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Topic 3.1 — Seed-banking and germplasm research strategy

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Topic 3.1 — Seed-banking and germplasm research strategy

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Executive summary

The current threat of myrtle rust (*Austropuccinia psidii*) to Myrtaceae species including a number of New Zealand's indigenous and socio-economically important species requires that *ex situ* conservation is used to complement in situ collections. However, New Zealand's Myrtaceae have received little attention in terms of long-term *ex situ* conservation. Seed of some Myrtaceae species are considered orthodox and can be stored relatively easily in conventional seed banks. Nevertheless, some Myrtaceae species exhibit recalcitrant seed storage behaviour, e.g. *Syzygium maire,* and therefore require cryopreservation of isolated embryos. Theme 3 looked at seed banking and germplasm research strategies for selected New Zealand Myrtaceae species. This part particularly investigated storage behaviour of selected Myrtaceae species seeds, establishment of *in vitro* storage protocols for selected species, establishment of pollen cryopreservation protocols and establishment of cryopreservation protocol for selected recalcitrant Myrtaceae species. A desiccation trial was conducted on six species of Myrtaceae namely: *Lophomyrtus bullata, L. obcordata, Metrosideros diffusa, M. umblleta, M. bertletii* and *Syzygium maire*. *S. maire* seeds and embryos showed extreme sensitivity to desiccation, and lost viability completely following desiccation to a moisture content below 20%, confirming its recalcitrant behaviour. The rest of the species seeds are desiccation tolerant, i.e. orthodox. Tissue culture protocols were developed for *Lophomyrtus* 'Red Dragon' – a hybrid of *L. bullata* x *L. obcordata, Metrosideros excelsa*, *M. perforata*, *Leptospermum scoparium* and the highly endangered *Metrosideros bartlettii*. *In vitro* propagated *M. bartlettii* have been transferred to the greenhouse successfully (100% acclimation success) and are ready for repatriation. In addition, photo-autotropic micropropagation techniques were developed for *L. scoparium*. Pollen cryopreservation was tested for *Metrosideros excelsa*. Desiccation of the pollen to about 5% moisture content (achieved with equilibrium with 15% relative humidity) followed by rapid freezing and rapid thawing was found optimum for cryopreservation. As *S. maire* was established as a recalcitrant species, the zygotic embryos were cryopreserved using excised embryo, encapsulation-dehydration and plant vitrification solution 2 (PVS2) vitrification techniques. No survival was recorded following excised embryo and PVS2 vitrification cryopreservation. Cryopreservation using the encapsulation-dehydration technique resulted in embryo survival and root formation (30%), although complete regeneration was not recorded.

This report highlights the importance of integrated conservation strategies, i.e. involving seed storage, *in vitro* conservation, pollen storage and cryopreservation of recalcitrant species for conservation to ensure future access to New Zealand's unique Myrtaceae germplasm as a key component of long-term management response to the threat posed by *Austropuccinia psidii*.

Recommendations

1. Having identified the optimum storage conditions, the germplasm of species at risk need to be conserved immediately and this requires conservation facilities and physical resources, e.g. cryo-tank and humidity-controlled freezers and growth rooms that operate at international standards.
2. This study also identified the importance for a centralised management and curation system for collected germplasm resources. This will include a register of collections held by various groups, and where and how the conserved material is stored to ensure that a standardised operating procedure is adapted.
3. To improve cryopreservation success for *S. maire* by incorporating modified techniques. Further optimisation of zygotic embryo cryopreservation protocol for *S. maire* using biochemical markers and anti-oxidant assays.

4. To incorporate more species for pollen cryopreservation and to assess the long-term effect of cryopreservation on their viability.
5. To establish *in vitro* collections of different provenances and to develop cryopreservation protocols for shoot tips using the growing plantlets of both *M. bartletii* and *S. maire*.

1 Introduction

The current threat of myrtle rust (*Austropuccinia psidii*) to Myrtaceae species including a number of New Zealand's indigenous and socio-economically important species requires that *ex situ* conservation is used to complement *in situ* collections. To mitigate the effects of biotic and abiotic threats, *ex situ* germplasm conservation is widely applied in many species using protocols for conservation of pollen, seed and clonal germplasm, complemented by *in vitro* propagation and cryopreservation (Sakai 2004, Pence 2014). As the myrtle rust threat is advancing at an alarming rate in New Zealand (MPI Myrtle Rust Update April 2019), there is an urgent need to investigate *ex situ* conservation strategies for New Zealand's Myrtaceae species as little information is available on long-term *ex situ* conservation.

Seed is the most preferred plant propagule for *ex situ* germplasm conservation because they are easy to handle, relatively inexpensive to store and have the advantage of regenerating whole plants from genetically diverse materials. Gene banking of seeds is aimed at preservation of genetic variations within and among populations for future use in breeding programmes and for germplasm conservation purposes. The internationally recommended standard for seed banking is –18°C and 15% relative humidity (FAO 2014). However, it is also important to note that not all seeds can be dried and stored at cold temperatures. Seeds are divided into three main categories (orthodox, intermediate and recalcitrant) based on their storage behaviour and their sensitivity to desiccation and temperature (Roberts 1973; Ellis et al. 1993). Orthodox seeds are those that can tolerate drying to very low moisture contents (≤3–7% fresh weight), and their longevity increases as moisture content and temperature are reduced (Roberts 1973). Intermediate seeds tolerate partial desiccation (~10% moisture content) but longevity is reduced at low moisture content in low temperature storage. On the other hand, recalcitrant seeds are very sensitive to desiccation and will lose viability after only the slightest amount of drying. Therefore, conventional seed banking can only be used to store orthodox and intermediate (to some extent) seeds but not recalcitrant seeds. For long-term storage of recalcitrant seeds, cryopreservation, a process by which living tissues are conserved in liquid nitrogen at -196°C, is recommended.

Only a few protocols for *ex situ* conservation of germplasm are available for New Zealand Myrtaceae species. However, no reports are available on cryopreservation of recalcitrant Myrtaceae species. Hence, germplasm conservation technologies developed in other families either as *in vitro* cultures, or as cryo-preserved shoot tips, seeds, embryos and pollen will be adopted in this study. Seed of some Myrtaceae species are considered orthodox and can be stored relatively easily, i.e. in conventional seed-banking environments. However, some members of this family do exhibit recalcitrant seed storage behaviour, e.g. *Syzigium maire* as indicated in our pilot study on `Assessing the cryopreservation potential of recalcitrant Myrtaceae germplasm` (*MPI 18061*) and must be conserved in cryopreservation as isolated embryonic axes. For some species there will be requirements for *in vitro* or tissue culture storage of valued genotypes because of poor seed set or a very low number of known individual genotypes.

The aim of this theme is to develop a research-led strategy to efficiently and cost-effectively conserve maximum allelic diversity with the ultimate future goal of repatriation to the environment. A Myrtaceae Germplasm Collection & Storage Strategy is being developed (DOC) and the distribution of high priority species and individuals is being mapped (MPI RFP 18607). This project will identify or verify optimum *ex situ* conservation strategies for seed storage, *in vitro* propagation (including producing shoot tips for cryogenic storage experiments), and cryopreservation (seeds and/or embryonic axes, and pollen) for selected Myrtaceae species.

2 Research strategies and critical steps

There were four research strategies or critical steps identified under this theme: 1) New Technology Development; 2) Knowledge Exchange; 3) Technology Transfer and 4) Dissemination of Research Outputs. The activities related to each of these research strategies or critical steps are listed below:

1. **New technology development**
    a. Development of seed storage protocols
    b. Development of embryo and shoot tip cryopreservation protocols
    c. Development of *in vitro* culture technologies
    d. Development of pollen storage and viability assessment technologies.
2. **Knowledge exchange**
    a. Consultation with local and central governmental agencies to ensure a coordinated approach is used
    b. Collaborations with other conservation groups
    c. Consultation with iwi
    d. Research collaboration with Wellington City Council, Massey University, Royal Botanic Gardens Kew, UK, Plant Bank Australian Botanical Gardens Networks.
3. **Technology transfer**
    a. PhD student training and teaching (Massey University)
    b. Delivery of training in seed conservation techniques.
4. **Dissemination of research outputs**
    a. Seminars and workshops with participating groups
    b. Conference presentations
    c. Project reports
    d. Scientific paper publications.

3 Experimental procedures and results

3.1 Mānuka seed research

3.1.1 Seed material and experimental work

Seed collection from 64 populations (25–35 collections covering 10 tress per population). Seeds were removed from their capsules, cleaned and stored at 15°C/35% relative humidity while being processed. Seed germination on water agar was conducted on 29 randomly selected populations. The seeds were incubated at alternating temperature 13/25°C with a 12-h photoperiod. For these 29 populations, seed storage was compared at –20°C and in cryopreservation after 1 year of storage. Representative seeds samples were dispatched to Brisbane in batches.

