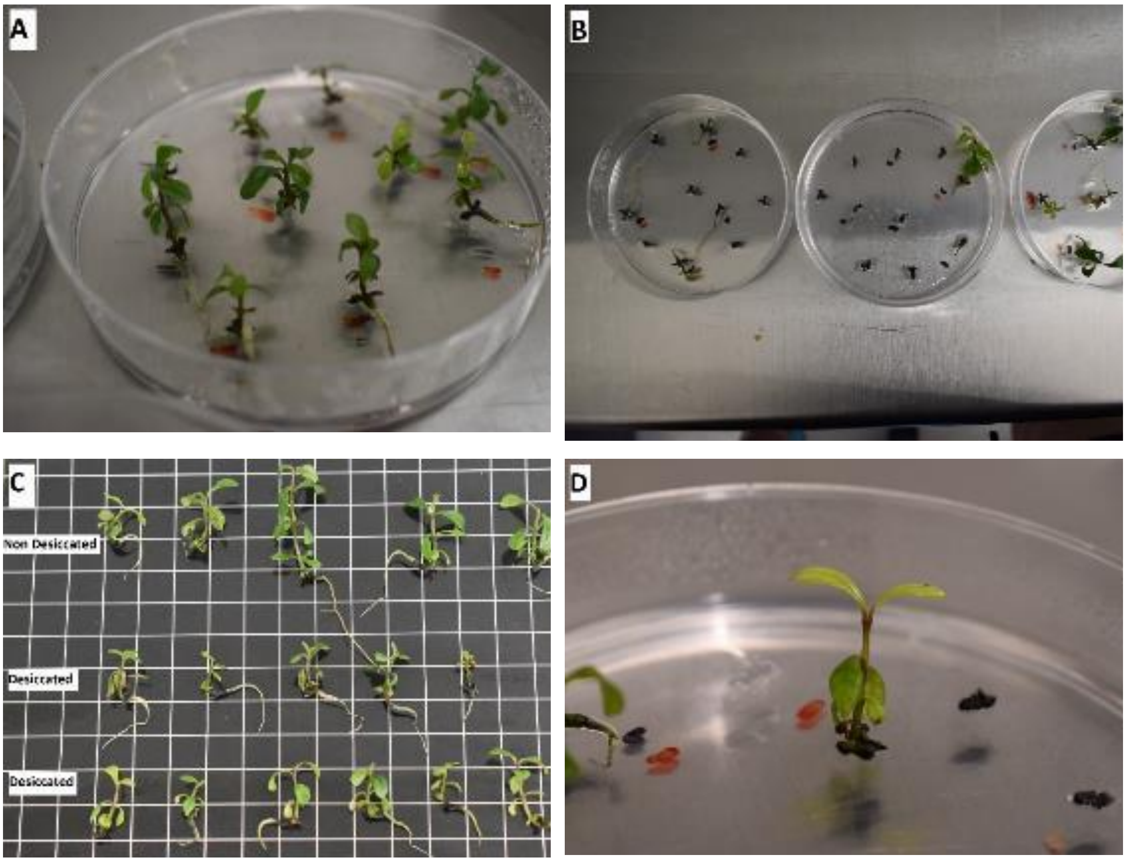


Figure 5.3: Compared to untreated EAs (A), desiccation to MC below 0.28 g/g significantly reduced the formation of cotyledons (B). After 4 months *in vitro* plantlets from all treatments were of similar size (C) although a few plantlets produced shoots but no root following desiccation (D).

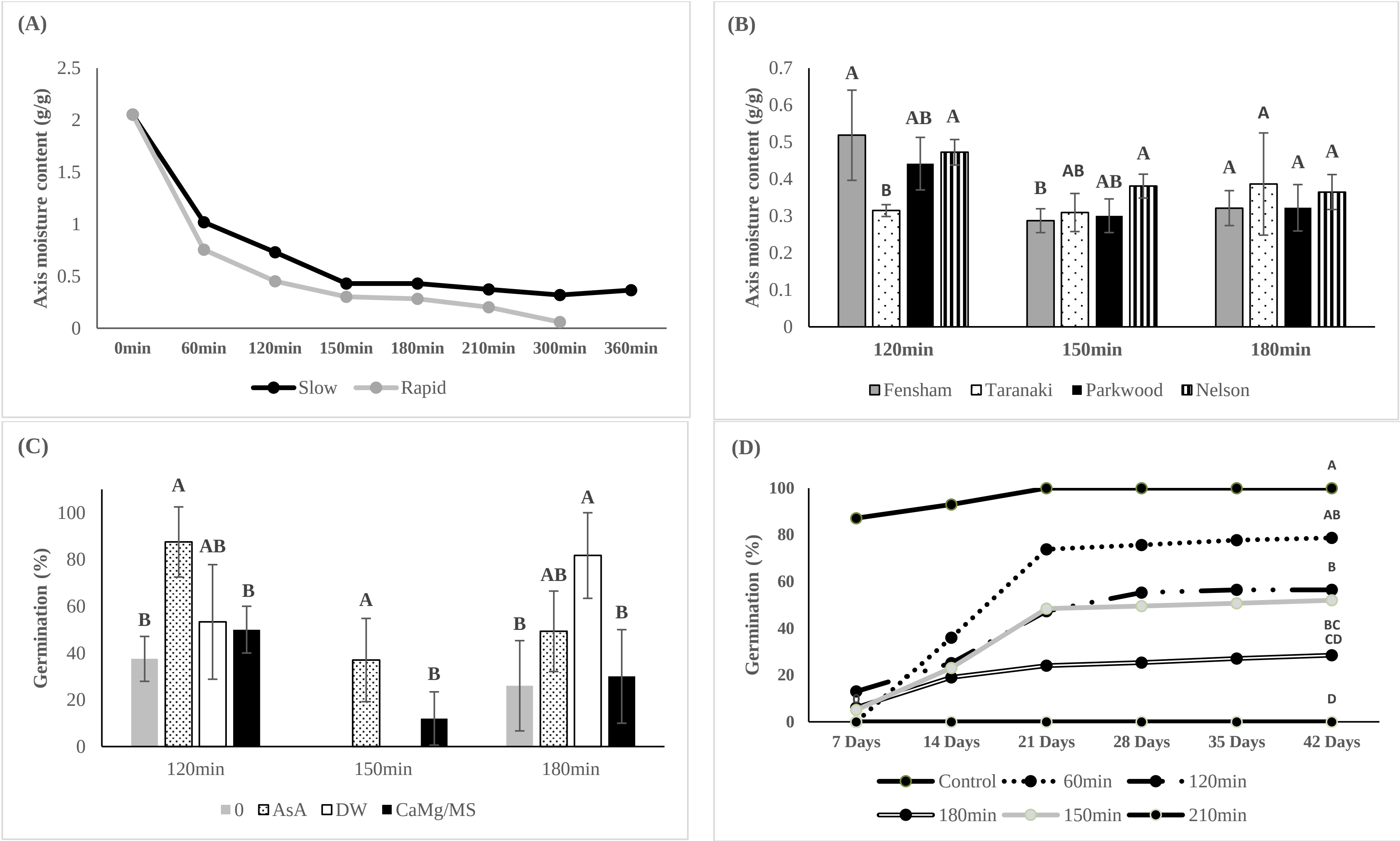
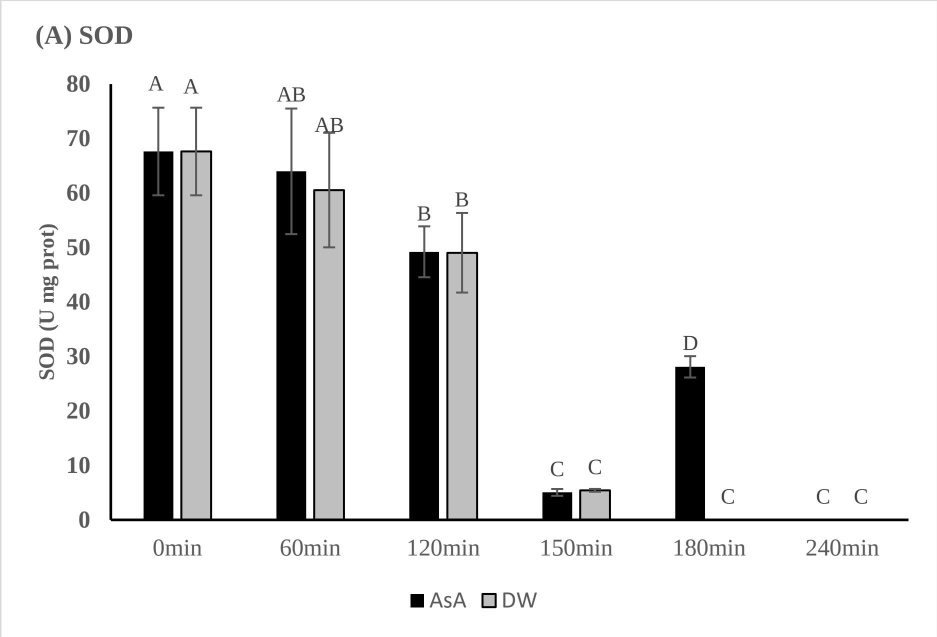


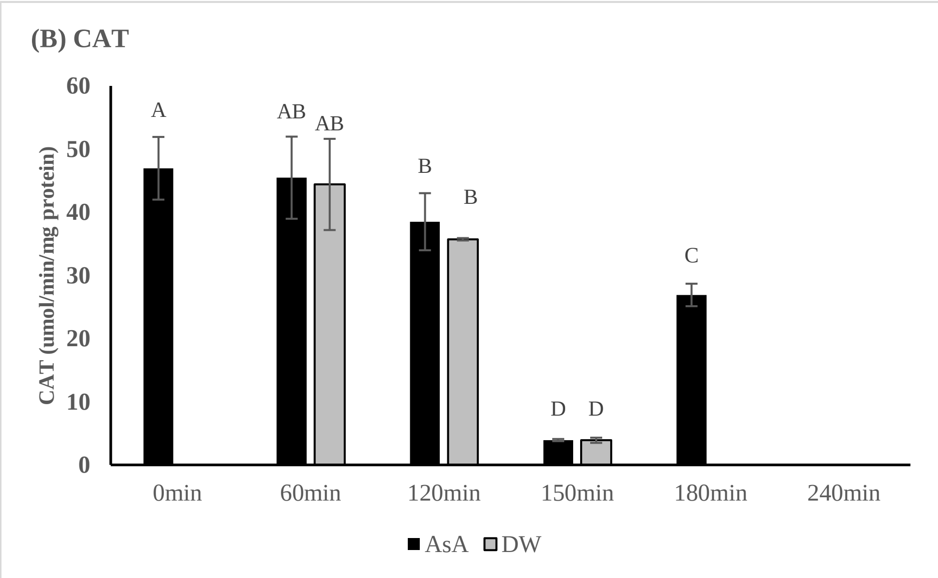
Figure 5.4: Loss of moisture from axes was significantly faster during rapid desiccation (A). Each datum point is the mean of 40 individual axes. Significant differences in moisture loss between seed lots were recorded during rapid desiccation for 120 min and 150 min, but MCs were similar after 180 min (B). Rehydration with AsA improved embryo survival following desiccation for 120- and 150 min (C). Desiccation delayed onset of radicle emergence (indicating survival) across all desiccation treatments with final survival significantly lower for longer desiccation treatments (D). Numbers above bars and line graphs for each desiccation treatment indicate significant difference at $P < 0.05$ based on Tukey's HSD. Error bars represent SD of the mean

5.3.2 Oxidative stress

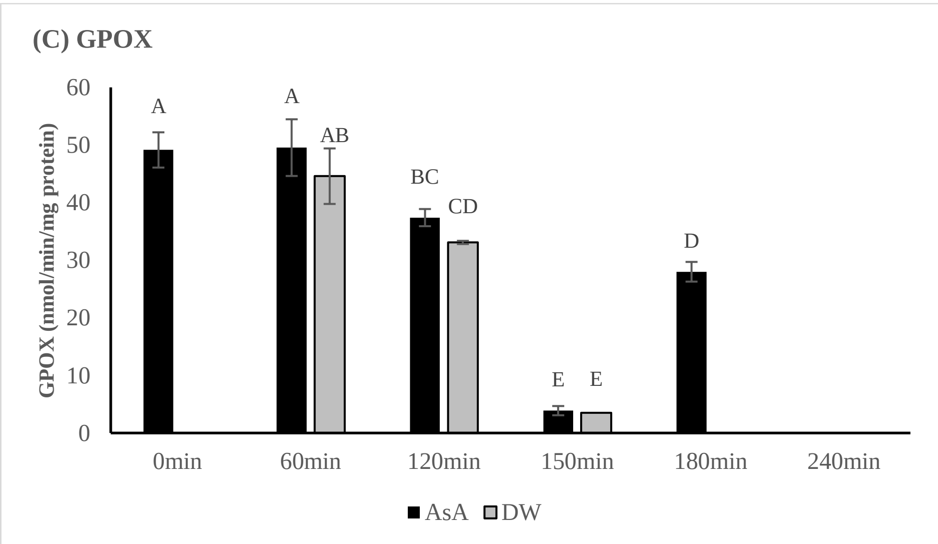
In the present study, except for 180 min desiccation followed by rehydration with AsA, desiccation of EAs to MC below 0.3 g/g resulted in a ten-fold decrease in all three enzymatic antioxidants (SOD, CAT and GPOX) with a five-fold increase in PC and LPOx (Table 5.2). No enzymatic activity was detected at MC of < 0.25 g/g, and this corresponded with a total loss of viability. There was a significant positive correlation between EA viability and SOD (r = 0.998), CAT (r = 0.78) and GPOX (r = 0.79; p = 0.01) activities. Desiccated *S. maire* EAs rehydrated in AsA had slightly higher SOD, CAT and GPOX activities compared to EAs rehydrated in DW, though this difference was not significant (Fig. 5.5). A significant accumulation of LPOx and protein carbonyl contents was associated with a decrease in MC (Fig. 5.5).



(A) SOD
SOD (U mg prot)
80
70
60
50
40
30
20
10
0
A A
AB
AB
B B
C C
D
C
C C
0min
60min
120min
150min
180min
240min
AsA DW



(B) CAT
CAT (umol/min/mg protein)
60
50
40
30
20
10
0
A
AB AB
B B
D D
C
0min
60min
120min
150min
180min
240min
AsA DW



(C) GPOX
GPOX (nmol/min/mg protein)
60
50
40
30
20
10
0
A
A AB
BC CD
E E
D
0min
60min
120min
150min
180min
240min
AsA DW

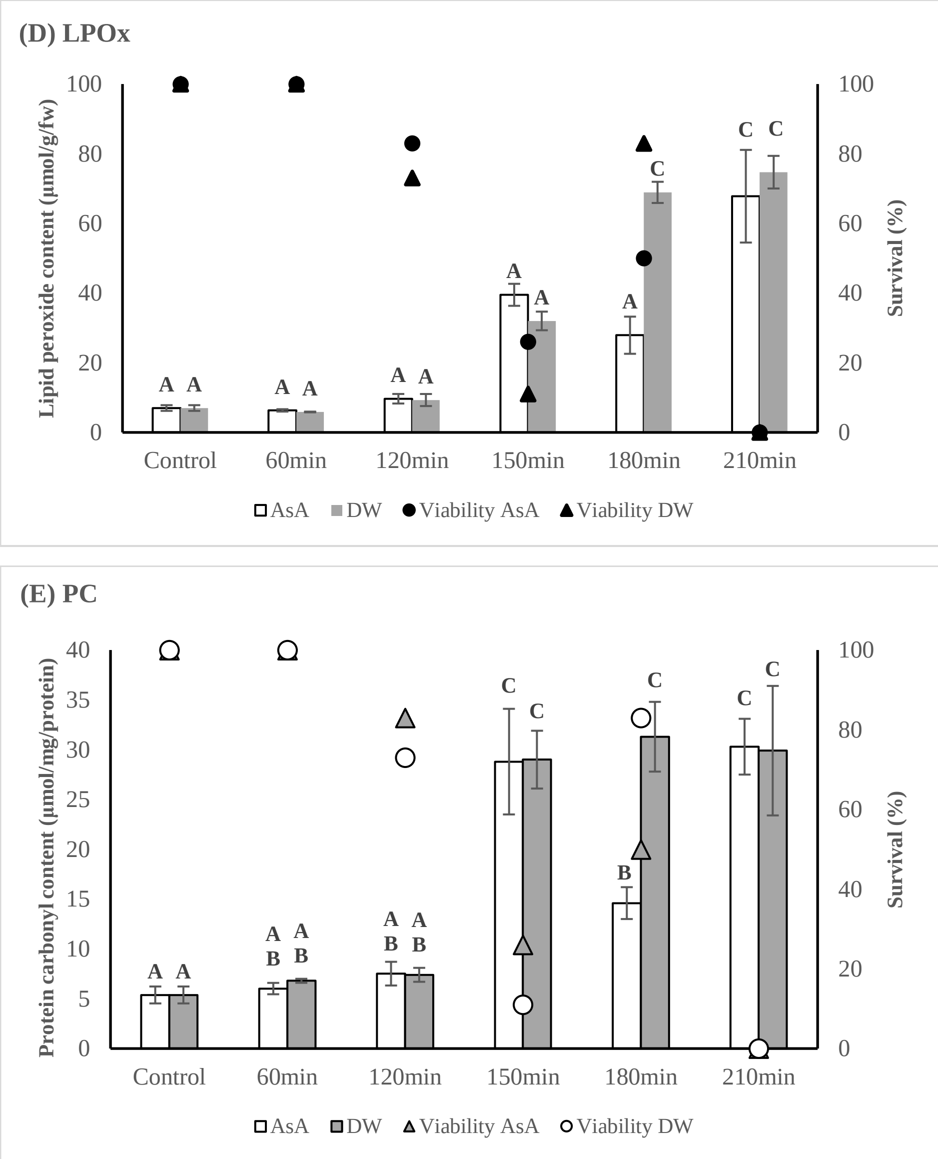


Figure 5.5: SOD (A), CAT (B) and GPOX (C) activities in EAs of *Syzygium maire* at various desiccation treatments following rehydration in ascorbic acid (AsA) or distilled water (DW) for 30 min. The impact of desiccation on lipid peroxide content (D) and protein carbonyl content (E) of *Syzygium maire* EA. Symbols represent mean viability for each treatments, error bars represent SD of the mean. Letters above bars indicate significant difference based on Tukey's HSD ($p < 0.05$).

