Comparison of Seed Traits of Tropical (Indonesia) and Temperate (New Zealand) Orchid Species to Support Orchid Seed Conservation

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Abstract

Most orchid species are endangered, and a basic understanding of their seed biology is required to support conservation efforts. In particular, more knowledge of orchid seed characteristics would be useful for both *in situ* and *ex situ* conservation. This comparative study used morphological and biochemical approaches to characterise and compare orchid seed qualitative and quantitative traits, to explore biochemical changes during natural seed ageing and to investigate different asymbiotic *in vitro* seed germination media and cryopreservation techniques for *ex situ* conservation. This study included six orchid species with different growth habits and distribution ranges (*Dendrobium strebloceras, D. lineale, D. cunninghamii* (epiphytic), *Gastrodia cunnninghamii*, *Pterostylis banksii* and *Thelymitra nervosa* (terrestrial)) from temperate New Zealand and tropical Indonesia.

Morphometric analyses revealed similarities in the qualitative traits of seeds and capsules at the genus level (*Dendrobium*). However, high variability in micro-morphological seed characteristics were observed in the orchid species in this study which were unrelated to their taxonomy, biogeographical origin, or growth habit, suggesting different ecological adaptations possibly reflecting different modes of dispersal.

An investigation of the seed fatty acids showed that linoleic, oleic and palmitic acids (polyunsaturated, monounsaturated and saturated fatty acids, respectively) were the most abundant fatty acids in the seeds of all species and that rapid degradation of unsaturated acids occurred during ageing. Three epiphytic *Dendrobium* species had similar lipid composition but *Gastrodia cunnninghamii*, *Pterostylis banksii* and *Thelymitra nervosa* (terrestrial species) were characterised by the absence of erucic and palmitoleic acid. Changes in the proportion of oleic and linolenic acid were strongly correlated with seed viability loss. The ageing pattern was species-specific with *D. strebloceras* being more vulnerable to lipid degradation.

Epiphytic *Dendrobium* orchids had a less stringent media formulation requirement for seed growth than terrestrial orchids, and similarly, seeds of tropical species *D. lineale* and *D. strebloceras* germinated more easily than those of temperate species in this study. Cryopreservation with direct immersion of dry orchid seeds in liquid nitrogen is suggested as the most effective and efficient method for orchid seed conservation.

Keywords: air space; asymbiotic *in vitro* germination; cryopreservation; direct-freezing cryopreservation; epiphytic; Fatty Acid Methyl Ester (FAMEs); Gas Chromatograph-Mass Spectrometry (GCMS); liquid nitrogen; protocorm; PVS2 vitrification cryopreservation; orchid; seed micro-morphometrics; seedling development; seed natural ageing; terrestrial; tropical; temperate, vacuum infiltration

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Abbreviation

ANOVA	:	Analysis of variance
AS	:	Air space
BHT	:	Butylated hydroxyltoluene
BM1	:	Basic culture Medium-1
DMRT	:	Duncan's New Multiple Range Test
EL	:	Embryo length
EV	:	Embryo volume
EW	:	Embryo width
FAMEs	:	Fatty Acid Methyl Esters
GCMS	:	Gas Chromatography-Mass Spectrometry
GLM	:	General Linear Model
LS	:	Loading solution
LSD	:	Least Significant Difference
LiCl	:	Lithium chloride
m.a.s.l.	:	meter above sea level
MPD	:	Morphophysiological dormancy
MS	:	Murashige and Skoog

MUFA	:	Mono unsaturated fatty acid
NaCl	:	Sodium chloride
NaClO		Sodium hypochloride
PUFA	:	Poly unsaturated fatty acid
PVS2	:	Plant Vitrification Solution 2
RH	:	Relative humidity
SAFA	:	Saturated fatty acid
SD	:	Standard deviation
SE	:	Standard error
SL	:	Seed length
SV	:	Seed volume
SW	:	Seed width
S:U	:	Saturated : Unsaturated ratio
TCC	:	Tri-phenyl tetrazolium chloride
UnLS	:	Unloading solution
VW	:	Vacin and Went
v/v	:	volume/volume
w/v	:	weight/volume

CHAPTER I

Introduction:

Background, Aims and Research Framework

1.1. Introduction

Orchid seed conservation has been a significant concern for many decades in many scientific orchid communities. These communities include the Orchid Specialist Group (OSG) under The International Union for Conservation of Nature/Species Survival Commission (IUCN/SSC), the Multilateral Seedbank Project (The Global Darwin Initiative Project-Orchid Seed Science and Sustainable Use-OSSSU), national government or research institutes working in conservation, and also privately funded initiatives through orchid societies, for example the New Zealand Native Orchid Group (New Zealand), Perhimpunan Anggrek Indonesia (Indonesia) Orchid conservation alliance (South America initiative), Orchid Conservation Coalition (mostly from the US) and other Regional or National Orchid Societies found in many countries.