3.1.2 Results

Germination percentage of mānuka seeds varied with populations. Figure 1 below summarises the initial germination of 29 randomly selected populations. Overall germination recorded was very low with the highest being 26% and the lowest was 5%.

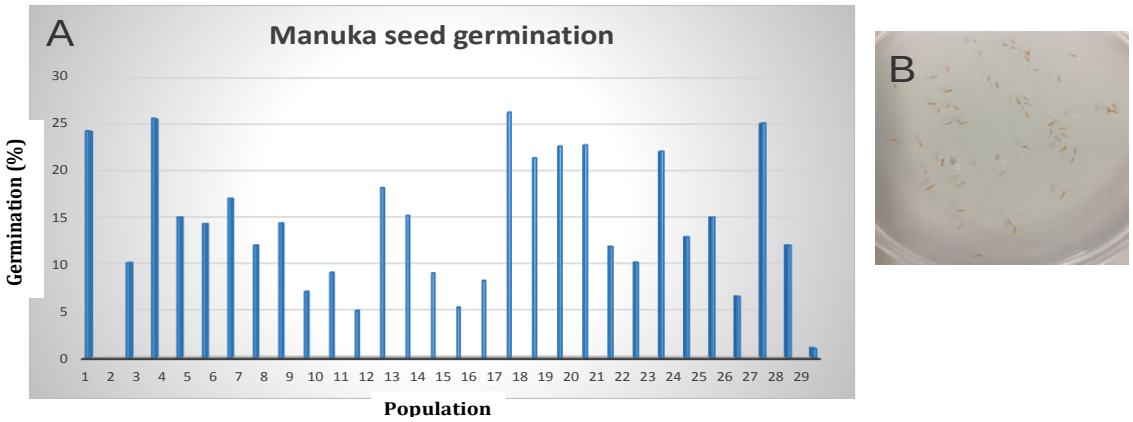


Figure 1. Comparative germination of mānuka seeds collected form 29 different population (A) and mānuka seed germination on water agar (B)

All the seeds did not survive –20°C storage after a 1-year period. Following cryopreservation, the germination did show a slight decline from initial germination but did retain the viability better than –20°C storage (Figure 2).

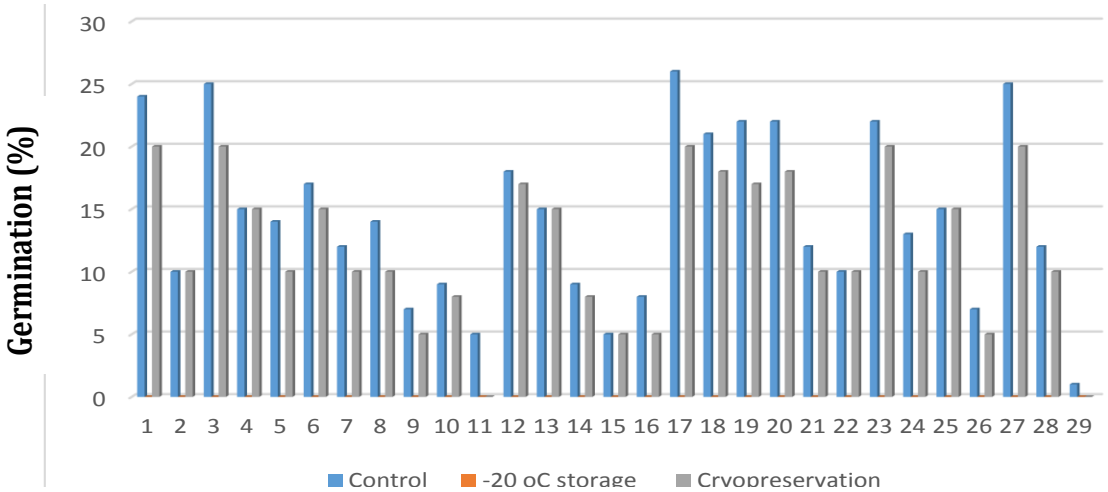


Figure 2. Comparative 1-year storage results at –20°C and cryopreservation for mānuka seeds collected from 29 different populations

3.2 Storage behaviour of selected Myrtaceae species

Seeds of *Lophomyrtus bullata, L. obcordata, Metrosideros diffusa, Metrosideros umblleta* and *Metrosideros bartletii* were collected and supplied by DOC and Wellington Botanic Garden. The seeds were processed from their capsule and tested for initial germination. Following that, the seeds were equilibrated to various relative humidity environments ranging from 5, 15, 30, 55, 75 to 100%. The relative humidity environments were created using lithium chloride salt solutions with different concentrations. Around 100 to 200 seeds were used for each humidity environment treatment. Before placing the seeds in an air-tight container (Figure 3), the initial moisture content of the seeds was determined and initial seed weight was also recorded. Seed weight loss was monitored at regular intervals until there were no changes in the weight, indicating the seeds had reached their equilibrium with their environment. Upon reaching equilibrium, seed moisture content and germination were assessed.

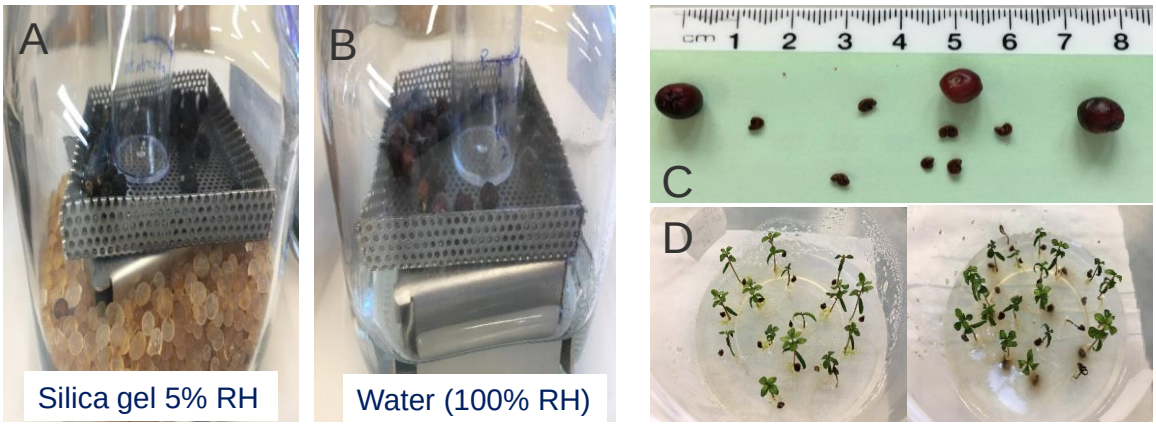
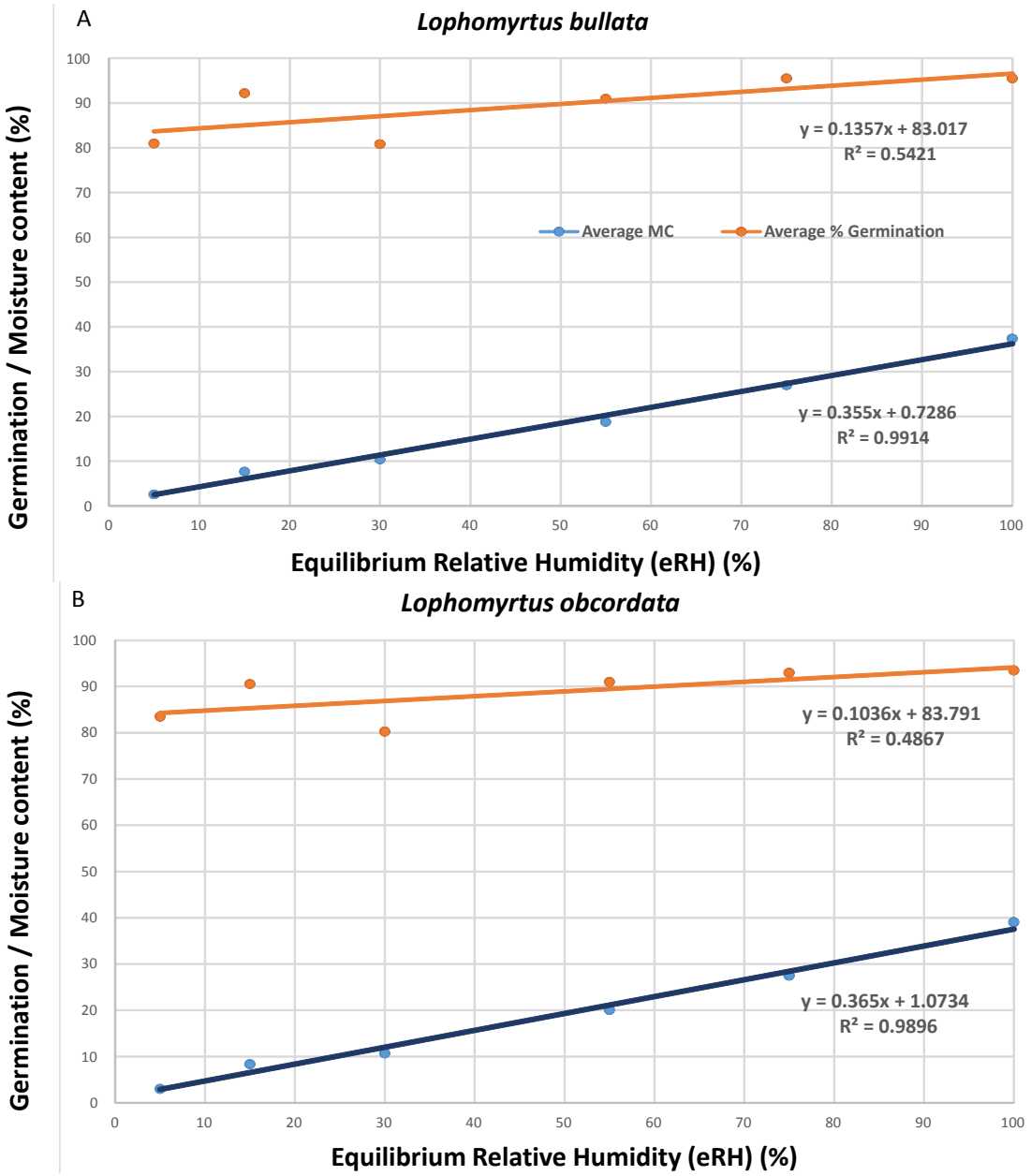