5.3.3 Differential Scanning Calorimetry (DSC)

The DSC thermograms provided measurements for parameters including enthalpy of melting, amount of osmotically inactive (un-freezable / bound) water and amount of osmotically active (freezable) water. Onset and end temperatures of ice nucleation and ice melt, as well as areas of crystallization/melt and enthalpies are summarised in Table 5.3. The melt onset temperature was significantly lower in EAs desiccated to reach MC of < 0.44 g/g (120 min or longer) but there was no significant difference in the melt onset temperature in samples desiccated for between 120 min and 180 min. No thermal event was evident in EAs desiccated for 210 min (<0.20 g/g) (Table 5.3). Osmotically inactive (unfrozen) and osmotically active (frozen) water contents declined in response to desiccation times (Table 5.3). Enthalpies of melt were significantly reduced to smaller / insignificant thermal events recorded between 5°C–10°C following desiccation of 120 min or longer (Table 5.3; Fig. 5.6). Ice nucleation was inhibited after 210 min desiccation to reach a moisture content of c. 0.21 g/g with no thermal events recorded on rewarming (Fig. 5.6).

Table 5.2: The effect of rapid desiccation and rehydration solution on embryo germination, SOD, CAT, GPOx activities and PC and LPOx levels. Data are presented as mean ± SD (P < 0.05) for six different treatments. Superscript of different letters within a column indicates significantly difference at p < 0.05 based on a one-way ANOVA analysis (Tukey HSD test).

Dry Time (min)	**MC (g/g)**	**Rehydration**	**Embryo Survival (%)**	**Enzymatic antioxidants**			**Damage Markers**	
				SOD (U mg^{-1} prot)	**CAT (umol/min/mg prot)**	**GPOx (nmol/min/mg prot)**	**PC (nmol/mg prot)**	**LPOx (nmol/g/FW)**
0	1.94 ± 0.2	n/a	100 ± 0.0	67.62 ± 8.04^{a}	46.96 ± 4.96^{a}	49.19 ± 3.06^{a}	5.38 ± 0.85^{a}	7.00 ± 0.82^{a}
60	0.75 ± 0.12	AsA	100 ± 0.0	63.98 ± 11.52^{ab}	45.47 ± 6.50^{ab}	49.54 ± 4.92^{a}	6.02 ± 0.57^{ab}	6.33 ± 0.34^{a}
		DW	100 ± 0.0	60.54 ± 10.51^{ab}	44.58 ± 5.40^{ab}	44.58 ± 5.40^{ab}	6.18 ± 0.18^{ab}	5.87 ± 0.10^{a}
120	0.45 ± 0.17	AsA	87.5 ± 15	49.21± 4.67^{b}	38.50 ± 4.52^{ab}	37.39 ± 1.49^{bc}	7.52 ± 1.19^{ab}	9.67 ± 1.37^{a}
		DW	53.0 ± 24.5	49.03 ± 7.37^{b}	35.72 ± 0.19^{bc}	33.08 ± 4.82^{cd}	7.39 ± 0.65^{ab}	9.37 ± 1.73^{a}
150	0.30 ± 0.01	AsA	37.0 ± 17.8	5.04 ± 0.63^{d}	3.86 ± 0.17^{d}	3.88 ± 0.80^{e}	28.80 ± 5.30^{c}	39.57 ± 3.16^{b}
		DW	n/a	5.42 ± 0.26^{d}	3.90 ± 0.41^{d}	3.28 ± 0.28^{e}	29.03 ± 2.94^{c}	32.04 ± 2.67^{b}
180	0.28 ± 0.07	AsA	49.3 ± 17.2	28.09 ± 1.96^{c}	26.91 ± 1.78^{c}	27.99 ± 1.71^{d}	14.59 ± 1.63^{b}	27.90 ± 5.33^{b}
		DW	81.7 ± 18.3	ND	ND	ND	31.27 ± 3.50^{c}	68.94 ± 3.04^{c}
210	0.20 ± 0.03	AsA	0	ND	ND	ND	30.32 ± 2.81^{c}	67.98 ± 13.30^{c}
		DW	0	ND	ND	ND	29.87 ± 6.50^{c}	74.68 ± 4.68^{c}
F-value				69.39	98.63	162.5	43.6	95.98

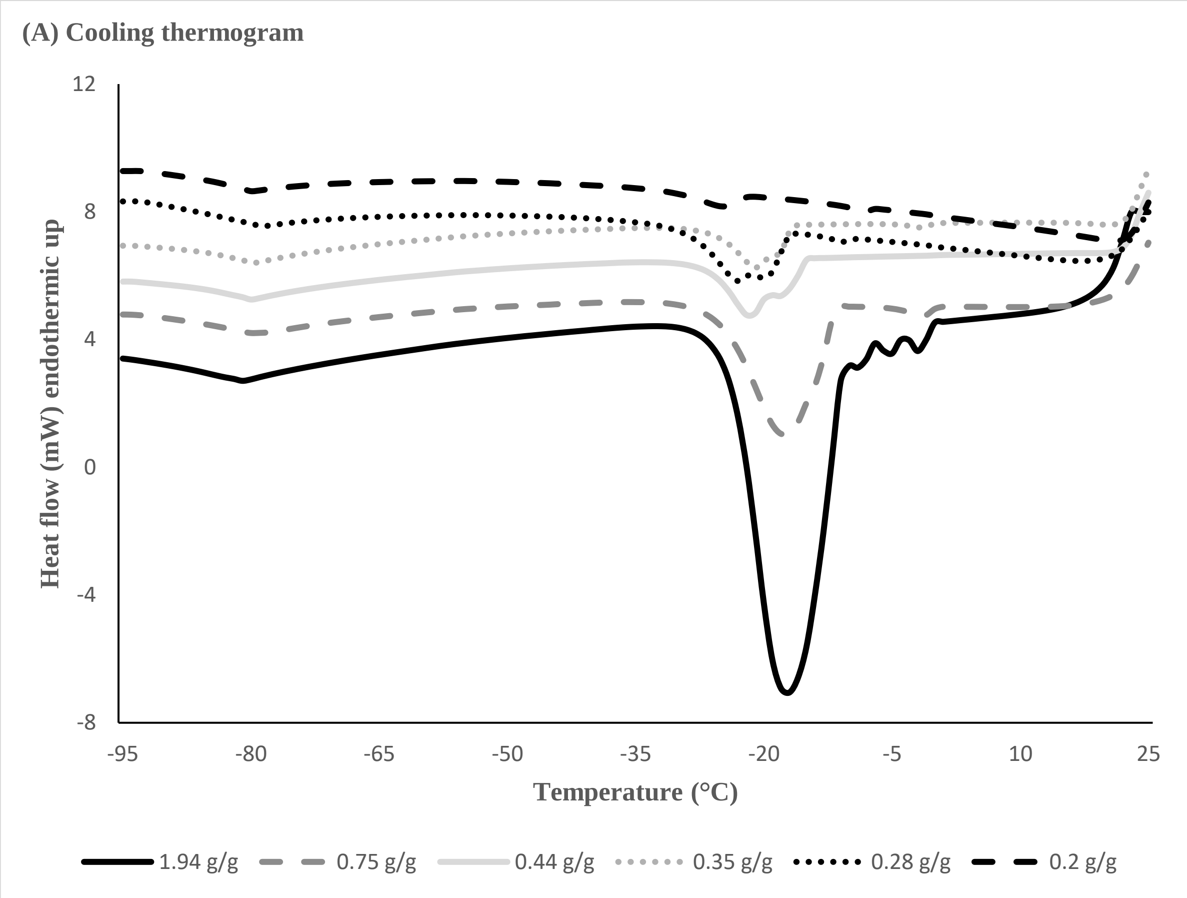
ND = not detected

Table 5.3: Desiccation time (0–210 min), embryo weight (mg), total moisture content (MC), water composition (frozen and unfrozen water) as well as cooling and warming thermodynamic properties of *Syzygium maire* embryonic axes (EAs). Superscript of different letters within a column indicates significantly difference at $p < 0.05$ based on a one-way ANOVA analysis (Tukey HSD test). Data are presented as mean ± SD ($P < 0.05$) for three different treatments.

Dry Time (min)	Embryo Weight (mg)	Total MC (g/g)	Water Composition		Cooling				Warming			
			*Osmotically active water content (Frozen water) (g/g)	**Osmotically inactive water content (unfrozen water) (g/g)	Onset Temperature of Crystallization (°C)	End Temperature of Crystallization (°C)	Area of crystallization (mJ)	Enthalpy of Melt (J/g)	Onset Temperature of Melt (°C)	End Temperature of Melt (°C)	Area of Melt (mJ)	Enthalpy of Melt (J/g)
0	5.21 ± 0.6	1.94 ± 0.2	2.36 ± 1.0	0.38 ± 0.05	–3.05 ± 3.1^{a}	–22.8 ± 1.0^{a}	–571.0 ± 189.9^{a}	109.5 ± 20.2^{a}	9.3 ± 2.2^{a}	21.8 ± 3.0^{a}	603.6 ± 122.2^{a}	128.0 ± 16.3^{a}
60	2.96 ± 0.8	0.75 ± 0.12	0.71 ± 0.2	0.18 ± 0.05	–4.21 ± 6.8^{a}	–23.6 ± 3.5^{a}	–213.8 ± 86.6^{b}	70.1 ± 10.6^{ab}	8.3 ± 4.4^{a}	19.6 ± 2.4^{ab}	180.5 ± 87.2^{b}	60.4 ± 18.0^{b}
120	2.74 ± 0.4	0.45 ± 0.17	0.38 ± 0.07	0.04 ± 0.03	–18.74 ± 2.7^{a}	–26.0 ± 1.9^{a}	–74.5 ± 36.2^{b}	27.5 ± 13.0^{bc}	0.9 ±1.3^{b}	16.4 ± 1.3^{b}	35.2 ± 32.0^{b}	13.9 ± 10.0^{c}
150	2.13 ± 0.1	0.30 ± 0.01	0.33 ± 0.1	0.04 ± 0.03	–12.85 ± 10.0^{a}	–25.7 ± 1.7^{a}	–49.7 ± 23.7^{b}	23.7 ± 12.5^{bc}	–1.3 ± 0.5^{b}	16.0 ± 1.0^{b}	28.4 ± 17.8^{b}	13.3 ± 9.2^{c}
180	3.20 ± 0.5	0.28 ± 0.07	0.21 ± 0.01	0.06 ± 0.01	–13.5 ± 4.6^{a}	–28.6 ± 1.4^{a}	–80.9± 52.6^{b}	24.3 ± 12.7^{bc}	–2.0 ± 2.6^{b}	16.5 ± 0.7^{b}	68.5 ± 21.7^{b}	20.3 ± 4.4^{c}
210		0.20 ± 0.03	Not detected	Not detected	–7.77 ± 0^{a}	–30.3 ± 0^{a}	–12.8 ± 0^{b}	7.0 ± 0^{c}	ND	ND	ND	ND

ND = not detected

* Osmotically active water content of each EA was calculated from the endothermic-heat changes derived from the melt endotherm during the warming cycle and the total water content of the sample as 1 g of water releases 334.5 joules of heat energy during the conversion to and from ice.
** Osmotically inactive water content was the difference between total water and osmotically active water contents.



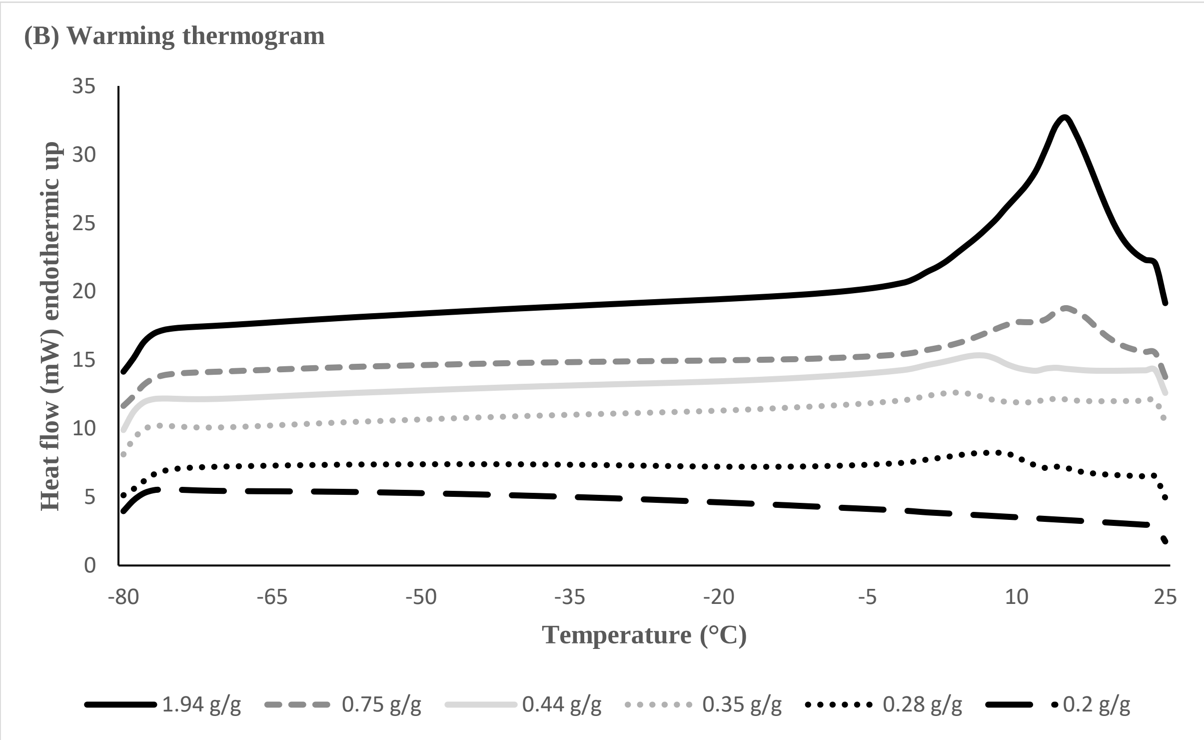
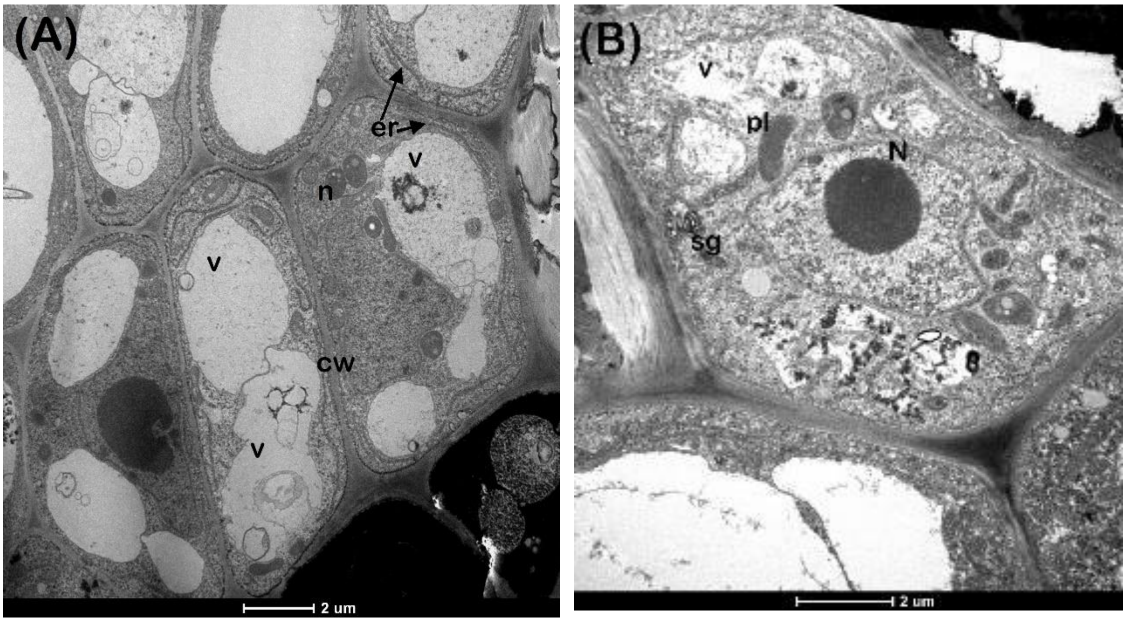


Figure 5.6: DSC warming (A) and cooling (B) thermograms for *Syzygium maire* EAs rapidly desiccated to various moisture contents. A single EA was rapidly desiccated for 0 min (solid black line), 60 min (broken grey line), 120 min (solid light grey line), 150 min (light grey dotted line), 180 min (black dotted line), or 210 min (broken black line) to achieve indicated MC levels.