Much of the literature on orchid seed covers morphological characters, germination and conservation (section 1.2), but there are fewer publications available on orchid seed germination and cryostorage that cover a range of habitats and biogeographical distributions. Nor have the biochemical characteristics of orchid seed, particularly those associated with seed deterioration, been well-studied. Consequently, more comparative studies of orchid are needed to gain a better understanding of:

a. orchid seed morphology and its adaptation to habitats and biogeographical distribution,
b. seed biochemical characteristics and relation to seed deterioration symptoms during natural ageing,
c. asymbiotic *in vitro* seed germination on epiphytic and terrestrial orchids,
d. potential cryopreservation methods for storing orchid seeds.

1.2. Research background

Many species of orchid are susceptible to environmental changes (Barman and Devadas 2013; De and Medhi 2014). This urges for an enhanced effort toward *ex situ* conservation as an insurance policy against loss of plant populations *in situ*. The orchid family, Orchidaceae, is the second largest among flowering plants (angiosperms), after Asteraceae. Orchidaceae comprise c. 26,000-28,000 species in 736-749 genera (Chase *et al.* 2015; Chase *et al.* 2017). In addition to having great diversity over the world except in Antarctica (Givnish *et al.* 2016), the importance of orchids is also due to the significant economic value of the flowers. Orchids are traded both as potted plants and cut flowers. Around 85% of *Dendrobium* genera account for 10% of the international fresh cut flower trade (De *et al.* 2015).

In addition, orchids are also widely used for multiple ethno-botanical purposes, for instance as food, flavourings, medicines, ornaments, perfumes, and their symbolism in myths has inspired cultural ancient works of art, literature, and poetry (Popova *et al.* 2016). The utilization of *Dendrobium* orchids had also been reported since ancient times in association with religious, cultural, medicinal and ornamental purposes (Lavarack *et al.* 2000).

Yet, Orchidaceae is the most vulnerable family with all its species recorded in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2017; Wilis 2017). According to Zotz (2013), most orchids are epiphytic (69% of all orchid species) living in the tropics, while most terrestrial species are found in temperate regions. A study to rank the world's 228 countries based on their environmental impact found New Zealand had the highest proportion of threatened species, followed by Colombia and Indonesia (Bradshaw *et al.* 2010). Moreover, Bradshaw *et al.* (2010) also identified Indonesia, as one of the locations of the richest biodiversity in the world and ranked second (after Brazil) for natural forest loss.

The wide diversity of habitats and biogeographical distribution of orchid species present challenges for orchid seed conservation. Plant adaptation to specific or limited geographical distribution may stimulate the evolution of morphological

characters, often of structures related to dispersal mechanisms (Emadzade *et al.* 2010). Biogeographical distribution is also known to influence seed biochemistry, for example, by causing variation in seed oil contents for many plant species (Linder 2000). Moreover, living habitats also relate to specific nutritional needs and environmental requirements for orchid seed germination (da Silva 2013; Rasmussen *et al.* 2015).

Considering various plant responses and adaptations to different living habitats and the wide biogeographical distribution of orchid species, studies comparing species from different habitats (epiphytic and terrestrial) and biogeographical distributions (temperate and tropical) are needed to support orchid seed conservation. This study focused on comparing selected terrestrial and epiphytic New Zealand orchids (temperate region) with epiphytic orchid from tropical Indonesia, to investigate if their habitat and biogeographical differences result in different seed morphological and biochemical characteristics, and to investigate if these differences also influence the success of seed germination. Furthermore, we also developed cryopreservation protocols to support orchid seed conservation for the orchid species in this study.