Figure 3. Seeds were equilibrated in different humidity environments (A & B), capsules and seeds of *Lophomyrtus bullata* (C), seeds of *L. bullata* seeds germination following desiccation (D).

The seeds had around 20% initial moisture content and 90% germination. Figure 4 below summarises the moisture content and germination results following equilibration at the selected relative humidity. The moisture content of the seeds ranged from 2 to 46% following equilibration at 5% and 100% relative humidity, respectively. However, the germination was still high (>80%) even for the seeds dried to around 2% moisture content for all these five species, indicating that all these species are orthodox i.e. desiccation tolerant and therefore can be stored in a conventional seed bank for a long term.



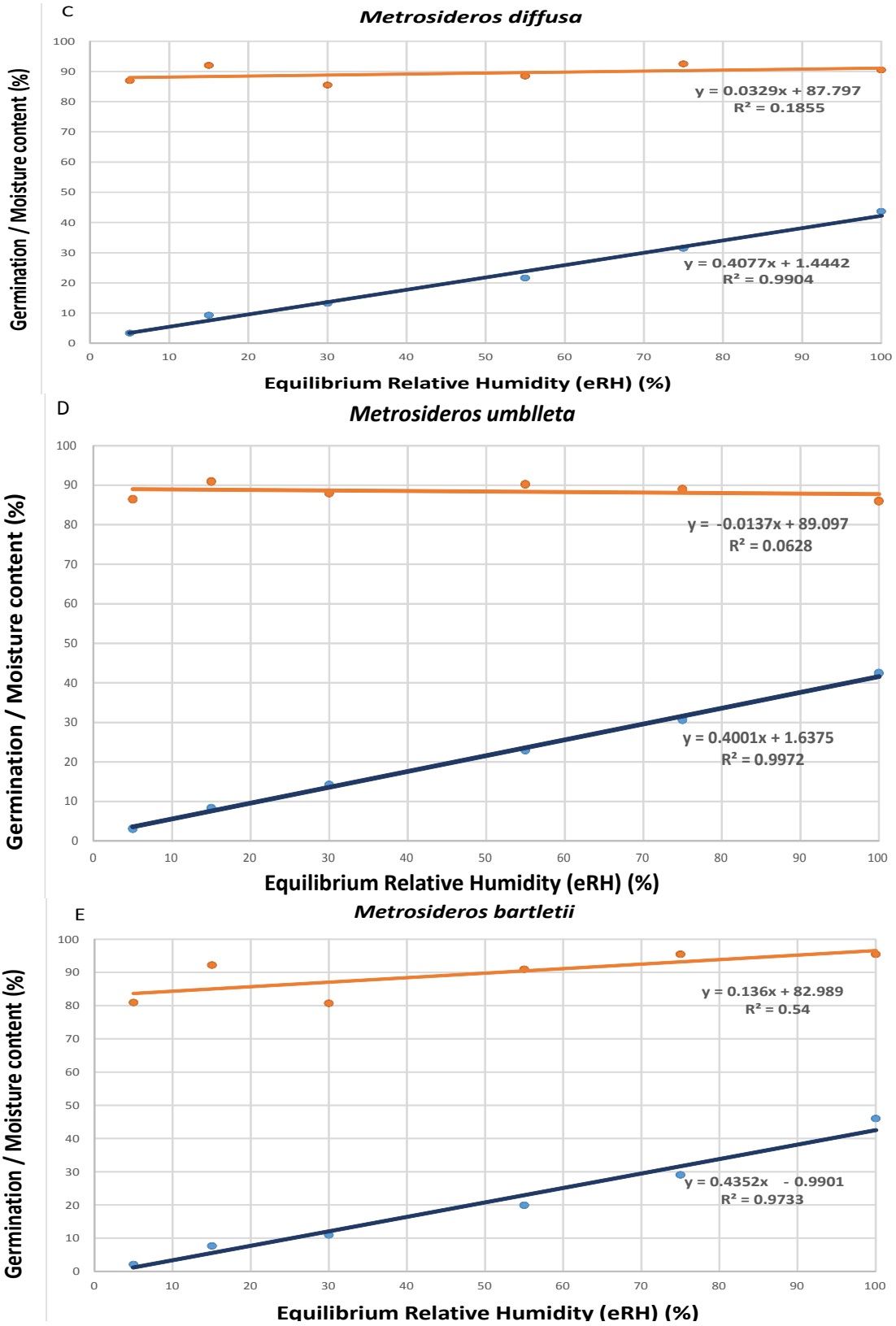


Figure 4. Desiccation tolerant profile for *Lophomyrtus bullata* (A), *Lophomyrtus obcordata* (B), *Metrosideros diffusa* (C), *Metrosideros umblleta* (D) and *Metrosideros bartletii* (E).

3.3 *In vitro* culture protocols for selected Myrtaceae species

Genebanking through *in vitro* culture is another option being tested. Vegetative propagation using cuttings of several Myrtaceae species has been demonstrated but is slow, often difficult, and season and genotype dependant. Therefore, micropropagation is the preferred method of propagation. Once optimised, these methods can be used to establish *in vitro* repositories for conservation and also can be used to source explants for cryopreservation for long-term conservation. Our objective was to test both photo-autotropic and *in vitro* propagation methods for selected species of Myrtaceae.

This includes initiation of seedlings and shoots into tissue culture and their propagation using *in vitro* and photo-autotropic micropropagation techniques. In addition, we have successfully transferred tissue cultures of *Metrosideros bartletii* to greenhouse conditions for further growing as it is the most endangered of the species.

Seedlings of *Metrosideros bartlettii* and *Lophomyrtus obcordata* in water agar plates were received from Otari Botanic Gardens, Wellington. These cultures were not sterile. Therefore, shoots were excised and further surface sterilised by immersion in 75% ethanol followed by a solution of sodium dichloroisocyanurate (NaDCC, 5 g/L) containing 0.1% (v/v) Tween 20® for 30 min, washed in sterile water (3x) and cultured on a medium consisting of Murashige and Skoog (1962) (MS) half-strength macro elements, full strength MS microelements, B5 (Gamborg et al. 1968) vitamins with 3% (w/v) sucrose solidified with agar (7.5% w/v). If further contamination was observed, the shoot tips were rinsed in 75% ethanol for 40 sec and cultured in same medium as above, supplemented with NaDCC 70–100 mg/L for 2–4 days after which the plantlets were transferred to NaDCC-free medium.

Lophomyrtus 'Red Dragon' (a hybrid of *L. bullata* x *L. obcordata, Metrosideros excelsa* and *M. perforata*) were received from Ardmore Nurseries Ltd, Papakura, Auckland, and maintained in the greenhouse at The New Zealand Institute for Plant and Food Research Limited (PFR), Palmerston North (Figure 5). *Leptospermum scoparium* was sourced from the PFR collection at Palmerston North (Figure 6). Newly formed shoots (3–5 cm) were brought to the laboratory, leaves were clipped and 3–5 nodal pieces were used for surface sterilisation using the same method described for seedlings.

Figure 5. *Lophomyrtus* 'Red Dragon' (A), *Metrosideros excelsa* (B) and *M. perforata* (C) growing in the PFR greenhouse in Palmerston North.

The explants used for tissue culture initiation and *M. bartlettii* seedlings in water agar are shown in Figure 7.

Figure 6. *Leptospermum scoparium* collection at PFR, Palmerston North that served as initial material for tissue culture experiments.