5.3.4 Ultrastructural observations

Meristematic cells of hydrated EAs, as illustrated in Fig. 5.7A, were compact and characterised by large vacuoles. Mitochondria were frequent and had clearly defined cristae, with little polysome formation. Plastids contained well defined starch grains and internal membranes were well-developed. Cell walls were smooth and did not show any protrusions or disruptions with cells containing large nucleus. In all cells, the plasmalemma was closely appressed to the cell walls. Good ultrastructural preservation was still evident after desiccation to < 0.3 g/g MC with regular shaped nuclei (N) and dense plastids (p) although occasional abnormality in the form of plasmolysis (arrows) was encountered with an increase in the number of vacuole (Fig. 5.7B & C). Overall deterioration of ultrastructure (Fig. 5.7D) was apparent when EAs with MC > 0.75 g/g were exposed to liquid nitrogen with cell wall disruption evident (Fig. 5.7E) while cell membranes appeared intact following exposure to LN at MCs of < 0.3 g/g (Fig. 5.7F).



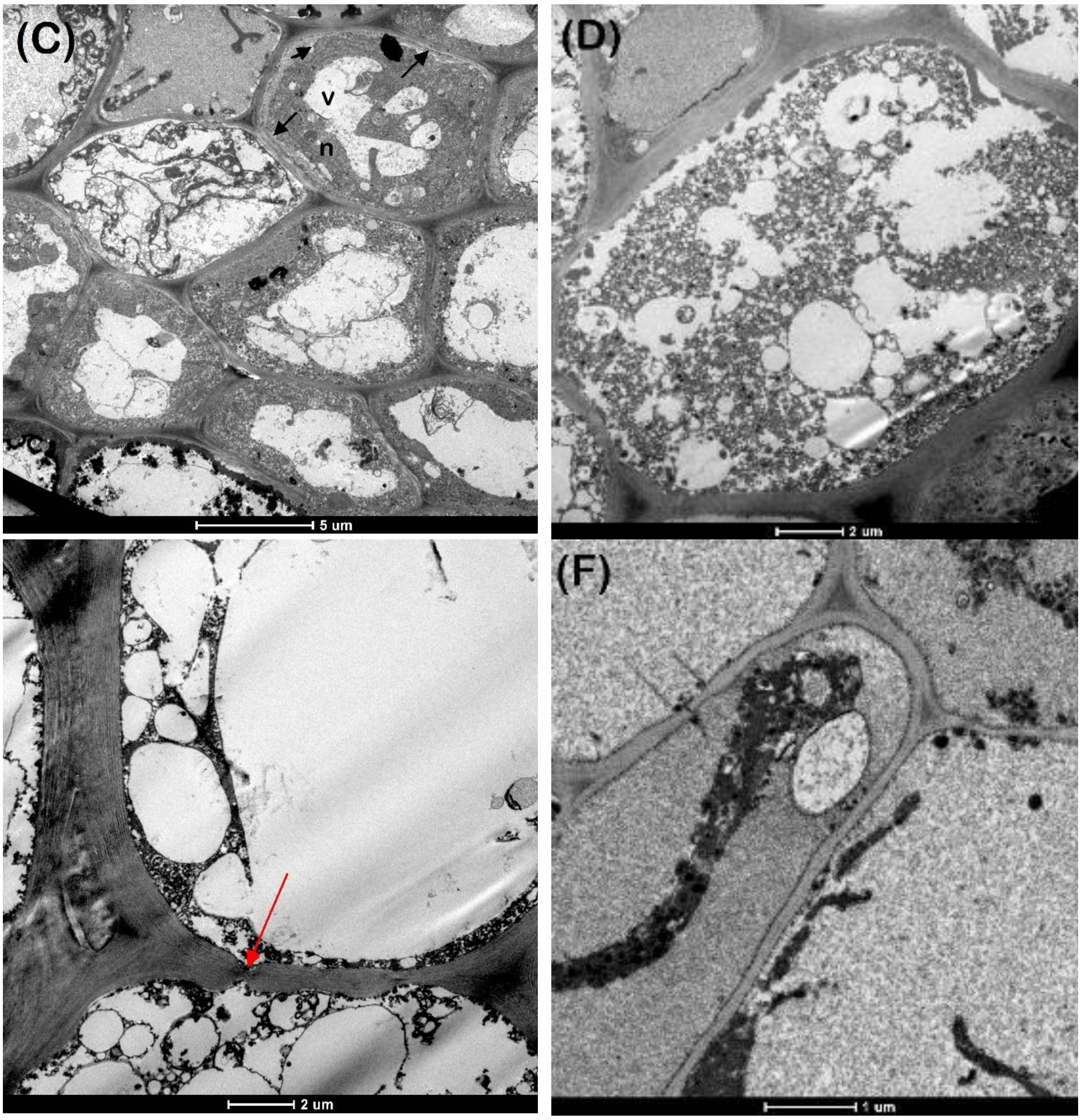


Figure 5.7: The ultrastructure of fresh *Syzygium maire* EAs typically had large vacuoles (V), irregularly shaped nuclei (N) and well developed endoplasmic reticulum (er) (A). Good ultrastructural preservation was evident following desiccation to < 0.3 g/g (B) although plasmolysis (arrows) were evident togheter with an increase in vacuole (v) (C). Disintegration of cells (D) as well as damage to cell membranes (E) were evident following freezing of EAs with MC > 0.75 g/g while cell membranes appeared intact after freezing of EAs desiccated to < 0.3 g/g (F).

5.4 Discussion

Similar to many recalcitrant-seeded species, *S. maire* seeds are shed at high MC with seeds and EAs remaining sensitive to desiccation (Van der Walt *et al.*, 2021b). It is now generally accepted that rapid drying of EAs permits survival to lower MC (reviewed by Berjak *et al.*, 2008) due to less time available for aqueous-based deleterious reactions to occur (Pammenter *et al.*, 1998; Walters *et al.*, 2001; Walters *et al.*, 2008). The loss of enzymatic antioxidant capacity is often accompanied by a decline in viability following desiccation or cryopreservation (Hendry *et al.*, 1992). This is due to elevated levels of

ROS which overwhelms the antioxidant system eventually leading to cellular death (Halliwel, 2006). CAT and GPOx are involved in the metabolism of hydrogen peroxide (H_2O_2) which is a product of SOD catalysed dismutation of $•O_2$ (Fuller *et al.*, 2004). Since the accumulation of H_2O_2 in cells becomes toxic, its removal is a vital component of antioxidant protection. Enzymatic antioxidant activity (SOD = 67.6; CAT = 49.6 and GPOX = 49.2) in EAs with high MCs (> 0.75 g/g) were similar to those recorded in mature zygotic embryos of *Juglans regia* (Juglandaceae) (Jariteh *et al.*, 2015). Initial decrease in embryo viability in *S. maire* was recorded at MCs of below 0.44 g/g which was achieved after approximately 120 min of rapid desiccation. Further desiccation resulted in more survival loss accompanied by a significant drop in the activities of all three enzymes. This was however not unexpected, a drop in protective enzymes during the desiccation of recalcitrant EAs has been reported for *Madhuca latifolia* (Sapotaceae) (Chandra *et al.*, 2021), *Quercus robur* (Fagaceae) (Hendry *et al.*, 1992; Ntuli *et al.*, 2011) and *Trichillia dregeana* (Meliaceae) (Varghese *et al.*, 2011). SOD, CAT and GPOx all continued to decline progressively with desiccation of *S. maire* EAs, this was in contrast with studies on *T. dregeana* in which APX, which also metabolises H_2O_2, initially declined but then increased in the response to rapid desiccation (Varghese *et al.*, 2011). The reduced or loss of SOD, CAT and GPOx activities could indicate that these enzymes were damaged due to high ROS levels and oxidative damage, and so are no longer able to effectively scavenge free radicals, and/or the synthesis of new proteins was inhibited. The high levels protein carbonyls observed as SOD, CAT and GPOx activities declined supports the view that oxidative damage enzymatic proteins were occurring.

Cell membranes comprise of two components, a lipid bilayer and membrane proteins, and these membranes are usually the first target for stress during excessive ROS levels (Xu *et al.*, 2017). The ROS attack unsaturated fatty acids which results in the peroxidation of membrane lipids and eventually to cell death (Walters *et al.*, 2001). This was evident in *S. maire* EAs in which the first significant increase in LPOx was at MC of 0.3 g/g with close to a 10-fold increase in LPOx following desiccation to less than 0.2 g/g MC. Interestingly, there was a negative correlation between the increase in LPOx and EA viability (r = -0.655) indicating that viability loss is likely not only due to membrane disintegration.

Exogenous application of AsA to plants under abiotic stress has resulted in positive, negative and neutral responses (Xu *et al.*, 2017). This wide range of response to AsA can be due to tissue type, stress type, duration of stress, AsA concentration and duration of the AsA application (Xu *et al.*, 2015). This study found that desiccated *S. maire* EAs rehydrated in AsA had slightly higher SOD, CAT and GPOX activities compared to EAs rehydrated in distilled water, though this difference was not significant. It is possible that 30 min rehydration in AsA did not allow sufficient time for cellular uptake thereby minimizing the impact AsA had on antioxidant activities. Surprisingly in EAs desiccated to 0.28 g/g, SOD, CAT and GPOX activity was significantly higher in EAs rehydrated with AsA compared to distilled water, it also had higher antioxidant activities compared to EAs desiccated to 0.3 g/g. The most likely explanation for this is that EAs used in the 0.28 g/g MC treatment was larger and therefore did not consistently reach low MCs resulting in less oxidative stress. Unlike somatic embryos, zygotic embryos are not uniform in size and consists of heterogenous tissue and this likely influences desiccation intensity and duration (Pammenter *et al.*, 1998). Embryo anatomy and tissue topography have also been implicated in low seedling recovery or abnormal growth following desiccation and/or cryopreservation (Sershen *et al.*, 2008; Wesley-Smith *et al.*, 2001; Wesley-Smith *et al.*, 2015). Despite these variances, MCs selected for desiccation and cryopreservation are based on embryo bulk water content which is obtained from mass weighted averages (Ballesteros *et al.*, 2014). Similar challenges were found in the present study for example, MCs between replicates following 150 min rapid desiccation ranged from 0.27 to 0.46 g/g and this was also reflected in viability tests where replicates from longer desiccation treatments sometimes had higher survival compared to shorter treatments (Fig. 4C). It is postulated that not all EAs within the same desiccation treatment reached MCs which were based on bulk water contents and larger embryos therefore showed higher antioxidant activity and viability. This was also evident in thermograms of individual EAs which showed a ten-fold difference in the onset temperature of ice crystals between various EAs desiccated for 60 min (data not shown).

The MC in biological material is a critical factor for successful cryopreservation of living tissue (Reed, 2008; Walters *et al.*, 2013) with MCs of 0.40 – 0.25 g/g reported in the successful cryopreservation of recalcitrant embryos (Berjak *et al.*, 2011; Wesley-Smith *et al.*, 2001). Thermal transitions of *S. maire* EAs were used to determine the amount of freezable water at various MC levels. In non-desiccated EAs there was a large endothermic peak with an onset temperature close to 0°C indicating that this endothermic peak was due to ice formation during the cooling phase. The endothermic events in desiccated EAs became progressively smaller with EAs desiccated to < 0.44 g/g displaying melt enthalpies of 37.78 to 65.91 J/g. Small endothermic events are commonly observed in the thermograms of oily seed such as *Cuphea carthagenensis* (Lythraceae) and *Coffea* spp. (Crane *et al.*, 2006; Dussert *et al.*, 2001). However, since the total lipid content in *S. maire* embryos are < 2%, the endothermic events are likely caused by osmotically active water. The extent of devitrification observed during small exothermic transitions could be manipulated by cooling and warming rates. Moderate to low cooling and warming rates are likely to result in thermal transitions (crystallization, recrystallization, melting) since these rates are too slow to outrun the kinetics of ice formation (Choi & Bischof, 2010; Fahy *et al.*, 2014). The DSC analysis in this study was done at cooling rates of 10°C min^{-1} while plunging EAs in LN will significantly increase freezing rates (Wesley-Smith *et al.*, 1995). The unfrozen water fraction was determined by using enthalpies as a function of water content (Vertucci, 1989; Vertucci, 1992); the regression value obtained for *S. maire* EA was around 0.2 g/g which is achieved by rapidly desiccating EAs for more than 210 min.

In the absence of cryoprotection it is critical that MC is lowered sufficiently to prevent the formation of lethal ice crystals (Wesley-Smith *et al.*, 1995). Ultrastructural observations of *S. maire* EAs at various steps in the desiccation and freezing process indicate that EAs with MCs > 0.75 g/g suffered complete disintegration of internal structures and major disruptions to the cell membranes. Rapid desiccation to MCs < 0.3 g/g resulted in plasmalemma irregularities while frozen EAs with MCs < 0.3g/g inspected in this study had intact cell membranes. It is however not clear whether the damage observed in this study was due to the desiccation or due to subsequent fixation for TEM. Lack of resin infiltration when using standard fixation and embedding protocols has been reported for embryos of some species even when specimen size was dramatically reduced (Naidoo, 2010; Ramsay & Koster, 2002). Ultrastructural observations of *S. maire* EAs also revealed a variety of responses, from ultrastructurally intact to total lyses of the cells, even within the same treatment. Similar results have been reported for potato (Kaczmarczyk *et al.*, 2008), *Cosmos atrosanguineus* (Asteraceae) (Wilkinson *et al.*, 2003), *Pisum sativum* (Myrtaceae) (Wesley-Smith *et al.*, 1995) and Amaryllid species (Sershen, Berjak, *et al.*, 2012a). These authors highlight that for an accurate assessment of the ultrastructural impacts associated with desiccation and cryopreservation it is important that a large number of samples are assessed to determine the response of the explants to the treatment. Due to limited seed availability and access to electron microscopy, only five embryos per treatment was processed for ultrastructural studies of *S. maire* embryos. The images used in this study represented the general appearance of the cells for each treatment but for a more accurate assessment of the ultrastructural response of *S. maire* to desiccation and freezing, a significantly larger samples size is required.

5.5 Conclusion

This study investigated the reasons for desiccation sensitivity in *S. maire* EAs and whether the formation of intracellular ice causes freezing damage in EAs desiccated to various MC levels. The decline in enzymatic antioxidant activities and the increase in lipid peroxidation suggests that oxidative stress, rather than structural damage, causes viability loss in EAs desiccated to MCs < 0.3 g/g. This was supported by thermal analysis which showed that endothermic peaks are significantly reduced at lower MCs but MCs of <0.2 g/g is required to eliminate intracellular ice formation, but this level of desiccation was found to the lethal to EAs. It is recommended that faster cooling and warming rates coupled with the exogenous application of antioxidants are investigated to increase EA survival following desiccation and cryopreservation. It is furthermore likely that rapid desiccation in combination with cryoprotective agents such as PVS2, and addition of antioxidants could promote embryo survival following freezing.

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Chapter 6.

Evaluation of encapsulation-dehydration, droplet-vitrification, and vacuum-infiltration vitrification for the cryopreservation of *Syzygium maire* zygotic embryos

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Abstract

Syzygium maire is a threatened Myrtaceae tree species with limited information on long-term seed storage options for its recalcitrant seed. This study investigated cryopreservation of *S. maire* embryonic axes (EAs) using encapsulation-dehydration as well as Plant Vitrification Solution (PVS2) vitrification. PVS2 was applied through droplet vitrification (DV) and droplet vacuum infiltration vitrification (DVIV) methods. Embryo survival and plantlet formation were compared following encapsulation-dehydration and exposure to PVS2 through DV and DVID. Differential Scanning Calorimetry (DSC) was used to investigate the thermal behavior in EAs treated with PVS2 using DV (30, 60, 90 and 120 min) and DVID (3, 6, 9 and 12 min). Moisture loss for encapsulated EAs was slower compared to non-encapsulated EAs but accompanied with lower survival. Following cryopreservation, encapsulated EAs desiccated to 0.61 and 0.48 g/g MC showed initial radicle emergence but failed to complete the germination process and form plantlets. Exposure to PVS2 using the DV method had a negative impact on embryo survival and plantlet formation, while DVIV resulted in improved results for non-cryopreserved EAs. Neither PVS2 vitrification method resulted in embryo survival following cryopreservation. DSC thermograms revealed that PVS2 moderated the thermal characteristics of EAs. Endothermic events were significantly reduced following incubation in PVS2, but ice nucleation was not completely eliminated in the treatment times tested. The smallest melt enthalpies were associated with 60 min and 90 min DV followed by 6 min DVIV. Differences in individual embryo size impacted DSC analysis with significant variations in thermal behavior observed between replicates of the same treatment. Longer PVS2 incubation times using DVID, coupled with optimization of cooling and warming rates and the use of antioxidants in embryo recovery could increase embryo survival following cryopreservation.