1.2.1. Orchid classification

Historically, there had been a challenge to classify the large diversity of orchids. According to Dressler (1979), orchid species had been previously classified into six subfamilies on the basis of floral morphological characters: pollen, anther, rostellum, subsidiary cells, and subsidiary cells of stomatal leaves as the list below:

Family : Orchidaceae
Subfamilies : Apostasioideae Reichenbach
Cypripedioideae Lindley
Spiranthoideae Dressler
Orchidoideae
Epidendroideae
Vandoideae

Recent studies on most morphological classifications of orchids are also focused on floral characteristics and vegetative traits, either on their quantitative or qualitative traits (Kumar and Rajkumar 2012; Moniruzzaman and Ara 2012; Aybeke 2012; Jakubska-Busse *et al.* 2017). Additionally, molecular phylogenetic data has been found to have a strong relationship with morphological traits (Xiang *et al.* 2013; Cameron *et al.* 1999; Cameron 2005; Mytnik-Ejsmont *et al.* 2015), plant evolutionary history (Chase and Hills 1992; Yu and Goh 2000; Chase *et al.* 2003; Inda *et al.* 2012; Berg *et al.* 2005; Givnish *et al.* 2015), historical geography and dispersal (Givnish *et al.* 2016) also living habit (Cameron 2005; Freudenstein and Chase 2015).

Recent advances in molecular biology and morphological traits have enabled an update of orchid classification, as well as the description of new genera and species (Cameron *et al.* 1999; Chase *et al.* 2015; Chase *et al.* 2003; Givnish *et al.* 2015; Inda *et al.* 2012; Aybeke 2012; Gorniak *et al.* 2010; Berg *et al.* 2005; Felix and Guerra 2010; Bianco *et al.* 2017). Chase *et al.* (2017) classified Orchidaceae into five subfamilies based on DNA study and morphology of both vegetative and generative features, as the list below:

Family : Orchidaceae

Subfamilies : Epidendroideae
Orchidoideae
Vanilloideae
Cypripedioideae
Apostasioideae

Epidendroideae is the largest sub family in the Orchidaeae, followed by Orchidoideae (Chase *et al.* 2015). Details of subfamilies, tribes, and subtribes are provided in Table 1.1. Geographical distribution has been a major factor influencing the diversity of those two sub-families. Most Epidendroideae (99%) are tropical species while most of temperate species are Orchidoideae (Freudenstein and Chase 2015). Moreover, variation in geographical distribution is also likely to be related

to living habits; most of tropical species are epiphytic and temperate species develop a terrestrial niche (Freudenstein and Chase 2015).

Table 1.1. Orchidaceae subfamilies, tribes and subtribes

Subfamilies	**Tribes**	**Subtribes**
Epidendroideae	Epidendreae	Laeliinae
Epidendroideae	Epidendreae	Pleurothallidinae
Epidendroideae	Epidendreae	Ponerinae
Epidendroideae	Epidendreae	Bletiinae
Epidendroideae	Epidendreae	Calypsoinae
Epidendroideae	Collabieae	Collabiinae
Epidendroideae	Podochileae	Podochilinae
Epidendroideae	Vandeae	Angraecinae
Epidendroideae	Vandeae	Aeridinae
Epidendroideae	Vandeae	Adrorhizinae
Epidendroideae	Vandeae	Polystachyinae
Epidendroideae	Cymbidieae	Cymbidiinae
Epidendroideae	Cymbidieae	Cyrtopodiinae
Epidendroideae	Cymbidieae	Stanhopeinae
Epidendroideae	Cymbidieae	Coeliopsidinae
Epidendroideae	Cymbidieae	Maxillariinae
Epidendroideae	Cymbidieae	Eriopsidinae
Epidendroideae	Cymbidieae	Zygopetalinae
Epidendroideae	Cymbidieae	Oncidiinae
Epidendroideae	Cymbidieae	Catasetinae
Epidendroideae	Cymbidieae	Eulophiinae
Epidendroideae	Malaxideae	Dendrobiinae
Epidendroideae	Malaxideae	Malaxidinae
Epidendroideae	Arethuseae	Coelogyninae
Epidendroideae	Arethuseae	Arethusinae
Epidendroideae	Thaieae	-
Epidendroideae	Nervilieae	Nerviliinae
Epidendroideae	Nervilieae	Epipogiinae
Epidendroideae	Gastrodieae	Gastrodiinae
Epidendroideae	Xerorchideae	-
Epidendroideae	Triphoreae	Triphorinae
Epidendroideae	Triphoreae	Diceratostelinae
Epidendroideae	Wullschlaegelieae	-
Epidendroideae	Tropidieae	Corymbidinae
Epidendroideae	Tropidieae	Tropidiinae
Epidendroideae	Sobralieae	-
Epidendroideae	Neottieae	-
Orchidoideae	Orchideae	Coryciinae
Orchidoideae	Orchideae	Disinae
Orchidoideae	Orchideae	Orchidinae
Orchidoideae	Orchideae	Brownleeinae