To induce axillary shoot formation for micropropagation, two-nodal shoots were transferred to MS medium supplemented with 6-benzylamino purine (BAP – 1 mg/L) and indole-3-butyric acid (IBA – 0.1 mg/L). The shoots arising were separated and transferred back to basal MS media for growth. Rooting of shoots was tested in two ways: a) by dipping cut ends of shoots in IBA (15–30 mg/L) for 30 sec and growing in basal MS media; and b) by supplementing the basal MS media with IBA (0.5–1 mg/L).

For developing photo-autotrophic micropropagation methods, sterilised shoot tips (2–5 cm) were embedded in sterilised rock wool cubes aseptically and placed in sterile 250-mL plastic tissue culture vessels. After adding 10 mL of sterile liquid medium without sucrose, the tubs were placed on a tilting device that enabled liquid feeding for 10 min followed by 20 min of draining (Figure 8). The cultures received 70–80 µmol s^{-1} m^{-2} light (16 h per day) supplied by Sylvania Grow-Lux 58w/GRO-T8 (Germany) Lights. Rooting of shoots was tested using 1.5 mg/L IBA in the same growth solution (Figure 8).

Surface sterilisation using NaDCC as the sterilant including the subsequent culture in media supplemented with NaDCC gave 20% clean explants of *Lophomyrtus* ‘Red Dragon’ and *M. perforata*, and 42% *L. scoparium*. Through shoot tip culture of *M. bartlettii* we could achieve almost 100% sterile plants because the initial contamination in the agar plates was not high (Figure 7 D and E).

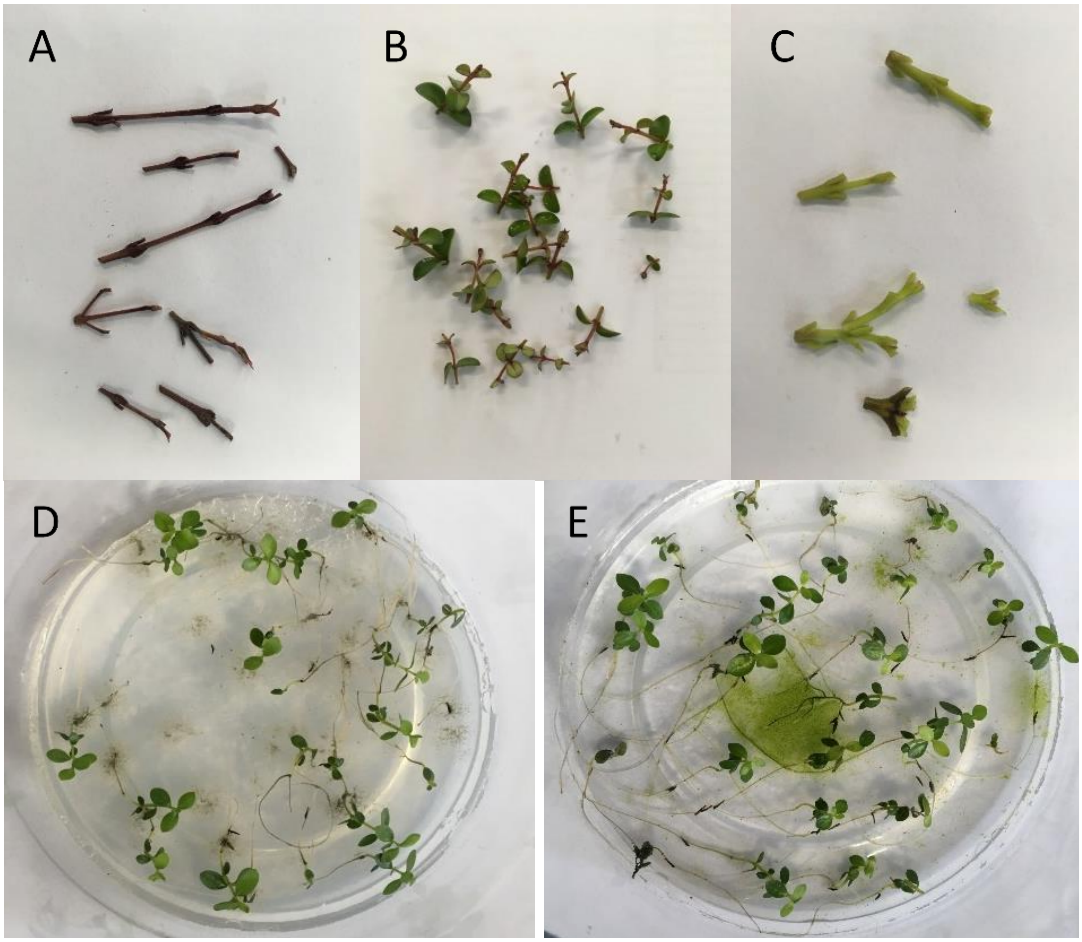


Figure 7. Initial material used for establishment of *in vitro* cultures and micropropagation experiments. A. *Lophomyrtus* 'Red Dragon' – a hybrid of *L. bullata* x *L. obcordata,* B. *M. perforata*, C. *Metrosideros excelsa* and D & E. *M. bartlettii* seedlings in agar plates.

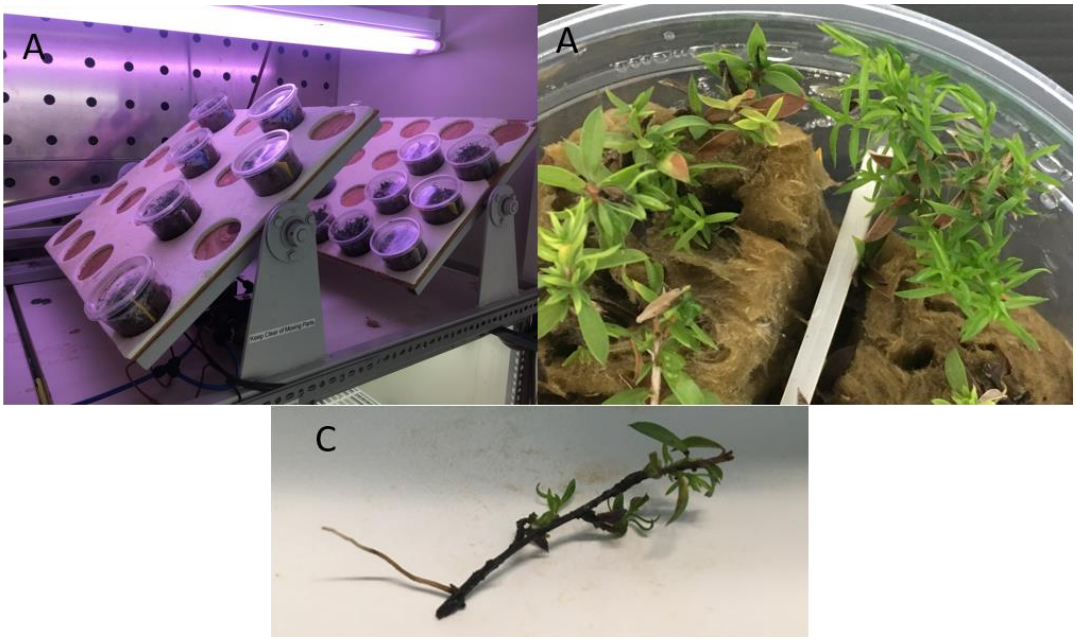


Figure 8. Photo-autotropic micropropagation of Myrtaceae. A) Tilting device that can be programmed for feeding time, B) *Leptospermum scoparium* growing in rockwool, C) Rooted cutting ready for greenhouse acclimation.

Inclusion of BAP and IBA at 10:1 ratio enabled proliferation of axillary shoots (Figure 9 B) and IBA alone was effective in producing roots (Figure 9C). These plantlets after rooting were easily acclimatised to the greenhouse by first holding them in non-soil media for 2 weeks in a bottom-heated (27°C) fog tent, followed by misting in a mist bed (Figure 9D). These plants can now be potted and repatriated to the wild.

Thus, we have so far developed *in vitro* culture methods for *Lophomyrtus bullata*, *Lophomytus obcordata*, *Leptospermum scoparium*, *Metrosideros excelsa* and the highly endangered *Metrosideros bartlettii*.