Keywords: Differential Scanning Calorimetry (DSC), embryo, Myrtaceae, Plant Vitrification Solution (PVS2), recalcitrant seeds, threatened, vitrification, zygotic.

6.1 Introduction

Syzygium maire (Myrtaceae; swamp maire, waiwaka) Gaertn, is a 15 m tree endemic to New Zealand. Significant loss of swamps, coupled with a threat from the fungal pathogen myrtle rust (*Austropuccinia psidii*), has resulted in the species being listed as Nationally Critical on the New Zealand Threat Classification System (de Lange, 2018). *Syzygium maire* berries are 10–15 mm in diameter and have a mean weight of 1.11 ± 0.3 g while the embryos are 4–5 mm in size (Chapter 3; Van der Walt *et al.*, 2021). Seeds and embryos are recalcitrant and do not tolerate desiccation to moisture contents (MC) below 20% (Nadarajan *et al.*, 2021; Van der Walt *et al.*, 2021). The impact of rapid desiccation on the oxidative status, ultrastructure and physiological functions of *S. maire* zygotic embryos were investigated in Chapter 5. Differential scanning calorimetry (DSC) scans illustrated that desiccation to <0.2 g/g is required to prevent the formation of ice crystals, but this is lethal to embryos. Thus, chapter 5 recommended that the use of cryoprotectants is investigated in the cryopreservation of *S. maire* embryonic axis.

For recalcitrant species, the only long-term germplasm storage option is cryopreservation, i.e. storage of plant material in liquid nitrogen. Despite the low, or lack of, tolerance to desiccation in recalcitrant species, it is essential that excess moisture is removed from cells to prevent the formation of lethal ice crystals during freezing. Ice nucleation can impact the structural and osmotic integrity of cells resulting in physical raptures and mechanical injury, while colligative damage may result from excessive solute concentrations (Benson, 2008b). During vitrification, when liquids are solidified without crystallization, the system is amorphous and comprises a glassy state which lacks organized structure but has the mechanical and physical properties of a solid (Taylor & Brockbank, 2004).

Physical desiccation or the application of chemical cryoprotectants have been used successfully to achieve vitrification in plant cells (Benson, 2008b; Sakai, 2004). Based on the molecular mass of the cryoprotective agent (CPA), it can be classified as penetrating or non-penetrating. Penetrating CPAs, (i.e., glycerol, dimethyl sulfoxide (DMSO), methanol), have low molecular mass and can therefore permeate cell membranes and cell walls while non-penetrating CPA's, (i.e., sucrose and mannitol), have high molecular mass and cannot move across membranes (Benson, 2008a; Fahy & Wowk, 2014; Naidoo, 2010). Tao and Li (1986) however argued that a third category of

CPAs are applicable to plants, these CPAs, which includes mannitol, oligosaccharides and PEG_{1000}, also have low molecular weight but can penetrate the cell wall only and not the cell membrane. Glycerol has been used successfully as a cryoprotective agent since the 1940's, but due to its slow membrane penetration rate, DMSO, which has high membrane penetrating properties, is been added in increase absorption (Meryman, 2007; Sakai, 2004). Although DMSO has been found to be non-genotoxic, there is a strong cytotoxic activity especially in concentrations of more than 10% or during prolonged or continuous exposure (Galvao *et al.*, 2014). This cytotoxic effect of DMSO on plant cells is due to the inhibition of mitochondrial respiration and increase of the cytosolic calcium during the rewarming phase (Galvao *et al.*, 2014). Plant Vitrification Solution 2 (PVS2), a low toxicity CPA capable of vitrifying plant cells was developed by Sakai *et al.* (1990). PVS2 consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and sucrose, and provides effective cryoprotection for a wide range of explants (Volk & Walters, 2006). Since PVS2 includes penetrating and non-penetrating cryoprotectants, it operates through two protective mechanisms: (1) it replaces cellular water, and (2) it changes the properties of remaining cellular water (Volk & Walters, 2006). A key aspect in the use of cryoprotectants is balancing the protection effect provided with the chemical toxicity (Fuller *et al.*, 2004). It is therefore essential that the PVS2 treatment time and temperature are optimised for each species and type of plant material (Fahy *et al.*, 2014).

To evaluate the efficiency of cryoprotectants in achieving a glassy state, thermal analysis using differential scanning calorimetry (DSC) can be used. Heat and water state transitions are monitored in DSC samples and analysed as a function of time and temperature (Benson, 2008b; Benson *et al.*, 1996). This enables an assessment of the efficiency of cryoprotectants to achieve a stable glassy state (Benson, 2008a) and demonstrates the relationship between freezing or melting transitions and damage at sub-freezing temperatures (Volk & Walters, 2006). PVS2 has been confirmed to prevent ice nucleation and form a glassy state during the cooling phase of some species such as *Ribes* (Grossulariaceae), garlic and mint shoots (Benson *et al.*, 1996; Volk & Walters., 2006). However, glass transition events were found to be unstable in some cases and resulted in devitrification during the warming phase, this was linked to the thermal behaviour of PVS2 (Benson *et al.*, 1996). Rewarming can affect ice stability and very small ice crystals may grow into larger, more damaging crystals if a frozen system is rewarmed slowly. In cases where cells have been vitrified at low water contents, fewer water molecules are available to participate in nucleation, devitrification and ice growth when rewarmed. However, cells vitrified by cryoprotectants such as PVS2 have been found to form less stable glasses on rewarming (Benson, 2008b). In order to achieve thermal and osmotic stability, it is important that addition and removal of cryoprotectants are stringently controlled (Benson, 2008b).

Vitrification procedure includes a number of successive steps such as preculture of plant material on sucrose-rich medium, treating plant material with a loading solution (LS) in preparation for the vitrification solution, exposure to PVS2, cooling/warming and washing to remove PVS2 before culture (Reed, 2008). During conventional vitrification (CV) the plant material, which was first treated with LS (generally consisting of Murashige and Skoog basal medium (MS) supplemented with 2.0 M glycerol and 0.4 M sucrose), is placed in PVS2 solution for a predetermined time ranging from 0 to 6 h (Gagliardi *et al.*, 2002; Nakkanong & Nualsri, 2018), although longer duration treatments of up to 36 h have been reported (Sajini *et al.*, 2011). Following the PVS2 treatment, the plant material is placed into a cryovial containing fresh PVS2 and submersed in liquid nitrogen (LN). Droplet-vitrification (DV), a modified version of CV, aims to increase the cooling/warming rates associated with CV. To achieve this, following treatment with PVS2, the plant material is placed onto aluminium foil strips and covered with a fresh drop of PVS2 before submersion in LN. DV ensures high cooling/warming rates because of the small volume of PVS2 and increased thermal conductivity by aluminium foil strips (Reed, 2008). Vacuum Infiltration Vitrification (VIV) is a novel method in which plant tissue is submerged in PVS2 solution and is put under a vacuum of 381 mm Hg (50 kPa) for 1–5 min (Nadarajan & Pritchard, 2014). VIV was shown to enable faster and more uniform uptake of cryoprotectants (Nadarajan *et al.*, 2014).

Another technique used for the cryopreservation of zygotic embryos is encapsulation combined with vitrification or dehydration (Sakai & Engelmann, 2007). Plant material, which can consist of meristems or embryos, are encapsulated in calcium alginate beads before physical or osmotic desiccation (Benson, 2008a). The hypothesis is that alginate encapsulation should allow recalcitrant tissue to withstand physical manipulation better (Peran *et al.*, 2006). Encapsulation-dehydration or encapsulation-vitrification is more commonly used for the cryopreservation of shoots (i.e. Benelli *et al.*, 2013; Ciringer *et al.*, 2018). However, successful cryopreservation of zygotic embryos using encapsulation has been reported for *Ekebergia capensis* (Meliaceae) (Peran *et al.*, 2006*)*, *Havea brasiliensis* (Euphorbiaceae) (Nakkanong *et al.*, 2018) and *Ilex* (Aquifoliaceae) species (Mroginski *et al.*, 2008). Encapsulation-dehydration of *Artemisia sieversiana* (Asteraceae) seeds were found to be unsuccessful, with seeds losing viability after encapsulation (Yong *et al.*, 2019).

In this study, the effectiveness of three methods, encapsulation-dehydration, droplet-vitrification (DV) and the novel combination of droplet-vitrification with vacuum infiltration vitrification (DVIV), for the cryopreservation of zygotic embryos of *S. maire* were compared. The aim was to gain insight into the impact encapsulation-dehydration and the use of PVS2 on the survival and plantlet formation in *S. maire* before and after exposure to cryogenic temperatures. Using DSC, the size and temperature of exothermic, endothermic and glass transitions within EA treated with cryoprotectants were measured. Comparison of thermal properties provided insight towards understanding the mechanism of cryoprotection for *S. maire* zygotic embryos and can be used to optimise cryopreservation protocols.

6.2 Materials and Methods

6.2.1 Plant material

Mature *S. maire* fruits were collected in March (2018 – 2019) from Taranaki in the North Island of New Zealand (39.2933°S, 174.2663°E) (Department of Conservation Permit number 69441-FLO). Fruits were collected from 10-20 trees throughout the population, combined, pooled, and randomly assigned to treatments. Fruits were transported to the Lions Ōtari Plant Conservation Laboratory in Wellington where they were mixed with two parts medium grade vermiculite (Ausperl, Australia) and stored in airtight containers at 5°C until use. Fruits were not surface cleaned before storage. Prior to embryo excision, fruits were surface sterilized by immersion in 50% commercial bleach (4% sodium hypochlorite) for 5 min and then rinsed three times with sterile distilled water. The fruit pulp and seed coat were removed aseptically to expose the radicle tip, enabling excision of the embryonic axis.

6.2.2 Embryonic axis excision and culture

Two EA excision methods were tested, the first removed all cotyledon material from the EA while in the second method embryos were excised by cutting through the cotyledon, leaving 1 mm of cotyledon still attached to the embryos (Fig. 6.1). EAs were 4–5 mm in size. The EAs were then sterilized by immersion in 5 g/L sodium dichloroisocyanurate (NaDCC) for 10 min and rinsed three times using sterile distilled water. EA were cultured on half strength Murashige and Skoog (MS) medium supplemented with 200 mg/L ascorbic acid and 3% (w/v) sucrose, hereafter referred to as growth medium (GM). Cryopreserved EAs were kept in the dark for 1 week prior to exposure to light. All incubations were done at 15/25°C with a 16 h light/8 h dark photoperiod provided by fluorescent tubes (PFD of 30–50 µmol/m²/s). Survival was recorded as radicle emergence (> 5 mm) and complete plantlet formation (the formation of both roots and shoots).

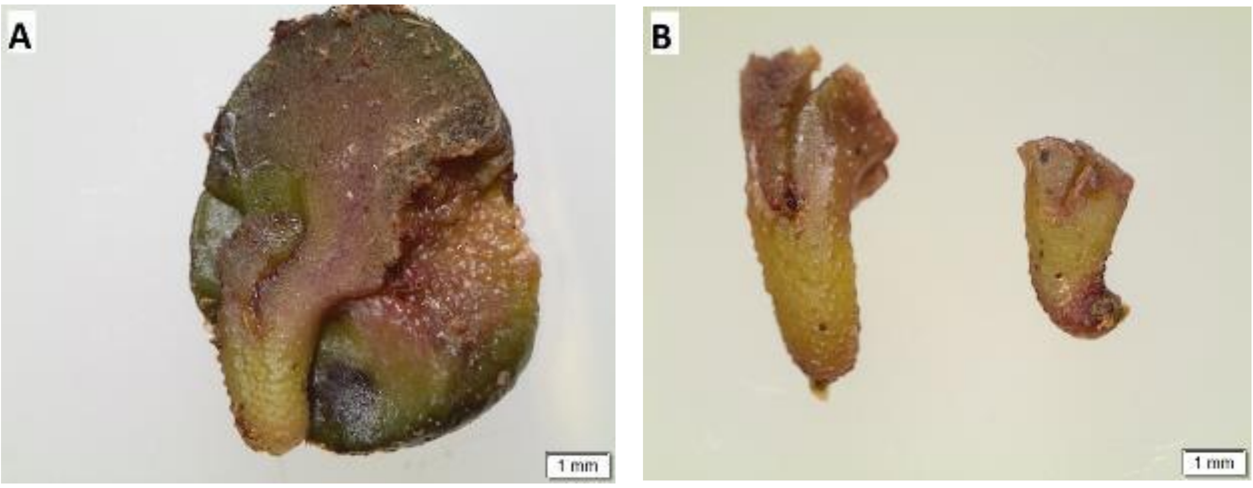


Figure 6.1: *Syzygium maire* (A) seed containing cotyledons and embryo and (B) excised embryo axes with (left) and without (right) cotyledon blocks.

Development of surviving EAs was assessed every 7 days, with normal plantlet formation established after 8 weeks in culture.

6.2.3 Moisture content determination

Moisture contents (MC) for all treatments were determined gravimetrically after drying at 103°C ± 1°C for 17 h. Values represent the mean MC of ten axes replicated four times and expressed on a g H_2O per g dry matter (g/g).

6.2.4 Encapsulation-dehydration

Sterile EAs were encapsulated by suspension in half-strength MS medium containing 3% (w/v) sodium alginate beads, a single EA encased in a drop of sodium alginate was dripped into a calcium solution consisting of half-strength MS medium with 1% (w/v) $CaCl_2$. Beads were polymerized for 20 min then blotted dry on sterile filter paper and dehydrated in the flow of a laminar flow cabinet for 0, 1, 2, 3, 4, 5, or 6 h. Following drying, encapsulated EAs were transferred to 1.8 mL sterile cryovials and plunged into LN. After at least 1 h in LN, EAs were rapidly thawed by stirring the cryovials in a water bath at 40 ± 2°C for 2–3 min. MC, survival, and plantlet development were determined as described above. MC and viability for encapsulated EA were compared to non-encapsulated EA (described in chapter 5).

6.2.5 Cryopreservation solutions

Solutions used for the vitrification protocol included loading solution (LS), PVS2 and unloading solution (ULS). The LS consisted of MS supplemented with 2.0 M glycerol and 0.4 M sucrose. The PVS2 consisted of MS supplemented with 0.4 M sucrose, 30% w/v glycerol, 15% w/v ethylene glycol and 15% w/v dimethyl sulfoxide (DMSO). The ULS consisted of MS supplemented with 0.75 M sucrose.

6.2.6 Droplet-Vitrification (DV)

Sterilized EAs were placed in LS for 20 min, then transferred to PVS2 for various incubation times (30, 60, 90 or 120 min). The LS and PVS2 treatments were conducted at either 20°C or 0°C. After PVS2 incubation period, EAs were either: a) soaked in ULS for 20 min and transferred to GM for viability assessment; or b) transferred onto small aluminum foil strips, covered with a drop of fresh PVS2 and then plunged into LN. After 1 h in LN the foil strips containing the EAs were transferred to sterile ULS warmed to 40 ± 2°C in a water-bath for 2–3 min and then soaked in ULS for 18 min at room temperature.

6.2.7 Droplet Vacuum Infiltration Vitrification (DVIV)

DVIV cryopreservation followed the same methodology described for DV except the EAs were treated with LS for 5 min under vacuum followed by PVS2 treatments (3, 6, 9 and 12 min) also under vacuum at 381 mm (15 in) Hg (50kPa). LS and PVS2 treatments were conducted at either 20°C or 0°C. Vacuum was applied using a single stage vacuum pump (model number: VP 130) and 5 L stainless steel vacuum chamber.

6.2.8 *Differential Scanning Calorimetry*

Thermal behaviour in EAs incubated in PVS2 were determined using a Perkin-Elmer differential scanning calorimeter (DSC 8500) (Shelton, USA), calibrated for temperature and heat flow with zinc (melting point 419.5°C) and indium (melting point 156.6°C). One EA per treatment were blotted dry and hermetically sealed into a large volume (60 µL) stainless steel capsule (sample pan) using an O-ring with the aid of a Perkin-Elmer Universal Crimper (Shelton, USA). Sample weight was measured using a micro-balance (Model: XPR6UD5; Mettler-Toledo, Switzerland), and subjected to calorimetric assessment within 5 min of sample preparation. Samples were cooled from 25°C to -100°C and held for 1 min before rewarming to 25°C at a rate of 10°C min^{-1}. Exothermic- and endothermic-heat changes were derived from the crystallization and melt endotherm during the cooling and warming cycles. The onset temperature of the transition was determined as the temperature at which the tangent of the sharpest portion of the first peak intersected the baseline. The onset, peak and end temperatures were calculated using PYRIS software. The areas of melt and crystallization peaks were calculated from the area above the baseline and expressed as millijoule (mJ). The enthalpies for these transitions are presented as joule per gram of sample weight (J/g). Calorimetric data were collected from three replicates per treatment.