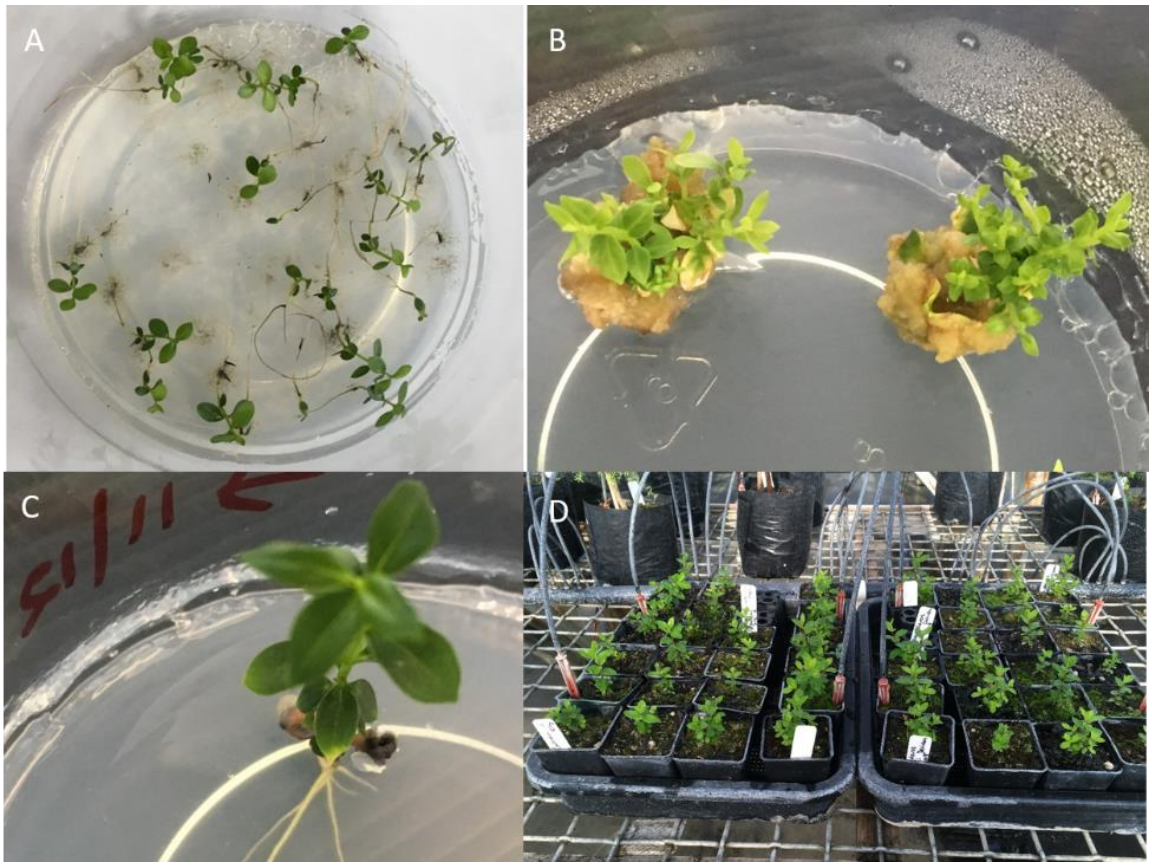


Figure 9. *In vitro* culture, micropropagation and greenhouse acclimation of propagated *Metrosideros bartlettii* material. Seedlings of *M. bartlettii* in agar/water plates as received (A). Multiple shoots induced (B), rooted plantlet ready for the green house (C) and healthy plants after acclimation in the greenhouse ready for repatriation (D).

Photo-autotropic micropropagation was successful for *L. scoparium* as shown in Figure 8. *Lophomyrtus* 'Red Dragon' was also partly successful but we need to improve the sterility as the initial explants seem to have higher microbial contamination.

3.4 Pollen cryopreservation for selected Myrtaceae species

Pollen samples of two Myrtaceae species, *Metrosideros excelsa* and *M. bartletii*, were collected from Victoria Esplanade, Palmerston North, and Otari Botanic Garden, Wellington, respectively (Figure 10). There was high viability at collection. However, *M. excelsa* pollen viability declined very rapidly at room temperature. Pollen cryopreservation was tested only for *M. excelsa as M. bartletii* pollen was not available in enough quantity for this assessment. Desiccation of the pollen to about 5% moisture content (achieved with equilibrium with 15% relative humidity) followed by rapid freezing into liquid nitrogen (stored for 1 h) and rapid thawing was found optimum (with viability retained as non-cryopreserved control pollen) for cryopreservation of *M. excelsa* pollen.

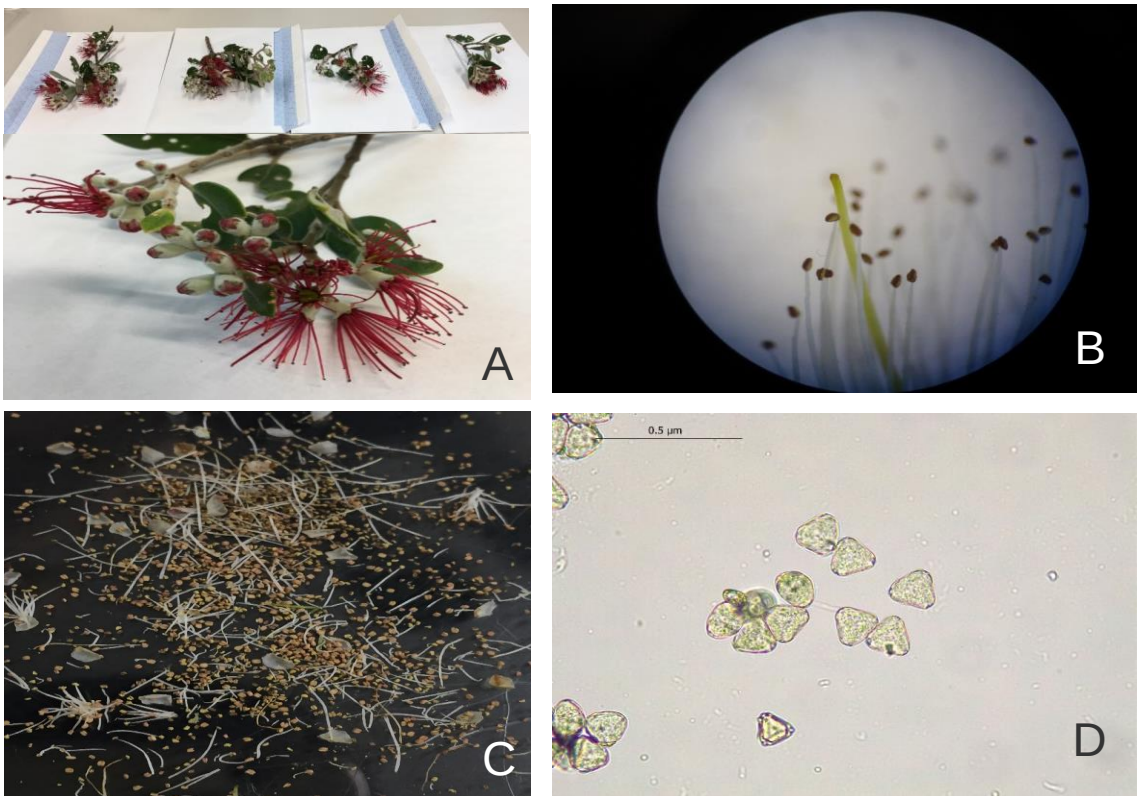


Figure 10. Flowers of *Metrosideros excelsa* (A), anthers of *Metrosideros bartletii* (B), pollen grains of *M. bartletii* (C), microscopic image of *M. bartletii* pollen (D).

3.5 Cryopreservation protocol for *Syzygium maire*, a recalcitrant Myrtaceae species

3.5.1 Seed collection and experimental procedures

This work was carried out in collaboration with Wellington City Council (WCC) and DOC, New Zealand. Seeds for this study were collected by WCC and DOC from various locations as described in Table 1. Where seeds were collected from privately owned land, landowner consent was obtained prior to collection (Appendix 1).

Table 1. *Syzygium maire* seed collection area.

Locality	Date collected	Estimated number of seeds	Collectors	Land owner
Taranaki	20 May 2017	2500	DOC	Private – Farm
Fensham Reserve	28 December 2017	3000	WCC	Forest and Bird Reserve
Nga Manu	17 January 2018	8000	WCC	Nga Manu Trust
Zealandia	15 February 2018	300	WCC	Zealandia Trust
Taranaki	21 March 2018	6000	WCC	Private land owner
Rotoiti – Bay of Plenty	19 February 2018	250	DOC	Tokerau Trust

DOC = Department of Conservation, New Zealand, WCC = Wellington City Council.

The typical natural habit of *S. maire* is a wetland as shown in Figure 11. Trees were monitored from the time of flowering through fruit set, seed maturation and until seeds were ready for dispersal (Figure 12).

Figure 11. Natural habitat of *Syzygium maire* (Photo by Karin van der Walt).

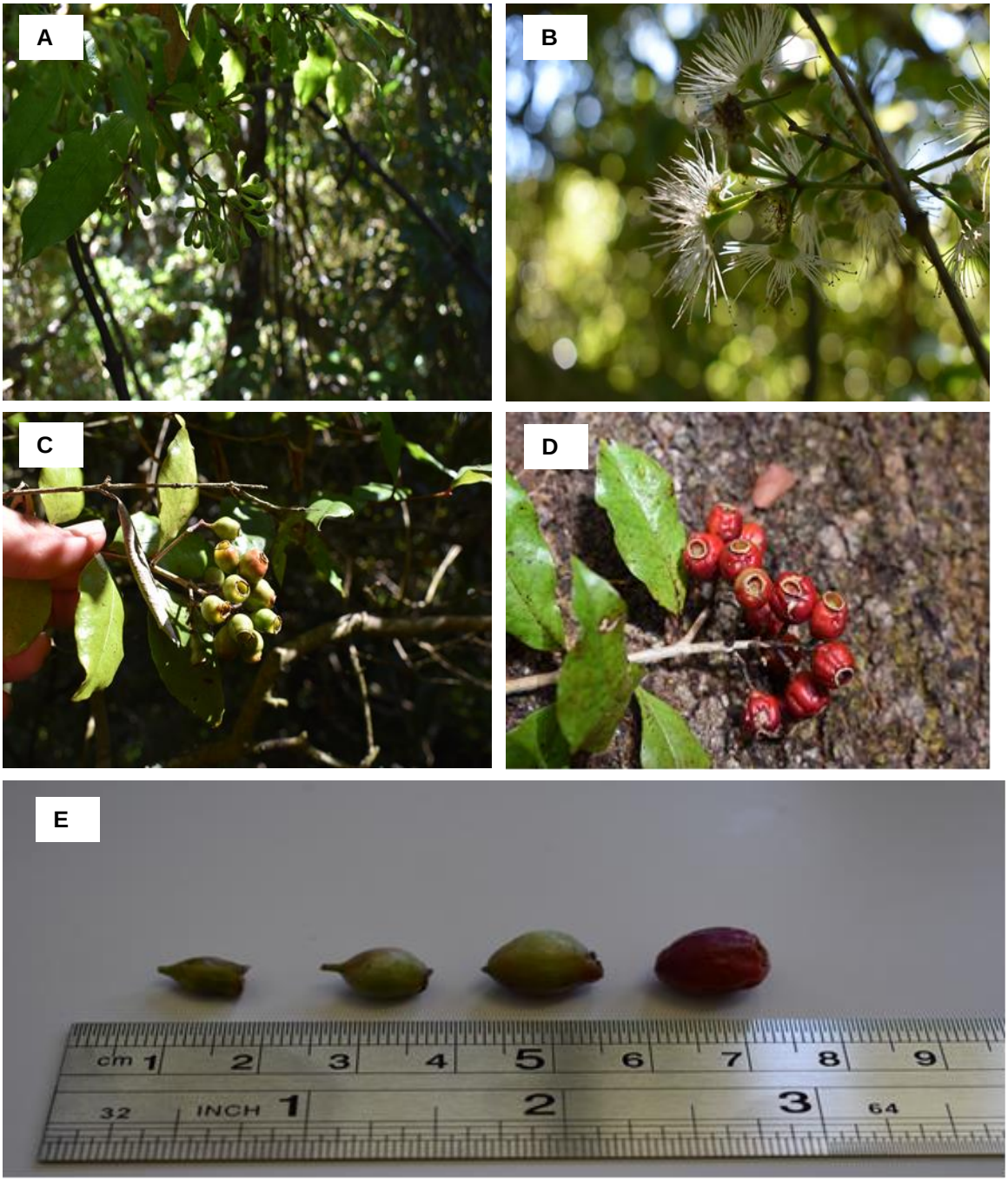


Figure 12. Seed development of *Syzygium maire* in their natural habitat. A: flower buds, B: flower at anthesis, C: fully matured seed, D: seed at shedding, E: seeds at various maturity stages (Photos by Karin van der Walt).

There were four project activities (milestones) under this project. The reporting for each activity will be covered separately below. Initial seed moisture content and germination tests were carried out according to the International Seed Testing Association (ISTA 2018) standards to determine seed quality before initiating the experiments. Moisture contents for different seed components, namely seed coat, pulp, cotyledon and embryo, were also measured. Seed desiccation sensitivity assessment was carried out to confirm the storage physiology of the seeds. For this assessment, the seeds were desiccated using silica gel to various moisture contents. On attaining these moisture contents, seed germination was tested by sowing them on 8% (w/v) water agar.

3.5.2 Initial moisture content, germination and desiccation sensitivity assessment for *S. maire* seeds

The initial seed moisture content was around 80% (fresh weight basis). All seed components had a moisture content above 80% with seed coat and pulp showing moisture contents above 90% (Table 2). The germination of fresh seed immediately after collection was 100% (Figures 12 and 13) indicating the seeds have a high viability at collection and before any experiments. Seed desiccation sensitivity assessment revealed that the seeds are extremely sensitive to drying with drying to 50% moisture content reducing the germination from 100% to 66%. Further drying to 40% and 30% reduced the germination to 50% and 25%, respectively. Drying the seeds below 30% resulted in 0% germination confirming the recalcitrant nature of *S. maire* seeds (Figure 13).

Table 2. Moisture content for different components of *Syzygium maire* seed immediately after harvest (n=25).

Seed component	Moisture content (%) (Average ± SD)
Seed coat	90.85 ± 0.73
Pulp	91.32 ± 1.03
Cotyledon	84.10 ± 1.85
Embryo	84.60 ± 0.80

A B C

Figure 12. Germination of fresh *Syzygium maire* seed. A) 5 days after sowing, B) 10 days after sowing, C) 15 days after sowing (Photos by Jayanthi Nadarajan).

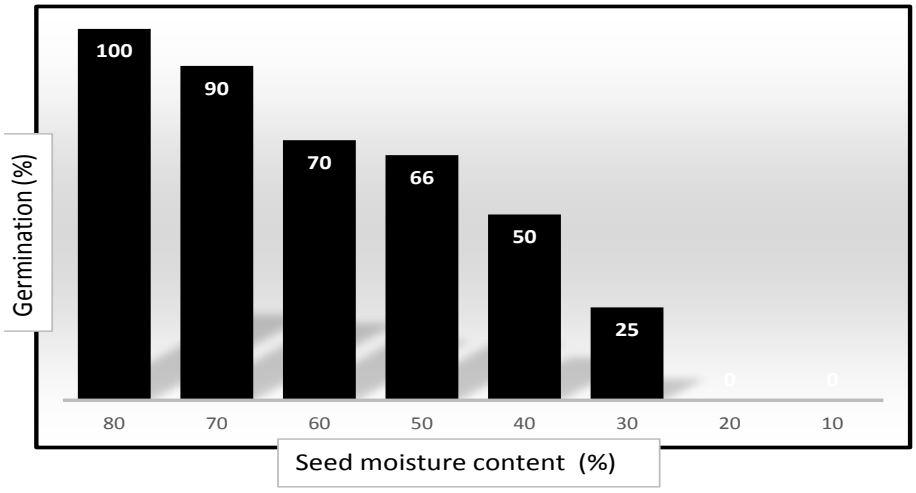


Figure 13. Germination of *Syzygium maire* seed at collection (80% moisture content) and following desiccation to various moisture contents (n = 100).

Desiccation sensitivity assessment for excised embryos of *S. maire*

Seeds collected by DOC from private land in Taranaki on 20 May 2017 were used in this experiment. The aim of this assessment was to test the desiccation tolerance level of *S. maire* embryos and to arrive at an optimum moisture range for cryopreservation of the embryos. As moisture content is a critical factor in developing a cryopreservation protocol, ensuring the optimum moisture content is achieved is essential. This is because under-drying will result in an excess of 'free' or 'freezable water' in the embryos that would lead to lethal ice crystallisation during freezing. On the other hand, over-drying will lead to embryo death, hence, finding the balance is the first step in a cryopreservation protocol development.

Seeds of *S. maire* were surface washed with 20% Janola® (2 min) and the embryos were excised aseptically in a laminar flow hood. The embryos were then surface sterilised with 20% commercial bleach (Janola®) and 50% ethanol followed by three rinses in sterile reverse osmosis water. The embryos were then desiccated for various durations (0, 1, 2, 3, 4, 5 and 6 h) in the air flow of a laminar flow bench to reach various moisture content levels. Moisture contents of the embryos were determined at the end of the desiccation period for each treatment. For each desiccation treatment, 10 embryos in four replicates were germinated on solid Murashige and Skoog (MS) (1962) medium supplemented with 3% (w/v) sucrose and incubated in a growth room set at 25°C with 16/8 h photoperiod provided by cool white fluorescent tube providing a PFD of 30–50 umol/m^2/s.

The embryo moisture contents following drying and their corresponding germination percentages are summarised in Table 3. The results reconfirm that *S. maire* is a recalcitrant species and the embryos are showing sensitivity to desiccation with viability severely reduced when the moisture content falls below 20% (Table 3).

Table 3. *Syzygium maire* embryo moisture content and their corresponding germination following desiccation (n=40).