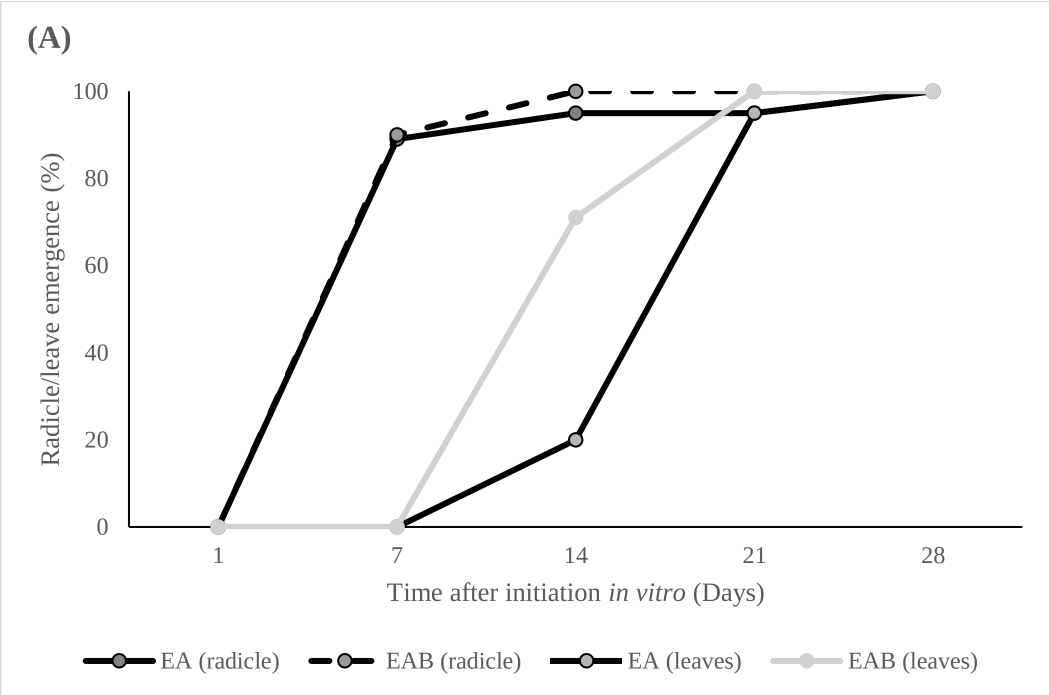
6.2.9 *Statistical analysis*

All experiments involved 10 EAs for each treatment, repeated four times. Survival and plantlet assessment results were compared using ANOVA, followed by Fisher's protected least significant difference test for significantly different means (at $P < 0.05$). A two-way ANOVA was used to test the interaction between exposure time and temperature on embryo survival. ANOVA followed by Tukey's HSD test was used to investigate differences in onset of melt temperature, freeze temperature and enthalpy of the melt. Data were checked for normality prior to analysis and transformed to arcsine values where needed. Results are expressed as mean ± SD. All statistical analysis was conducted using XLStat Software version 1.3-2020.

6.3 Results

6.3.1 Post-excision EA survival and plantlet formation

EAs with no cotyledon sections took longer to produce leaves. After 7 weeks in culture, they were visually smaller compared to EAs which contained small sections of cotyledons (Fig. 6.2). Based on these results, all EAs were excised with cotyledon blocks. Radicle growth from excised and sterilised EAs was observed after 7 days followed by shoot elongation and development of the first set of true leaves after 7–14 days. Production of the second set of leaves was observed after four weeks in culture. Survival and plantlet formation in untreated EAs (controls) were 100% with no contamination recorded.



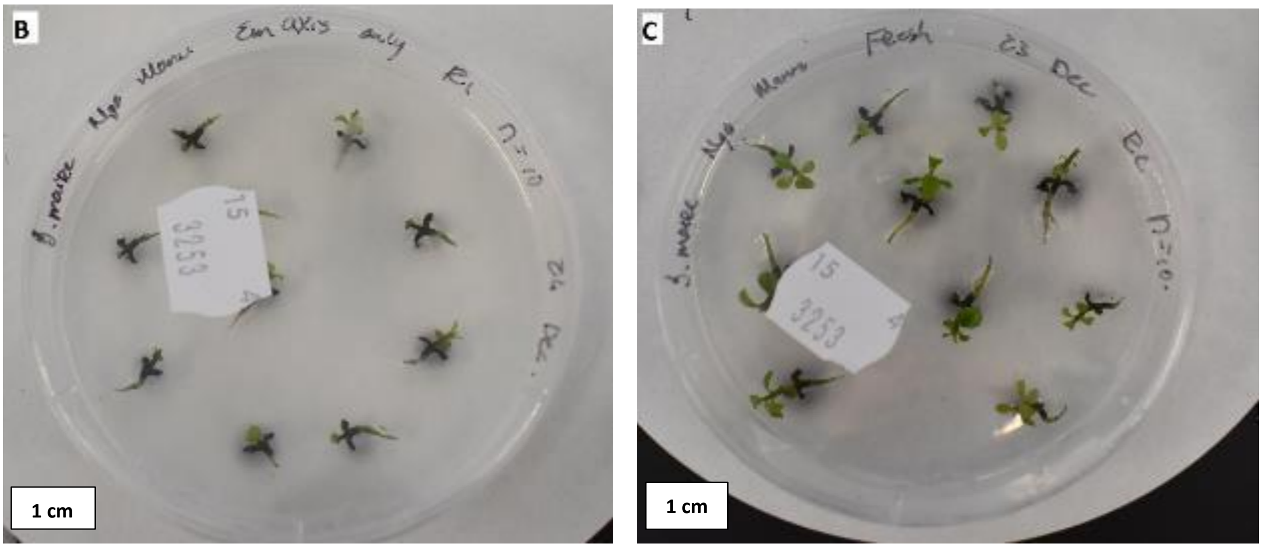


Figure 6.2: Radicle emergence and the development of leaves in embryonic axes with cotyledons attached (EAB) were faster compared to embryonic axes with no cotyledon blocks (EA) (A). After 8 weeks in culture, plantlets (B) from EA were visually smaller compared to EAB (C).

6.3.2 Encapsulation-dehydration

Initial MCs of non-encapsulated and encapsulated EAs were 2.05 ± 0.9 and 2.2 ± 0.06 g/g respectively. Significant rapid moisture loss from non-encapsulated EAs was associated with desiccation for up to 3 h ($P < 0.0001$) followed by gradual loss when desiccated further for up to 6 h (Fig 6.3A). Moisture loss in encapsulated EAs was slower but accompanied with lower survival (Table 6.1). Overall, encapsulation had a negative impact on survival of non-cryopreserved EAs (Fig. 6.3B). Following cryopreservation, encapsulated EAs desiccated to MCs of 0.61 ± 0.01 and 0.48 ± 0.02 g/g showed initial radicle emergence (survival) but failed to complete the germination process and form plantlets (Fig. 6.4).

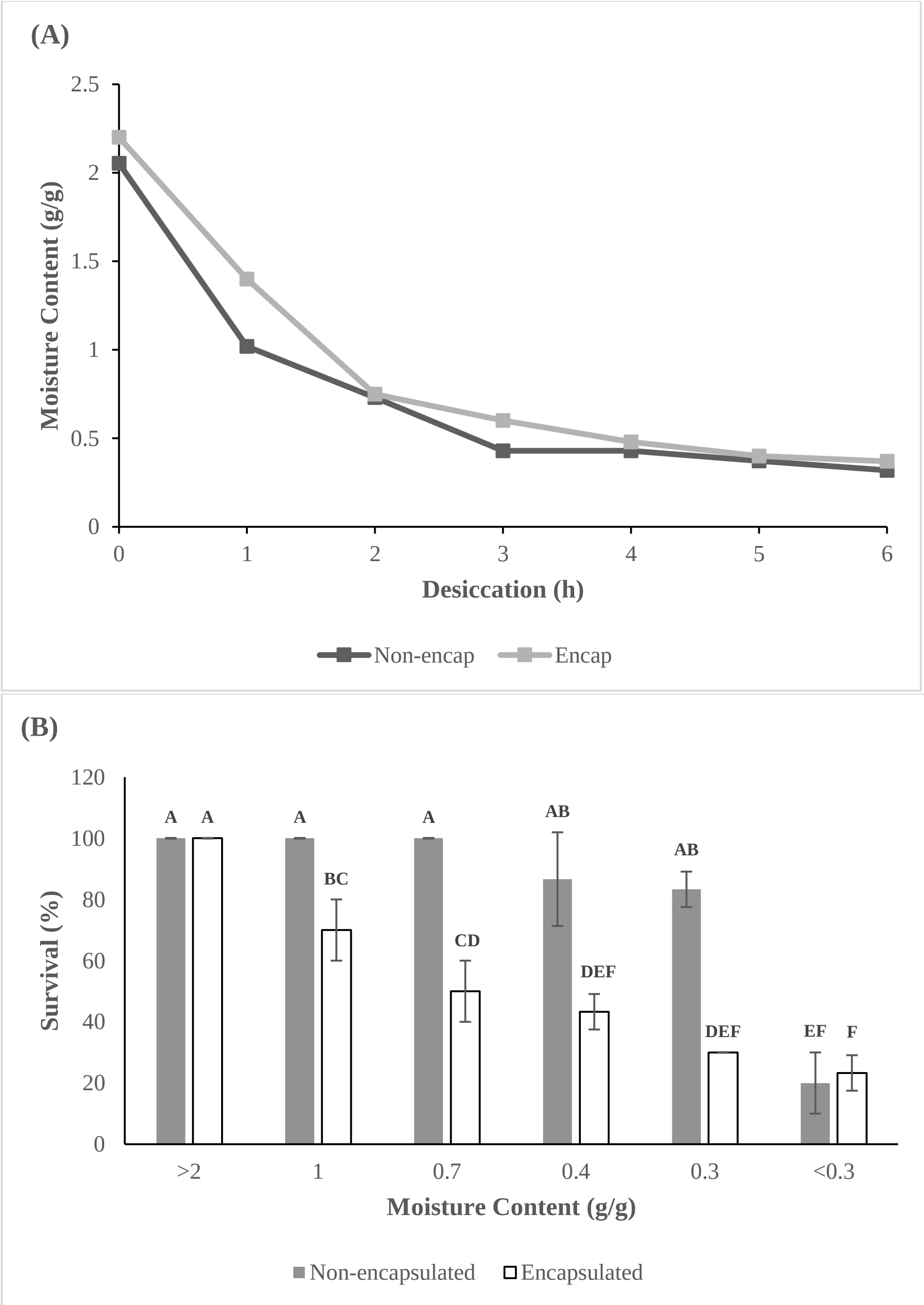


Figure 6.3: The rate of moisture loss during desiccation (0–6 h) under a laminar air flow cabinet for non-encapsulated and encapsulated *Syzygium maire* embryos (A). Encapsulation had a negative impact on non-cryopreserved encapsulated embryos (B). Results are displayed as mean ± SD. Bars with same letters do not differ significantly (Tukey HSD, $P \leq 0.05$; N = 10).

Table 6.1: Moisture content and germination (%) before (–LN) and after (+LN) cryopreservation for encapsulated embryonic axes (EA) air-dried for 0–6 h. Results are displayed as mean ± SD. Survival values in each column followed by the same letter do not differ significantly (Fisher's, P ≤ 0.05; N = 10).

Desiccation period (h)	Encapsulated EA		
	Embryo MC (g/g)	Survival (%)	
		-LN	+LN
0	2.2 ± 0.06	100 ± 0.0[a]	0[g]
1	1.4 ± 0.08	70.0 ± 10.0[bc]	0[g]
2	0.75 ± 0.03	50.0 ± 10.0[cd]	0[g]
3	0.61 ± 0.01	43.3 ± 5.8[de]	20.0±0[efg]
4	0.48 ± 0.02	30.0 ± 0.0[def]	26.7±5.8[def]
5	0.04 ± 0.01	26.7 ± 5.8[def]	0[g]
6	0.37 ± 0.02	23.3 ± 5.8[efg]	0[g]

Figure 6.4: Sodium alginate encapsulated embryos of *Syzygium maire* showing initial radicle emergence (survival) four weeks after cryopreservation.

6.3.3 Survival and plantlet development after DV and DVIV PVS2 vitrification

PVS2 vitrification method had a significant effect on non-cryopreserved EAs survival (p = 0.03) with 86.1 ± 21.2% of the EAs surviving after PVS2 exposure using DVIV compared with 71.1 ± 18.4% after DV (Fig. 6.5). During DV, EA survival was negatively (but not significantly) affected by exposure time (p = 0.061) and temperature (p = 0.023) with lowest survival (c. 43.3%) associated with 90 min and 120 min exposure at 0°C (Fig. 6.6A). PVS2 exposure time and temperature during DV had a significant negative impact on plantlet development (p = 0.007) with fewer plantlets developing during longer exposure times (60–120 min) at 0°C (Fig. 6.5; Fig. 6.6B). Neither exposure time (p = 0.660) nor temperature (p = 0.465) had a significant impact on non-cryopreserved EA survival or plantlet development during DVIV (Fig. 6.5; Fig. 6.6C & D). Neither DV nor DVID treatments resulted in EA survival following cryopreservation.

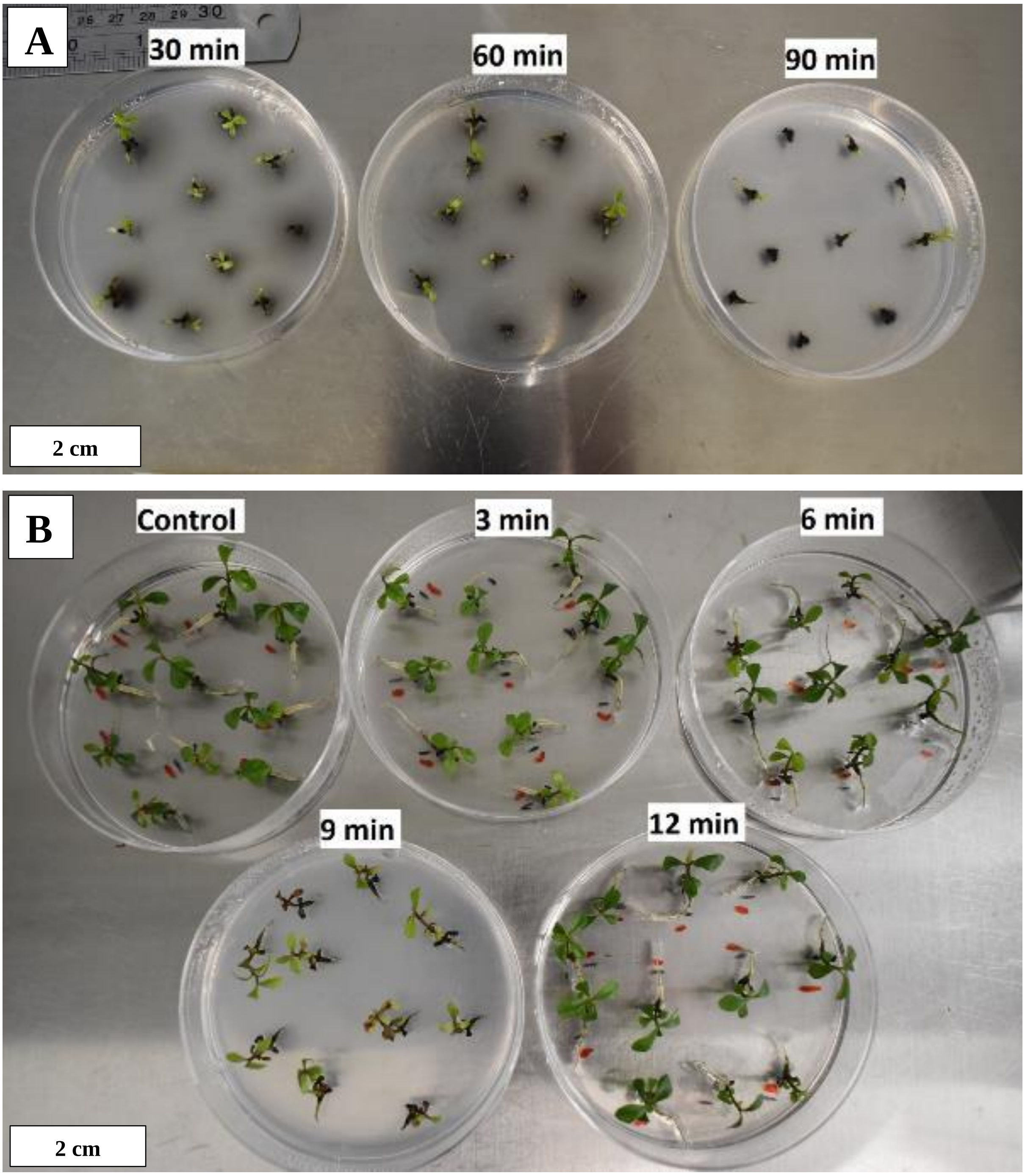


Figure 6.5: Seedling development after 10 weeks *in vitro* following exposure to PVS2 at room temperature through droplet vitrification (DV) (A) and droplet vacuum infiltration vitrification (DVIV) (B). Seedling development after 120 min DV not shown.