Desiccation duration (h)	Embryo moisture content (%) (Average ± SD)	Germination (%) (Average ± SD)
0	75.3 ± 2.5	100.0 ± 0.0
1	54.7 ± 1.5	72.0 ± 2.0
2	38.6 ± 1.1	52.0 ± 2.0
3	24.4 ± 1.0	38.0 ± 2.0
4	20.0 ± 1.0	29.3 ± 1.2
5	15.3 ± 0.6	28.0 ± 2.0
6	12.6 ± 1.0	17.5 ± 2.5

3.5.3 Excised zygotic embryo cryopreservation of *S. maire*

The aim of the assessment was to test if the excised embryos will survive cryopreservation following desiccation alone. Four desiccation periods (2, 3, 4 and 5 h) were selected based on the results from Milestone 1 showing that there is a very high moisture content for embryos desiccated for less than 2 h, and that for embryos desiccated longer than 5 h, the germination was very low. Hence, the optimum moisture content for cryopreservation was estimated at between 15 to 35%.

Protocols as described above were followed for seed surface washing, embryo excision and sterilisation. The embryos were desiccated for 2, 3, 4 and 5 h in the air flow of a laminar flow bench. After the desiccation period 10 embryos in four replicates were packed into cryo vials and cryopreserved by rapid freezing in liquid nitrogen. The embryos were stored in liquid nitrogen for 1 h and then thawed in a water bath at 40 ± 2°C. Following thawing, the embryos

were plated on solid MS medium supplemented with 3% (w/v) sucrose and incubated in the growth environment specified above to estimate their viability.

The results for excised embryos' desiccation and cryopreservation are summarised in Table 4. The results show that *S. maire* embryos are extremely sensitive to direct cryopreservation as there was no survival following cryopreservation of the excised embryos. The next step in this study was focused on encapsulation of the embryos in sodium alginate and cryopreservation.

Table 4. Germination before and after cryopreservation following desiccation for excised embryos of *Syzygium maire* (n=40).

Desiccation duration (h)	Embryo moisture content (%) (Average ± SD)	Germination before cryopreservation (%) (Average ± SD)	Germination following cryopreservation (%)
2	36.40 ± 1.05	55.00 ± 0.58	0
3	24.63 ± 1.0	40.0 ± 2.0	0
4	20.18 ± 0.39	35.00 ± 1.53	0
5	15.16 ± 0.63	30.00 ± 1.53	0

3.5.4 Encapsulation cryopreservation of *S. maire* embryos

The encapsulation-dehydration technique was developed on the basis of artificial seeds production, where explants are encapsulated into sodium alginate beads, pre-treated in sucrose rich medium, partially desiccated using a laminar air flow and then directly immersed in liquid nitrogen (Fabre & Dereuddre 1990). Not only have the embryos of numerous species been stored using this technique but shoot apices and embryonic axes of several temperate and tropical species have also been conserved (Engelmann 2004). Following the observation of 0% survival for excised embryo axis cryopreservation, in this part of the study the encapsulation-dehydration method of cryopreservation was tested for *S. maire* excised embryos.

Seeds from two different populations (Taranaki and Zealandia, Wellington), as described in Table 5, were used in this experiment. The encapsulation dehydration technique was applied to the excised embryos. Excised embryos were surface sterilised and encapsulated in 3% (w/v) sodium alginate. The encapsulated embryos were then desiccated to various moisture contents and cryopreserved. Following cryopreservation, germination was tested on MS medium supplemented with 3% (w/v) sucrose as described previously (Figure 14).

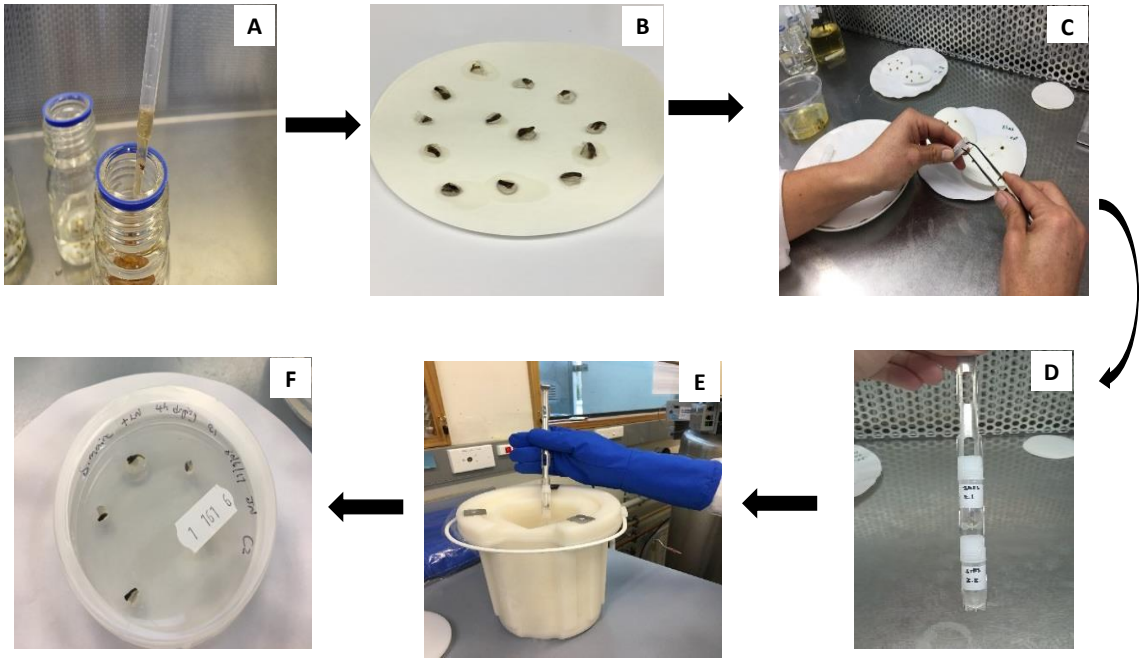


Figure 14. Sodium alginate (Na-alginate) encapsulation cryopreservation procedure. A) excised embryos were treated with 3% Na-alginate followed by 1% calcium chloride ($CaCl_2$) solution for polymerisation of the beads; B) embryos encapsulated in the beads were desiccated to various moisture contents; C) following desiccation, the embryos were packed in cryovials; D) cryovials were attached to the cryo-cane; E) the cryo-canes were rapidly cooled by submerging in liquid nitrogen (LN) and stored in LN for 1 hour; F) following LN storage, embryos were rapidly rewarmed and plated on Murashige and Skoog medium. (Photos by Jayanthi Nadarajan and Karin van der Walt).

Table 5. *Syzygium maire* seeds sampled from different populations used in the encapsulation cryopreservation.

Locality	Date collected	Estimated number of seeds	Collectors
Taranaki	20 May 2017	2500	DOC
Zealandia	15 February 2018	300	WCC
Taranaki	21 March 2018	6000	WCC

DOC = Department of Conservation, New Zealand, WCC = Wellington City Council

Seeds collected from Taranaki in May 2017 showed embryo survival around 20% and 30% following cryopreservation when the encapsulated embryos were dried to moisture contents of 37% and 30%, respectively (Figures 15 and 16). Moisture contents above 37% were lethal to the embryos as no embryos survived cryopreservation, possibly because of ice crystallization.

From our monitoring study on the seed development from flowering to seed maturity, one interesting observation we noted during this study was that flowering and seed production was much earlier in 2018 compared with 2017. Hence, the seed collection was carried out in March in 2018 compared to May in 2017. However, when the encapsulation cryopreservation trial was repeated using seeds collected from the same Taranaki population on 21 March 2018, there was no survival following encapsulation cryopreservation. This was also true for the seeds collected from the Zealandia population. One explanation for this observation could be that the seeds collected from the Taranaki population in May 2017 were at an optimum maturity level at which their moisture content could be manipulated without compromising their viability. Beyond this maturity stage, the seeds would enter a germination phase followed by a developmental/physiological stage, where drying the seeds could be lethal. We postulate that

the seeds from Taranaki population collected on 21 March 2018 and Zealandia population collected on 15 February 2018 would have passed this 'optimum maturity window' during this experiment.

As the germination post-cryopreservation is very low and inconsistent within the same (Taranaki) and between the two populations (Taranaki and Zealandia) tested, the next part of this study was focused on testing a more advanced cryopreservation protocol using vitrification cryoprotection.