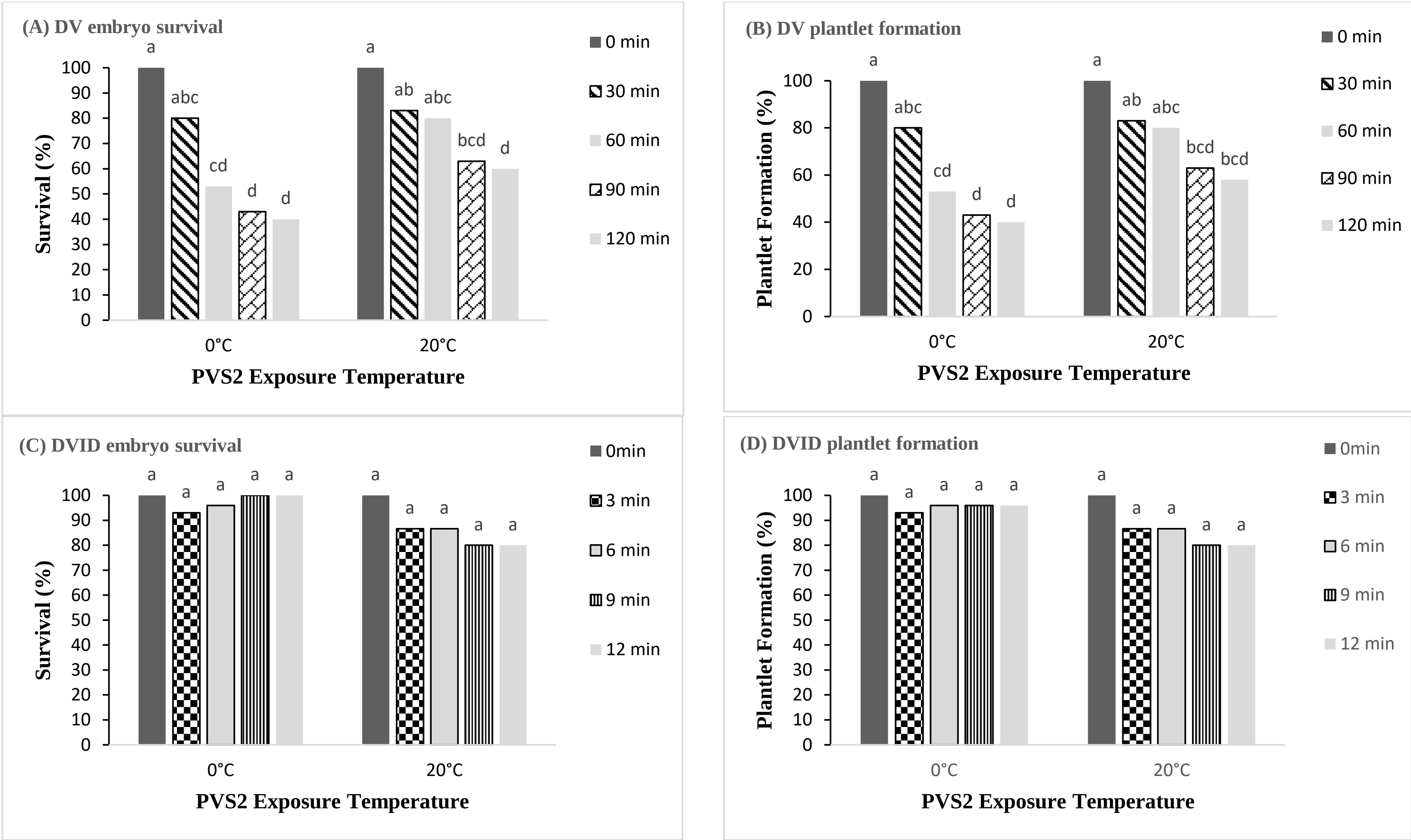


Figure 6.6: Embryo survival (%) and plantlet formation (%) following exposure to PVS2 using DV (A & B) and DVIV (C & D). Values followed by the same letter do not differ significantly (Fisher's, $P \leq 0.05$; $N = 10$).

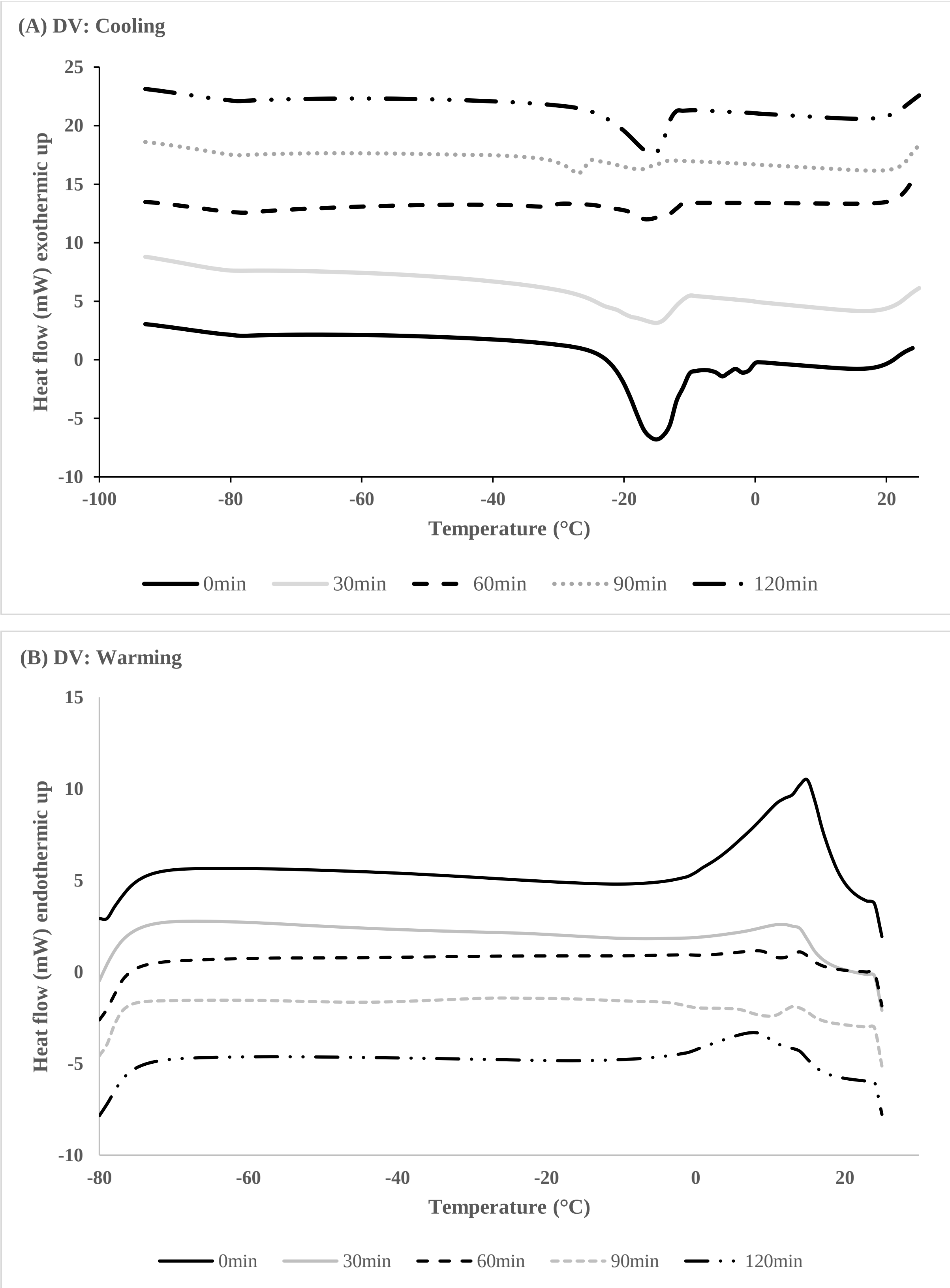
6.3.4 Differential Scanning Calorimetry

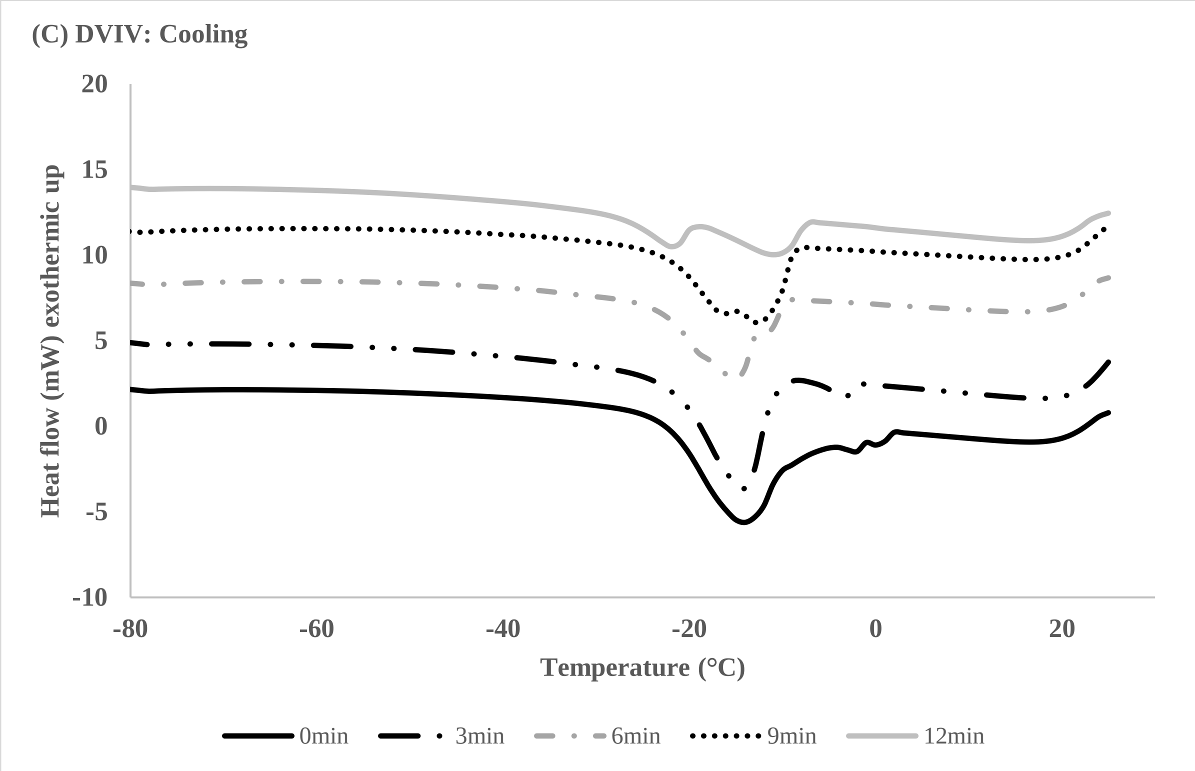
Thermograms were used to analyse critical cryopreservation parameters relating to onset/end temperatures for ice nucleation and melting events as well as enthalpies of crystallization and melting. PVS2 exposure time and method did not have a significant impact the onset temperature for melting transitions ($p > 0.776$) with temperatures ranging from -2.3°C to 4.6°C (Table 6.2).

Onset of crystallization was reduced from -0.9°C in control EA to -14.9 in EA treated for 90 min using DV (Table 6.2; Fig. 6.7A). The enthalpies of the melt were significantly reduced in EAs incubated in PVS2 for 6 and 12 min using DVIV and in all DV treatments (30, 60, 90 and 120 min) (Table 6.2). The smallest enthalpies were associated with 6 min DVIV (35.8 ± 37.5 mJ), 60 min DV (35.5 ± 46.9 mJ) and 90 min DV (32.0 ± 14.9 mJ) compared to 379.8 mJ in untreated EAs (Fig. 6.7). Substantial ice crystallization and melting transitions were observed in DSC scans of untreated EAs (Fig. 6.7A & C). Exothermic or crystallization events were detected during the cooling phase between -5°C and -30°C (Fig. 6.7A & C), while endothermic or melting events were observed in the warming phase between -6°C and 21°C (Fig. 6.7B & D). Large exothermic transitions preceded the freezing event in EAs treated for 3 min (VIV) (Fig. 6.7C) with smaller transitions also preceding freezing in EAs treated for 12 min (VIV) and 60 min (CV) (Fig. 6.7A & C). None of the incubation times or methods eliminated exothermic peaks indicating recrystallization events were still present at the cooling/warming rates tested.

Table 6.2: Cooling and warming thermodynamic properties of *Syzyium maire* embryonic axes (EAs) following PVS2 treatment for 3, 6, 9, and 12 min (DVIV) and 30, 60, 90 and 120 min (DV). Superscript of different letters within a column indicates significantly difference at p < 0.05 based on a one-way ANOVA analysis (Tukey HSD test). Data are presented as mean ± SD (P < 0.05) for three different treatments.

PVS2 Exposure time (min)	Embryo Weight (mg)	Cooling				Warming			
		Onset Temperature of Crystallization (°C)	End Temperature of Crystallization (°C)	Area of crystallization (mJ)	Enthalpy of Crystallization (J/g)	Onset Temperature of Melt (°C)	End Temperature of Melt (°C)	Area of Melt (mJ)	Enthalpy of Melt (J/g)
0	4.2 ± 1.0	-0.9 ± 2.4[a]	-21.1 ± 1.6[a]	-386.4 ± 132.5[a]	-89.7 ± 10.7[a]	1.7 ± 4.9[a]	19.3 ± 1.7[a]	362.3 ± 110.9[a]	84.6 ±6.3[a]
3	4.7 ± 1.2	-7.5 ± 4.6[ab]	-20.8 ± 1.6[a]	-305.3 ± 100.3[a]	-64.8 ± 9.4[ab]	4.6 ± 6.9[a]	17.6 ± 0.6[ab]	165.4 ± 16.6[ab]	39.1 ± 10.2[ab]
6	4.3 ± 0.3	-12.5 ± 2.1[b]	-20.6 ± 2.6[a]	-222.4 ± 61.3[a]	-51.0 ± 10.1[ab]	-1.7 ± 3.3[a]	16.6 ± 0.4[ab]	79.6 ± 39.1[ab]	18.4 ± 9.4[b]
9	4.2 ± 0.5	-11.2 ± 3.4[b]	-21.7 ± 3.1[a]	-251.9 ± 87.3[a]	-59.5 ± 14.5[ab]	-0.3 ± 0.8[a]	16.9 ± 0.5[ab]	160.0 ± 8.4[ab]	37.4 ± 17.4[ab]
12	4.2 ± 0.2	-8.8 ± 1.3[ab]	-23.9 ± 3.9[a]	-185.1 ± 109.4[a]	-43.1 ± 23.5[b]	1.9 ± 7.8[a]	18.3 ± 1.6[ab]	149.7 ± 78.3[ab]	35.0 ± 17.0[b]
30	4.9 ± 0.7	-13.9 ± 3.7[b]	-25.5 ± 2.2[a]	-158.0 ± 78.1[ab]	-32.9 ± 17.7[b]	-2.3 ± 2.3[a]	17.1 ± 0.2[ab]	147.4 ± 102.8[ab]	31.0 ± 23.7[b]
60	3.9 ± 0.2	-11.9 ± 4.2[b]	-29.8 ± 7.3[a]	-80.8 ± 85.4[b]	-20.7 ± 22.0[b]	1.1 ± 9.6[a]	16.2 ± 1.0[b]	43.7 ± 44.5[b]	11.2 ± 11.5[b]
90	4.3 ± 0.5	-14.9 ± 1.3[b]	-25.4 ± 6.2[a]	-73.0 ± 51.6[b]	-16.6 ± 11.3[b]	2.8 ± 8.6[a]	16.5 ± 0.9[b]	41.7 ± 10.7[b]	9.6 ± 4.5[b]
120	4.4 ± 0.3	-13.8 ± 1.1[b]	-24.6 ± 1.2[a]	-170.1 ± 127.1[ab]	-40.1 ± 30.9[ab]	-0.4 ± 4.6[a]	16.6 ± 0.2[ab]	132.3 ± 118.3[b]	31.4 ± 28.8[b]


(A) DV: Cooling
Heat flow (mW) exothermic up
25
20
15
10
5
0
-5
-10
-100
-80
-60
-40
-20
0
20
Temperature (°C)
0min
30min
60min
90min
120min
(B) DV: Warming
Heat flow (mW) endothermic up
15
10
5
0
-5
-10
-80
-60
-40
-20
0
20
Temperature (°C)
0min
30min
60min
90min
120min



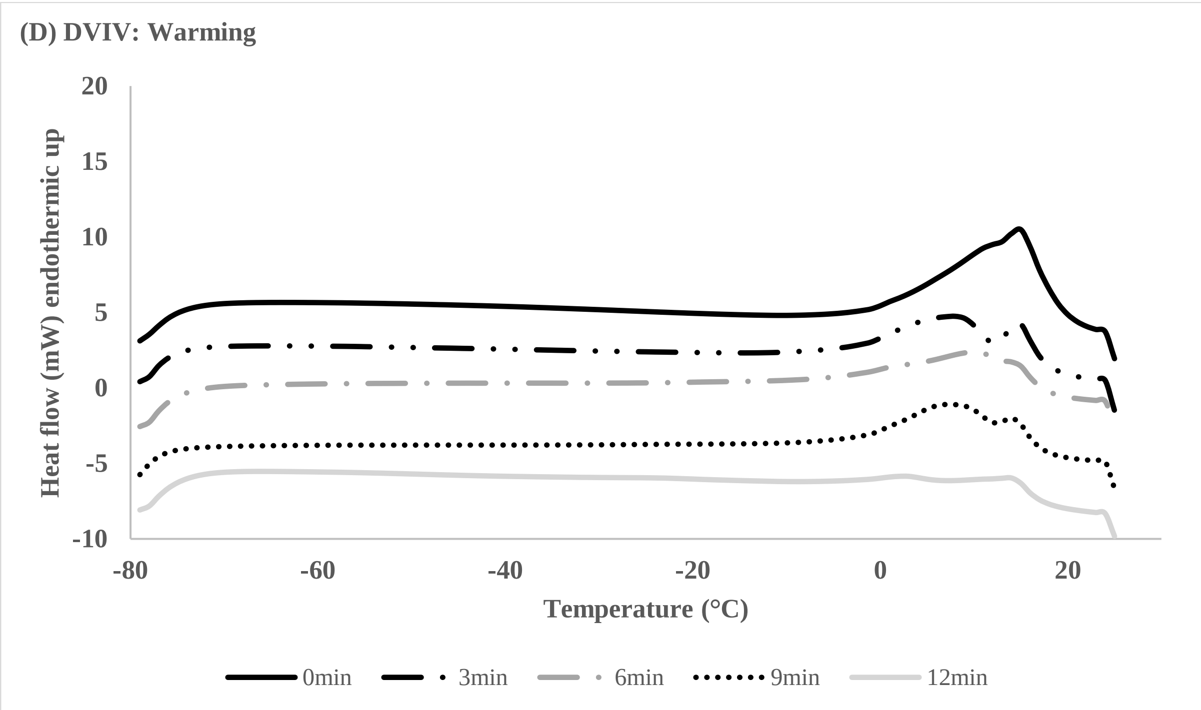


Figure 6.7: DSC thermograms for *Syzygium maire* showing first order transitions that occur during droplet vitrification (DV) cooling (A) and warming (B) and droplet vacuum infiltration vitrification (DVIV) cooling (C) and warming (D). A single embryonic axis (EA) was treated for various exposure times.