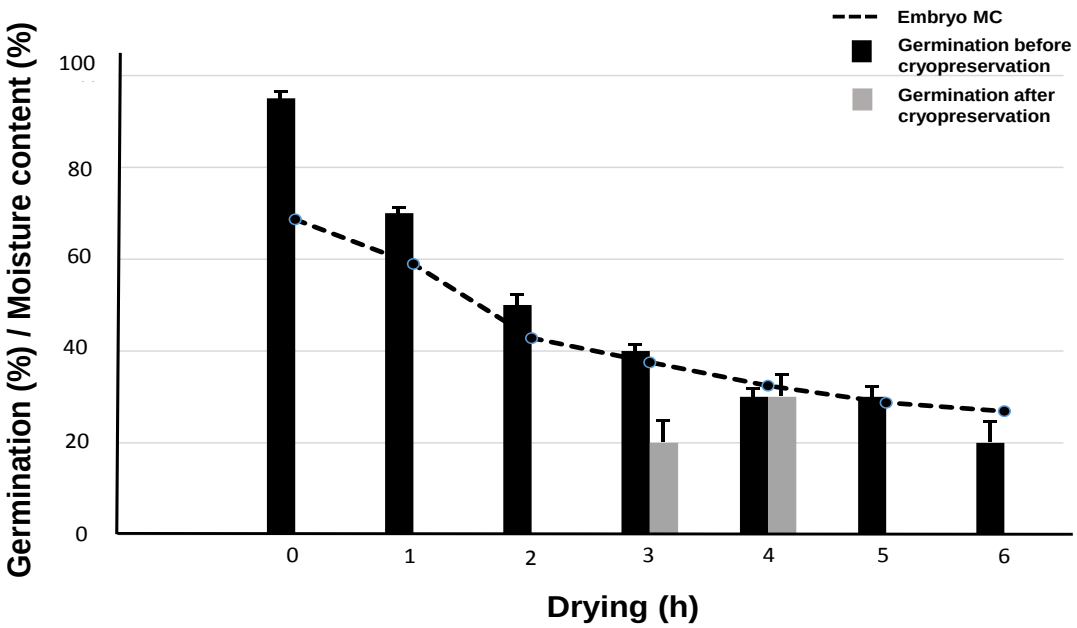


Figure 15. Germination of sodium alginate encapsulated embryos of *Syzygium maire* before and after cryopreservation following desiccation to various moisture contents.

Figure 16. Sodium alginate encapsulated embryos of *Syzygium maire* regenerating on Murashige and Skoog medium 4 weeks after cryopreservation. (Photos by Jayanthi Nadarajan).

3.5.5 Vitrification cryopreservation of *S. maire* embryos

Over the last two decades, vitrification cryopreservation using cryoprotectants has gained attention for improving survival of cryopreserved cells and tissues (Turner et al. 2001). In order to successfully cryopreserve tissues by vitrification, it is important to carefully control dehydration/cryo-protective procedures and to prevent injury by chemical toxicity or excess osmotic stresses (Sakai et al. 1990). Many different pre-treatment techniques have been developed to increase dry mass content, stabilise proteins and membranes, decrease freezing point, and enhance the glass forming tendency of the remaining water in the cryopreserved

tissues (Grospietsch et al. 1999) including loading with different cryoprotectant materials (sucrose, polyols, glycerol, proline, and dimethyl sulfoxide. Using these methods, combined with preculturing or preconditioning of donor plants, embryonic axes of several non-orthodox plants have been successfully cryopreserved using the vitrification technique (Engelmann 2004).

Seeds from the Taranaki population collected on 21 March 2018 were used for this experiment. The embryonic axes were pre-cultured for 24 h on solid MS medium supplemented with 0.75 M sucrose and incubated at 25 ± 2°C under a 16/8 h photoperiod. Following preculture, embryos were placed in a 2.0 ml cryovial and treated with loading solution (LS) for 20 min at room temperature (~25°C). LS is comprised of 0.4 M sucrose + 2 M glycerol in liquid MS medium (Sakai 2004). Following LS treatment, the embryos were treated with cryoprotectant before cryopreservation. PVS2 was used as a cryoprotectant. PVS2 was prepared in MS liquid medium, as: 30 % glycerol + 15 % ethylene glycol + 15 % DMSO + 0.4 M sucrose (Sakai 2004). The effect of the duration of application of the vitrification solution at two different temperatures was evaluated by treating embryos with PVS2 for 0, 30, 60 and 90 min at 0°C and at ambient temperature. At the end of the PVS2 treatment, the vitrification solution was renewed and the final volume in the cryotubes was adjusted to 1.5 ml. The embryos were now either unloaded and transferred to growth medium (solid MS medium supplemented with 3% (w/v) sucrose) (non-cryopreserved controls), or cryopreserved by rapid immersion of the cryotubes in LN. After 1 h of storage in LN, the embryos were rewarmed by placing the cryotubes in a water-bath at 40 ± 2°C for 5 min. The vitrification solution was removed and replaced by 1.5 ml of liquid MS medium supplemented with 1.2 M sucrose for 20 min (unloading treatment). The embryos were retrieved from the liquid medium, blotted dry on sterile filter paper and placed on growth medium. After 24 h, they were transferred on to fresh growth medium and germination was assessed as above.

There was no survival following cryopreservation after any of the three PVS2 treatment times at either temperature (Table 6). The embryos showed very low survival following PVS2 cryoprotectant treatment even before cryopreservation. The highest survival of around 27% was recorded for the 60 min PVS2 treatment before cryopreservation. Ambient temperature treated embryos showed much better survival compared with those treated at 0°C (Table 6).

Table 6. Summary results for *Syzygium maire* embryos following vitrification cryopreservation using four Plant Vitrification Solution 2 (PVS2) treatment periods at two different temperatures.

PVS2 Exposure time (min)	PVS2 Treatment temperature	Germination before cryopreservation (Average ± SD)	Germination after cryopreservation (%)
0	-	16.7 ± 5.8*	0*
30	Ambient	20.0 ± 14.1**	0*
30	0°C	15.0 ± 7.1**	0*
60	Ambient	26.7 ± 11.5*	0*
60	0°C	13.3 ± 5.8*	0*
90	Ambient	20.0 ± 10*	0*
90	0°C	10.0 ± 0**	0*

*n = 30; **n = 20.

4 Issues and mitigations

- Poor state of seeds collected was a concern and we addressed this issue by being proactive and passing on the protocols to seed collectors through Otari Native Botanic Garden.
- For *S. maire* seeds, seed collection at appropriate maturity stage was critical. We have been monitoring seed development from flowering to maturity in collaboration with Otari Native Botanic Garden.
- Seed collection from Māori and private lands was raised in the MPI Myrtle Rust Research Program workshop. We worked with PFR Māori Relationship Manager to ensure that the protocols for seed collections are adhered to and results are reported back and plants repatriated if necessary, in consultation with local iwi.

5 Conclusions and future work

This project gave us the opportunity to assess the seed storage physiology of selected New Zealand Myrtaceae species in order to define the optimum long-term conservation strategies for these species. From the six species studied, five species (*Lophomyrus bullata, L. obcordata, Metrosideros diffusa, M. umblleta* and *M. bertletii*) showed orthodox storage behaviour. Hence, these seeds can be stored relatively easily in conventional seed banks. However, *Syzygium maire* seeds and embryos showed extreme sensitivity to desiccation, and lost viability completely following desiccation to a moisture content below 20%, confirming its recalcitrant behaviour. Zygotic embryos of this species was successfully cryopreserved using encapsulation-dehydration technique with 30% survival. *Metrosideros excelsa* pollen cryopreservation was successful following desiccation of the pollen to about 5% moisture content followed by rapid freezing and rapid thawing.

The *in vitro* protocols for several Myrtaceae species described in this report were developed in one of New Zealand's leading tissue culture laboratories that houses over 1000 accessions of kiwifruit *in vitro*. The protocols developed, and experience and skills of staff should be utilised to conserve endangered Myrtaceae spp. in future programmes. The same lab houses New Zealand's first cryobank with more than 150 potato accessions and the core collection of apple germplasm. It is highly desirable to consider the use of facilities and staff skills for long-term conservation strategies of threatened and recalcitrant Myrataceae spp. such as *S. maire* using the developed cryopreservation protocols.

This report highlights the importance of integrated conservation strategies, i.e. involving seed storage, *in vitro* conservation, pollen storage and cryopreservation of recalcitrant species for conservation of Myrtaceae species to ensure future access to New Zealand's unique Myrtaceae germplasm as a key component of long-term management response to the threat posed by *Austropuccinia psidii*. This will ensure species diversity is not lost and that there will be a resource for future breeding programmes to deliver resistant plants back to landscape. The existence of other myrtle rust biotypes and the lack of knowledge of their potential effects on New Zealand Myrtaceae will require that material is conserved until this knowledge is available.

6 Major outputs

1. Knowledge/technology transfer and skill development through a PhD student supervision with Wellington City Council and Massey University.
2. Dissemination of research output through presentation at international scientific conferences:
    a. Nadarajan J, Van der Walt K & Pathirana R. Cryopreservation protocol development for *Syzygium maire*, a recalcitrant Myrtaceae species. COMBIO 2018 Sydney, Australia 26-28 September 2018.
    b. Nadarajan J. Plant cryopreservation: from axes to seeds. Society for Low Temperature Biology scientific meeting, Cambridge, UK, 19-20 September 2017 (Invited Talk).
    c. Pathirana R, Nadarajan J, Pathirana R. Plant germplasm conservation through cryo-preservation: challenges and opportunities. Invited Lecture at Acadia University/K.C.Irving Environmental Science Centre, Wolfville, Nova Scotia, Canada (5 September 2018).

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