6.4 Discussion

There is currently no published protocols for the cryopreservation of zygotic embryos of *Syzygium maire*. This study investigated the use of cryoprotectants for the cryopreservation of the embryonic axis (EAs) of *S. maire*. A common problem following embryo excision is the lack of, or poor capacity for, shoot formation due to excision injury caused to the embryonic apical meristem (Sershen *et al.*, 2008; Whitaker *et al.*, 2010). Embryo excision of *S. maire* was optimized by preventing injury to the radicle tip, which often protrudes through the seed coat, and by leaving small portions of the cotyledon attached to the EA. For species such as *Amaryllis belladonna* (Amaryllidaceae) (Shatnawi *et al.*, 2004), *Ekebergia capensis* (Meliaceae) (Peran *et al.*, 2006) and *Trichilia dregeana* (Meliaceae) (Whitaker et al., 2010), normal shoot production in non-cryopreserved embryos was only possible if small blocks of cotyledon tissues were left. However, leaving the cotyledon blocks might not be beneficial for cryopreservation as the increase in mass negatively affects thermal properties (Reed, 2008; Whitaker *et al.*, 2010).

In this study, encapsulated EAs suffered significant viability loss after only 1 h of desiccation despite MC of c. 1.4 g/g, which was not lethal for non-encapsulated EAs. This indicates that the conditions provided by encapsulating EAs were not optimal for embryo survival. Reduced germination of encapsulated, but not cryopreserved, somatic and zygotic embryos has been reported for *Ekebergia capensis* (Meliaceae) (Peran *et al.*, 2006), *Havea brasiliensis* (Euphorbiaceae) (Nakkanong *et al.*, 2018), *Santalum album* (Bapat & Rao, 1992), and two *Picea* (Pinaceae) species (Lulsdorf *et al.*, 1993). Microscopy studies of *Ekebergia capensis* embryos showed extensive shoot cell damage, while root cells appeared normal following encapsulation-dehydration, accounting for the lack of shoot development in germination experiments (Wesley-Smith *et al.*, 2015). These previous studies support the findings of this study in which *S. maire* encapsulated EAs showed initial survival, but not shoot formation, following cryopreservation. Although encapsulation of plant material might affect survival, it is also known that alginate beads protect tissue from damage during dehydration and cooling (Engelmann, 2004).

Despite the risk of phytotoxicity, the application of cryoprotective agents such as PVS2 is often vital for the successful development of cryopreservation techniques (Funnekotter *et al.*, 2015). The survival and regeneration capacity of plant material exposed to concentrated vitrification solutions will depend not only on the type of material used but also the composition of the vitrification solution, exposure time and temperature, and subsequent freezing (Xia *et al.*, 2014). DV has been found to increase explant survival through a) increased thermal conductivity because of the small amounts of cryoprotective agents used and the presence of aluminum foil; and b) decreased damage to samples from handling (Le *et al.*, 2019). Despite the advantages associated with DV, effective vitrification is still dependent on extended incubation times in PVS2. The application of a vacuum to the plant tissue is postulated to eliminate the pressure differential of the air spaces found in live plant tissue, and a 10-fold reduction in PVS2 exposure time was reported for *Carica papaya* (Caricaceae), *Passiflora edulis* (Passifloraceae) and *Laurus nobilis* (Lauraceae) embryos (Nadarajan & Pritchard, 2014). The novel DVIV method described in this study combines increased thermal conductivity associated with DV with significantly reduced exposure times to PVS2, through application of PVS2 under a vacuum. The current study showed that higher survival and plantlet formation was associated with DVIV irrespective of the temperature or exposure time. Applying PVS2 through DV not only resulted in longer exposure times, decreased EA survival and plantlet formation, but overall regrowth was slower, especially for the 0°C treatments (data not shown). The delayed onset of germination and lower survival percentages of the 0°C DV treatments in this study could also be a result of chilling stress (Fuller *et al.*, 2004), which was greater for the longer treatment times associated with DV. Incubation of plant materials in vitrification solutions at lower temperatures is often applied to reduce phytotoxicity, but this could also result in longer incubation times (de Freitas *et al.*, 2016). However, regrowth for tropical and subtropical plant species is negatively affected when PVS2 is applied at temperatures below 10°C (Reed, 2008). In the present study, except for 6 min PVS2 exposure at 0°C, all EAs showing radicle emergence (survival) after DVIV developed into normal plantlets, while significantly fewer plantlets developed following DV ($P = 0.023$; Fig. 6.3 & 6.4). The preculture step on sucrose enriched medium is known to be essential to mitigate the injurious effects of vitrification on the embryos of plant species such as coconut (Corredoira *et al.*, 2004; Sakai, 2004), but it has also been found to be ineffective for *Manilkara zapota* (Sapotaceae) and *Nephelium ramboutan-ake* (Sapindaceae) (Wesley-Smith *et al.*, 2015). Pilot studies conducted on preculture of *S. maire* EAs found reduced viability in EAs incubated on high sucrose MS medium at 25°C (data not shown).

Calorimetric measurements were assessed in *S. maire* EAs exposed to PVS2 solution using two methods (DV and DVIV). The aim was to determine if PVS2 reduced intracellular ice formation and secondly, to compare the effectiveness of DVIV and DV to reduce ice nucleation. Substantial freezing and melting transitions were observed in the scans of untreated EAs. PVS2 significantly reduced the size of the melting transitions expect for the shortest incubation time of 3 min DVIV. Although endothermic peaks were still evident in all the PVS2 incubation times tested in this study, the peaks were significantly smaller after 6 min DVIV, 60 min DV and 90 min DV. Regrowth of EAs after PVS2 exposure was significantly higher for all DVIV treatments, with c. 80% of EAs developing into normal plantlets following 9- and 12 min DVIV treatments. The DSC thermograms together with EA survival and plantlet formation indicated that longer PVS2 incubation through DVIV could potentially remove all freezable water from EAs.

Seeds from recalcitrant species are metabolically active with no arrest between embryo development and germination making them intolerant of desiccation (Baskin & Baskin, 2014). Cryopreservation provides a means of long-term storage for germplasm of recalcitrant species, although there are significant differences in survival potential between species (Berjak & Pammenter, 2008). The low survival following cryopreservation of *S. maire* EAs recorded during this study is not unique. Lack of success in cryopreserving EAs in woody species has been widely reported (Kistnasamy *et al.*, 2011; Normah *et al.*, 1986). Non-cryopreserved EAs of *Lansium domesticum* (Meliaceae) exposed to PVS2 for 20 min showed no reduction in viability but failed to germinate after exposure to LN (Noor *et al.*, 2010). In most cases, cryoprotection alone does not provide sufficient protection to survive cryopreservation in zygotic embryos (Naidoo, 2010). However, when cryoprotection is combined with other treatments such as partial dehydration, survival was increased after cryopreservation (Gaidamashvili *et al.*, 2019; Hajari *et al.*, 2011; Pence, 1991). This could be due to the reduced toxicity of additives while at the same time limiting the impacts of extreme evaporative drying (Benson, 2008a). Unsuccessful attempts at cryopreservation of embryos from recalcitrant species might be linked to the absence of clearly defined categories for critical moisture content and threshold moisture (Hor *et al.*, 2005) and successful cryopreservation will depend on optimizing the parameters required for each species and plant material (Fuller *et al.*, 2004).

6.5 Conclusion

In summary, this study investigated the feasibility of using encapsulation-dehydration and cryoprotectants for *S. maire* EA cryopreservation, including the novel approach of DVIV. The novel DVIV method increased EA survival and plantlet formation compared with

DV; however, neither of the vitrification method resulted in embryo survival following cryopreservation. DSC thermograms indicated that although endothermic peaks were significantly reduced after incubation of EAs in PVS2, none of the times tested in this study eliminated ice nucleation completely. Information on desiccation sensitivity, and toxic effects of exposure to PVS2 using DV and DVIV can be used to investigate additional steps to optimize EA survival after cryopreservation.

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Chapter 7.

General discussion and conclusion

7.1 General discussion

This study investigated the *ex situ* conservation options for five nationally critical Myrtaceae species in New Zealand. The three methods of *ex situ* conservation, living collections (for *Metrosideros bartlettii*), conventional seed banking (for *Lophomyrtus bullata*, *L. obcordata* and *Neomyrtus pedunculata*) and cryopreservation (for *Syzygium maire*) were investigated. The study presented novel information on the challenges for the conservation of Myrtaceae species, both in New Zealand and globally. It describes essential fruit and seed morphology for fleshy Myrtaceae species and provides detailed analysis of the challenges of seed banking these species. Four scientific publications, conference proceedings, book chapters, case studies and community posters published to date further highlights the contribution this study has made to *ex situ* plant conservation.

7.1.1 Living collections for the ex situ conservation of Metrosideros bartlettii

Living collections in arboretums, public and private gardens can be invaluable to the integrated conservation of threatened species (Asmussen-Lange *et al.*, 2011). In this study, six *M. bartlettii* trees in cultivation were used to study the breeding system, describe pollination methods and to establish pollen banking methods (chapter 2).

The study found that *M. bartlettii* was highly self-incompatible and required pollen from an unrelated individual to produce viable seed (Table 2.3). This is consistent with other studies which found similar breeding strategies in Myrtaceae species including *Metrosideros excelsa* (Schmidt-Adam *et al.*, 1999). The ability of *M. bartlettii* to hybridise with *M. excelsa* was a significant finding. Although the seedlings from hybridised treatments did not survive for longer than four weeks in this study, it is important to note that hybridisation treatments were limited to a single tree. It is recommended that future studies using larger samples, including other co-flowering species such as *M. robusta* are conducted.

Pollen conservation is becoming increasingly important in the conservation of highly threatened plant species, especially where males are geographically isolated from females or flowering is not synchronized (DeMauro, 1993; Towill, 2004). No information has been published on the biology, morphology, or conservation of *M. bartlettii* pollen. This study described the pollen germination protocol for *M. bartlettii* which can be used to assess pollen viability. Pollen viability of *M. bartlettii* differed between individual trees and between years on the same trees. Desiccating pollen did not have a significant impact on pollen viability, but desiccation was important when pollen was stored at -18°C and -196°C. Pollen stored at room temperature rapidly lost viability irrespective of the desiccation treatment.

The results from the study conducted on *M. bartlettii* are novel and will guide the management of *ex situ* collections of *M. bartlettii* to obtain viable seed which can be used for seed banking or restoration. Cross-pollination can also be used to increase reproduction in the wild, especially for isolated individuals. Long-term storage of pollen provides a tool to overcome challenges in cross-pollination of trees that are spatially and geographically isolated.

7.1.2 Seed storage physiology of Lophomyrtus and Neomyrtus

Seed banking, which involves storage at low moisture contents and -18°C, is often considered the most efficient form of *ex situ* conservation (Walters, 2004). However, conventional seed banking can only be used in species that tolerate the desiccation process (Sommerville *et al.*, 2020). Recent studies suggest that the number of angiosperms for which conventional seed banking is suitable might be less than previously predicted (Merritt *et al.*, 2014). Prior to this work, there has been no published information on the seed storage physiology of *Lophomyrtus bullata*, *L. obcordata* and *Neomyrtus pedunculata* (Sommerville *et al.*, 2017).

Fresh seed from both *Lophomyrtus* species, as well as seed collected from the natural hybrid between *L. bullata x L. obcordata*, had initial viability of > 80% with no dormancy associated with seeds collected at the optimum time. *Lophomyrtus bullata* and *N. pedunculata* were found to be sensitive to desiccation (~15% eRH) and freezing. *L. obcordata* seed was however not desiccation sensitive but two of the three seed lots tested in this study lost significant viability during sub-zero temperature storage. It is not uncommon for desiccation tolerant seed to display sensitivity to storage at sub-zero temperatures. For instance, more than 20% desiccation tolerant seed collected from rainforests in Australia, displayed sensitivity towards freezing (Sommerville *et al.*, 2021). The state of seed lipids at storage temperatures can be a critical factor in seed storage physiology (Crane *et al.*, 2003; Hamilton *et al.*, 2013). Lipid thermal fingerprints of *L. bullata, L. obcordata* and *N. pedunculata* indicate that the lipids are in a metastable state at conventional storage temperature i.e. around -18°C and seed will continue deteriorate when stored at this temperature.

Lophomyrtus species are highly susceptible to myrtle rust with local extinctions and reproduction failure in *L. bullata* (Sutherland *et al.*, 2020). With myrtle rust now established in all the climatically suitable areas in New Zealand (Toome-Heller *et al*., 2020), long-term seed storage is critical to prevent the extinction of these species (Sommerville *et al.*, 2020). The results from this chapter provide novel and crucial information on optimal fruit collection time, seed germination and seed storage physiology of *L. bullata* and *L. obcordata*. Both *Lophomyrtus* species, together with *N. pedunculata,* were found to be of intermediate storage physiology and conventional seed banking was not suitable for the collections tested. Since *L. bullata* and *N. pedunculata* were also desiccation sensitive, cryopreservation protocols using cryoprotectants should be investigated. It is further recommended that all three species are stored outside of their lipid transition temperatures and cooling rates are optimised for each species.

7.1.3 Phenology, fruit morphology and seed characteristics of Syzygium maire

Conservation efforts of the world's most threatened plant species are hindered by gaps in fundamental biological information (Cavender *et al.*, 2015) and their seed biology (Pritchard *et al.*, 2014). The seed biology of *S. maire* remains largely unstudied, hampering its effective conservation. In chapter 4, the seed developmental stages were identified for *S. maire* from differentgeographical areas. Seed germination protocols were developed, and seed desiccation tolerance investigated.

Seed development stage can influence desiccation sensitivity and it is therefore important that the optimal development time is identified (Daws *et al.*, 2006; Nadarajan *et al.*, 2006). This study identified 4 development stages of *S. maire* fruit: Stage 1 had no tissue specialization; stage 2 was characterised by swelling of the fruit as the cotyledons and embryo start to develop. Pulp started to expand in stage 3 and there is a slight change in pulp colour from green to light pink before the fruit becomes bright red berries at stage 4. Results from this study showed that *S. maire* seed was viable from stage 2 onwards and although there was no significant difference in final germination percentage of seed from stage 2, 3 or 4, seedlings from stage 4 developed faster and were more robust. The removal of fruit pulp was essential for seed germination in *S. maire*. This finding was supported by studies on fleshy Myrtaceae which found germination is dependent on the removal of the pericarp by weathering or ingestion by animals (Beardsell *et al.*, 1993).

Seeds of *Syzygium* species studied to date have been found to be recalcitrant (intolerant to drying) and to suffer from chilling injury when stored at temperatures below 10°C (Abbas *et al.*, 2003; Berjak & Pammenter, 2008; Nagendra *et al.*, 2019; Shü *et al.*, 2011). This study found that *S. maire* seeds can be stored for 6–8 weeks in a fully hydrated state at 5°C with limited viability loss (Table 4.4). However, after 12 weeks, in storage more than 50% of the seeds in storage had germinated.

In summary, chapter 4 defined the developmental stages of *S. maire* fruit while the seed germination and short-term storage evaluation of the seeds provided baseline information for chapter 5 which focussed on the desiccation sensitivity of *S. maire* seed and zygotic embryonic axis.

7.1.4 Desiccation sensitivity of Syzygium maire seeds and zygotic embryos

Cryopreservation provides a viable strategy for desiccation sensitive species, but its application is challenged by recalcitrant seeds generally being large, shed at high moisture contents and are metabolically active (Berjak *et al.*, 2008). Chapter 5 investigated cryopreservation of zygotic embryos as a long-term conservation strategy for *S. maire*.

In order for cryopreservation to be successful, it is essential that most of the freezable water from the plant tissue is removed to prevent the formation of lethal ice-crystals (Walters *et al.*, 2008). Physical removal of water through rapid desiccation was tested for the cryopreservation of *S. maire* embryonic axis (EAs). Untreated embryos had high moisture content (~1.93 g/g) and were sensitive to desiccation, but rapid desiccation resulted in survival to lower moisture contents compared to slow desiccation (Table 5.1). Embryonic axes did not survive exposure to liquid nitrogen irrespective of the MCs or desiccation methods used.

The effects of partial dehydration on oxidative stress in recalcitrant axes and zygotic embryos has been identified as a main source of cryoinjury in recalcitrant species (Benson *et al.*, 1992; Sershen *et al.*, 2012; Walters *et al.*, 2008). Desiccation of *S. maire* EAs to

MCs below 0.3 g/g resulted in a ten-fold decrease in all three enzymatic antioxidants (SOD, CAT and GPOX) with a five-fold increase in PC and LPOx (Table 5.2). No enzymatic activity was detected at MC < 0.25 g/g and this corresponded with a total loss of viability.

Lowering MC and exposure to liquid nitrogen in recalcitrant cells not only causes oxidative stress but the physical structure of cells can also be compromised during water removal (Pammenter *et al.*, 1998). Differential Scanning Calorimetry (DSC) thermograms indicated that enthalpies of melt were significantly reduced in *S. maire* EAs which were desiccated for 120 min or longer to reach MCs of less than 0.44 g/g (Table 5.3; Fig. 5.6). However, ice nucleation was only inhibited after 210 min of rapid desiccation (~0.21 g/g) which was fatal to *S. maire* EAs.

A significant challenge for the cryopreservation of *S. maire* EAs is the variety in embryo anatomy which influenced desiccation intensity of individual embryos. This has also been highlighted in other studies which implicates embryo anatomy and tissue topography in low seedling recovery following desiccation and cryopreservation (Sershen *et al.*, 2008; Wesley-Smith *et al.*, 2001).

Chapter 5 investigated the reasons for desiccation sensitivity in *S. maire* EAs and whether the formation of intracellular ice causes freezing damage in EAs desiccated to various MC levels. The decline in enzymatic antioxidant activities and the increase in lipid peroxidation suggests that oxidative stress, rather than structural damage, causes viability loss in EAs desiccated to MCs < 0.3 g/g. However, DSC thermograms confirmed the presence of small amounts of freezable water after rapid desiccation to MCs of less than 0.3 g/g which corresponded to EA survival of ~50%. Since additional desiccation was fatal to embryos, the use of vitrification solutions such as Plant Vitrification Solution 2 (PVS2) and encapsulation were investigated in chapter 6.

7.1.5 Cryopreservation of Syzygium maire zygotic embryos through droplet-vitrification, vacuum-infiltration vitrification, and encapsulation-dehydration

Physical desiccation or the application of chemical cryoprotectants have been used successfully to achieve vitrification in plant cells (Benson, 2008; Sakai, 2004). A key aspect in the use of cryoprotectants is balancing the protection effect provided with chemical toxicity (Fuller *et al.*, 2004). Chapter 6 evaluated the effectiveness of droplet-vitrification (DV), the novel combination of droplet-vitrification and vacuum infiltration vitrification (DVIV), and encapsulation-dehydration for the cryopreservation of zygotic embryos of *S. maire*.

Moisture loss from encapsulated EAs was slower compared to non-encapsulated EAs (Table 6.1) but was accompanied with lower survival. A reduction in germination of encapsulated embryos has been reported in other woody species (Nakkanong & Nualsri, 2018; Peran *et al.*, 2006). This reduced germination is likely due to unsuitable conditions provided by the encapsulation. Following cryopreservation, encapsulated EAs desiccated to c. 31% and 37% MC showed initial radicle emergence but failed to complete the germination process and form plantlets (Fig. 6.4).

Increased exposure times to PVS2 resulted in a drop in germination and negatively impacted the vigour of plantlets. The reduction of PVS2 exposure time using vacuum infiltration vitrification (DVIV) significantly increased embryo germination and plantlet formation (Fig. 6.5 & 6.6). Exposure to LN was fatal to all EAs treated with PVS2 irrespective of the application method or PVS2 exposure time.

DSC thermograms indicated that incubation in PVS2 significantly reduced melt enthalpies with the smallest enthalpies associated with 6 min DVIV, 60 min DV and 90 min DV (Table 6.2). None of the incubation times or methods completely eliminated exothermic peaks, indicating recrystallization events were still present at the cooling and warming rates tested (Fig. 6.7). These small endothermic events can however also be due to the devitrification of PVS2 during the warming phase (Benson, 2008).

In summary, this study investigated the feasibility of using cryoprotectants for *S. maire* EA cryopreservation, including the novel approach of DVIV. The novel DVIV method increased EA survival and plantlet formation compared with DV; however, neither of the vitrification methods resulted in embryo survival following cryopreservation. Thermograms indicated that although endothermic peaks were significantly reduced by incubation of EAs in PVS2, none of the times tested in this study eliminated ice nucleation. Information on desiccation sensitivity, and toxic effects of exposure to PVS2 using DV and DVIV can be used to investigate additional steps to optimize EA survival after cryopreservation.

7.2 Conclusions

This study investigated *ex situ* conservation strategies for five critically endangered Myrtaceae species in New Zealand. The species studied included four fleshy-fruited species (*L. bullata, L. obcordata, N. pedunculata* and *S. maire*) as well as *M. bartlettii,* a self-incompatible species reduced to five genotypes.

The population of *Metrosideros bartlettii* is limited to five genotypes represented by 14 individuals in the wild (de Lange, 2014). Without urgent conservation actions, extinction of this species is highly likely (Drummond *et al*., 2000). The study has shown that *M. bartlettii* favours cross-pollination and individuals who are geographically isolated will not be able to reproduce effectively (Chapter 2). Pollen handling and storage protocols described in this study makes it possible to overcome reproduction barriers due to flowering times and distance between trees. A significant finding was that *M. bartlettii* can hybridise with co-flowering *M. excelsa*, and this should be considered when seed is sourced from cultivated trees. Recommendations for the conservation of *M. bartlettii* include the establishment of a pollination network to increase reproductive output in both cultivated and wild species, securing germplasm of remaining genotypes and seed banking.

The investigation into conservation options for the four fleshy-fruited Myrtaceae revealed that seed of *L. bullata, L. obcordata* and *N. pedunculata* should be classified as intermediate storage physiology (Chapter 3). *L. bullata* and *N. pedunculata* did not tolerate desiccation down to low MCs required for conventional seed banking (~15% eRH) and cryopreservation protocols should be investigated as a matter of urgency. Although *L. obcordata* seed was found to be desiccation tolerant, together with *L. bullata* and *N. pedunculata,* it was sensitive to storage at sub-zero temperatures (-18°C and -196°C). Lipid thermal fingerprints revealed that all three species have lipids that are in a metastable state between c. -18°C and -23°C and lipid crystallization will likely cause continuous decline in seed viability. The seed of all three species should be stored outside of the lipid transition temperatures while cooling rates for cryopreserved seed should be optimised.

Whole seeds and excised embryos of *Syzygium maire*, was found to be highly recalcitrant (Chapter 4). Although rapid desiccation of embryonic axis (EAs) enabled survival to lower moisture contents, exposure to liquid nitrogen was fatal irrespective of the MC level or method used (Chapter 5). DSC analysis revealed that ice crystallization in *S. maire* EAs is eliminated at MC < 0.2 g/g but desiccation to this level was lethal to both EAs and enzymatic antioxidants. It is postulated that although faster cooling and warming rates might outrun ice nucleation when desiccated embryos are exposed to cryogenic temperatures, the decline in enzymatic antioxidant activities and increase in lipid peroxidation suggests that EA viability loss is due to oxidative stress. It is recommended that the cryopreservation protocol for *S. maire* embryos is further investigated through the optimisation of exogenously applied antioxidants or the use of cryoprotectants (Chapter 6).

Despite the risk of phytotoxicity, the application of PVS2 is essential for the successful cryopreservation of many plant species (Funnekotter *et al.*, 2015). The novel approach in this study which used droplet vacuum infiltration vitrification (DVIV) increased EA survival and plantlet formation compared to droplet vitrification (DV) in unfrozen embryos. However, neither method resulted in embryo survival following cryopreservation (Chapter 6). Thermograms indicated that PVS2 significantly reduced endothermic peaks, but none of the incubation times tested completely eliminated ice nucleation. It is recommended that extended PVS2 incubation times and a combination of DVID-rapid dehydration are investigated. The encapsulation-dehydration method resulted in c. 20% survival, but no plantlets were formed. Interestingly, prior to exposure to cryogenic temperatures, the survival of desiccated encapsulated embryos was overall lower than non-encapsulated embryos. This could indicate sub-optimal alginate beads and further studies into the optimisation of the beads is recommended.

Emerging pathogens such as myrtle rust are of great concern especially in New Zealand and the wider Pacific because of the detrimental impact on taonga species (treasured entities) (Marsh *et al.,* 2017). Seed banking, as a response against myrtle rust, is largely accepted as one of the best strategic insurances and Māori seed banks were initiated in 2018 (Black *et al.* 2019). With Indigenous communities in New Zealand the second biggest owners and managers of conservation land, a future based Māori seed conservation strategy is a critical element, especially for species threatened by pathogens and climate change. However, this study highlighted the challenges of conserving non-orthodox seeded species in conventional seed banks. This emphasizes the importance of incorporating western science and mātauranga Māori (traditional Māori knowledge) in an integrated conservation strategy for all threatened species.

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Appendices

Appendix 1: Published paper for Chapter 2

Pacific Conservation Biology

Cross-pollination and pollen storage to assist conservation of *Metrosideros bartlettii* (Myrtaceae), a critically endangered tree from Aotearoa New Zealand

Journal:	*Pacific Conservation Biology*
Manuscript ID	Draft
Manuscript Type:	Full Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	van der Walt, Karin; Otari Native Botanic Garden; Massey University, School of Agriculture and Environment Alderton-Moss, Jennifer; Victoria University of Wellington Lehnebach, Carlos; Museum of New Zealand Te Papa Tongarewa
Keyword:	breeding, extinction, hybridisation, threatened species

Appendix 2: Statement of contribution to doctoral thesis containing publication for Chapter 2 (form DRC 16)

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Name/title of Primary Supervisor:	Dr Svetla Sofkova-Bobcheva
In which chapter is the manuscript /published work:	Chapter 2

Please select one of the following three options:

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Pacific Conservation Biology

- The percentage of the manuscript/published work that was contributed by the candidate: 80.00

- Describe the contribution that the candidate has made to the manuscript/published work:
KvdW designed and performed the experiments, analysed the data and wrote the manuscript in consultation for Dr. Carlos Lehnebach.

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Candidate's Signature:	karin.vanderwalt
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Appendix 3: Authorship and copyright information for publication in Pacific Conservation Biology

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Pacific Conservation Biology **Manuscript No.** PC21054

Title of the paper (the 'Work') Cross-pollination and pollen storage to assist conservation of <i>Metrosideros bartlettii </i>(Myrtaceae), a critically endangered tree from Aotearoa New Zealand

Author(s) van der Walt, Karin; Alderton-Moss, Jennifer; Lehnebach, Carlos

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Appendix 4: Published paper for Chapter 4 (New Zealand Journal of Botany)

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Seed development, germination, and storage behaviour of *Syzygium maire* (Myrtaceae), a threatened endemic New Zealand tree

Karin Van der Walt, Peter Kemp, Svetla Sofkova Bobcheva, David J. Burritt & Jayanthi Nadarajan

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ABSTRACT

Syzygium maire is an endemic tree in New Zealand and classified as critically endangered. The *in situ* populations of this species are further threatened by the incursion of myrtle rust. The biology and ecology of *S. maire* have not been well documented and limited information on seed development, effective germination and seed storage behaviour of *S. maire* are hampering *ex situ* conservation and restoration. In this study, we investigated fruit and seed development on *S. maire* populations growing at four sites and in different seasons, and used short-term seed storage protocols to investigate the storage potential of harvested seeds. Fruit development took 9–11 months with three of the four identified stages occurring during the warmer spring and summer months. Embryos were found to be viable from stage two, but seedlings from final stage embryos were more robust. The moisture content of the fruit pulp varied between populations and within populations between seasons, but embryo moisture content remained more

Appendix 5: Statement of contribution to doctoral thesis containing publication for Chapter 4 (form DRC 16)

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Name of candidate:	Karin van der Walt
Name/title of Primary Supervisor:	Dr Svetla Sofkova-Bobcheva
In which chapter is the manuscript /published work:	Chapter 4

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Karin van der Walt, Peter Kemp, Svetla Sofkova-Bobcheva, David J. Burritt & Jayanthi Nadarajan Ka (2020). Seed development, germination, and storage behaviour of Syzygium maire (Myrtaceae), a threatened endemic New Zealand tree. New Zealand Journal of Botany, DOI: 10.1080/0028825X.2020.1794911

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- Describe the contribution that the candidate has made to the manuscript/published work:

KvdW did the experimental design, data analysis and compiled the ms for input from co-authors

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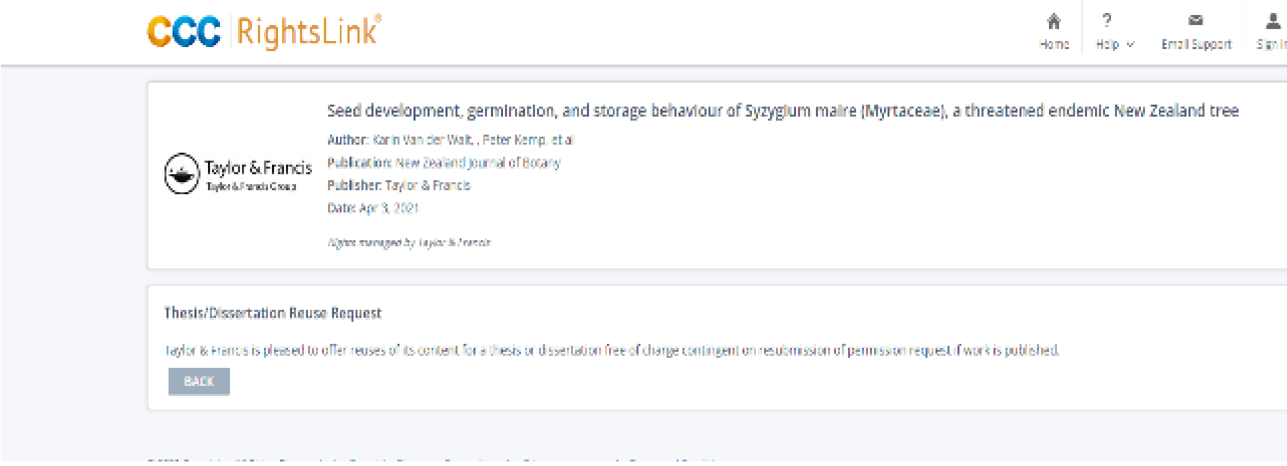
Appendix 6: Authorship and copyright information for publication in New Zealand Journal of Botany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Appendix 7: Published paper for Chapter 6 (CryoLetters)

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Evaluation of Droplet-vitrification, Vacuum Infiltration Vitrification and Encapsulation-Dehydration for the Cryopreservation of *Syzygium Maire* Zygotic Embryos

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BACKGROUND: *Syzygium maire* is a threatened tree species with limited information on long-term storage options for its recalcitrant seed. **OBJECTIVE:** To evaluate the cryopreservation of *S. maire* zygotic embryo axes (EA) using dehydration, encapsulation-dehydration as well as PVS2 vitrification using droplet vitrification (DV) and the novel droplet vacuum infiltration vitrification (DVIV) methods. **MATERIALS AND METHODS:** Excised naked and sodium alginate encapsulated EA were desiccated to various moisture contents (MC) using a laminar flow cabinet. Moisture content, embryo survival and plantlet formation, before and after cryopreservation, were assessed at 1 h intervals during the desiccation period (0 – 6 h). The influence of PVS2, using DV and DVIV, was assessed for various desiccation times and temperatures. **RESULTS:** Encapsulated EA desiccated to 31% and 37% MC survived but no plantlets formed following cryopreservation. Exposure to PVS2 using the DV method had a negative impact on embryo survival and plantlet formation, while DVIV resulted in improved results for non-cryopreserved EA. However, neither PVS2 vitrification method resulted in embryo survival following cryopreservation. **CONCLUSION:** *S. maire* embryos are sensitive to desiccation and likely require physical, chemical or a combination of protection methods to increase embryo survival and plantlet formation following cryopreservation

Keywords: MYRTACEAE; NEW ZEALAND NATIVE SPECIES; PVS2; RECALCITRANT SEEDS; VITRIFICATION
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Sent: Monday, 1 November 2021 9:45 pm
To: Karin Van der Walt <Karin.VanderWalt@wcc.govt.nz>
Subject: RE: CryoLetters

Hello Karin

Absolutely no problem with you putting your own data in your thesis. You could just cite the published work in the legend to the Figures or Tables.

I enjoyed hearing Jayanthi present your *Syzygium* work at the Exceptional Species meeting a couple of weeks ago.

Kind regards
Hugh

From: Karin Van der Walt [mailto:Karin.VanderWalt@wcc.govt.nz]
Sent: 01 November 2021 00:44
To: Hugh Pritchard <h.w.pritchard@btinternet.com>
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Kind regards

Karin van der Walt
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