Orchidoideae	Codonorchideae	-
Orchidoideae	Diurideae	Thelymitrinae
Orchidoideae	Diurideae	Megastylidinae
Orchidoideae	Diurideae	Drakaeinae
Orchidoideae	Diurideae	Cryptostylidinae
Orchidoideae	Diurideae	Diuridinae
Orchidoideae	Diurideae	Prasophyllinae
Orchidoideae	Diurideae	Caladeniinae
Orchidoideae	Diurideae	Acianthineae
Orchidoideae	Diurideae	Rhizanthellinae
Orchidoideae	Cranichideae	Chloraeinae
Orchidoideae	Cranichideae	Pterostylidinae
Orchidoideae	Cranichideae	Goodyerinae
Orchidoideae	Cranichideae	Galeottiellinae
Orchidoideae	Cranichideae	Manniellinae
Orchidoideae	Cranichideae	Discyphinae
Orchidoideae	Cranichideae	Cranichidinae
Orchidoideae	Cypripedioideae	-
Orchidoideae	Vanilloideae	Pogonieae
Orchidoideae	Vanilloideae	Vanilleae
Orchidoideae	Apostasioideae	-

Source: Chase *et al*. 2015

One of the most well-known Epidendrodeae is the subtribe of Dendrobiinae (including *Dendrobium*), which is one of the largest genera having over 1,500 species (The International Plant Names Index and World Checklist of Selected Plant Families 2020). Based on their vegetative and floral characters, the *Dendrobiinae* itself was subdivided into 35 sections and 12 genera which most are epiphytic, some are lithophytes, but rarely terrestrial plants (Lavarack *et al.* 2000).

Dendrobiinae species are found in diverse habitats from the Southern to Northern Hemispheres in South East Asia, Australia to Stewart Island in New Zealand and Tahiti in the Pacific (Lavarack *et al.* 2000; The International Plant Names Index and World Checklist of Selected Plant Families 2020). Tropical Indonesia possesses high diversity of *Dendrobium* species, and almost a quarter of Indonesian orchids of conservation priority are *Dendrobium* species (Irawati 2013). On the other hand, *Winika* is the only native *Dendrobium* species found in New Zealand; it is endemic and therefore found nowhere else (Lavarack 2010).

Winika was proposed as a monotypic genus by Clament *et al.* (1997) and followed by Lavarack *et al.* (2000), but this changes has not been accepted by the World Checklist of Selected Plant Families - WCPS (Govaerts (2003), https://wcsp.science.kew.org/qsearch.do). The latest reports by Burke *et al.* (2008) and Garnock-Jones (2014) presented evidence to keep *Winika* in *Dendrobium* genus. Hereafter, we adopted *Winika cunninghamii* as *Dendrobium cunninghamii*.

Even though most of Epidendroideae are epiphytic, a few species are terrestrial, including those in Gastrodieae tribe. Gastrodieae is amongst the most primitive of the epidendroids consisting of leafless species (Freudenstein and Chase 2015). The phylogenetic sisters of Gastrodieae are still unclear but they may be between Triphoreae to either Neottieae or Nervilieae (Gorniak *et al.* 2010). Gastrodieae has a wide range of geographical distribution from tropical Africa (Cribb *et al.* 2010), East (Hsu and Kuo 2010; Hu *et al.* 2014) to South (Suetsugu 2017) and South East Asia (Pelser *et al.* 2016), temperate Australia (McLennan 1959; Clements 1993; Gray and Low 2017) to New Zealand (Lechnebach *et al.* 2016).

The biggest genus in Gastrodiinae subtribe (Gastrodieae) includes *Gastrodia* species, also known as potato orchid. Most species in this genus are an obligate holomycotropic (Hsu and Kuo 2010) and are non-photosynthetic plants without chlorophyll (Lechnebach *et al.* 2016). The tuber or rhizoid is edible and *G. elata* is used in traditional medicine due to its pharmacological effect as anti-angiogenic, anti-inflammatory (Ahn *et al.* 2007) and anti-cancer (Shu *et al.* 2013.) agent. New Zealand possesses at least five *Gastrodia* species (Lechnebach *et al.* 2016): *Gastrodia sesamoides, G. molloyi, G. cunninghamii, G. cooperae,* and *G. minor*.

The second largest orchid subfamily is Orchidoideae, which comprises of 200 genera, found mostly in temperate areas (Chase *et al.* 2017). Of the 160 species found in New Zealand, the largest genus is *Pterostylis* (Pterostylidinae) with 35 species, followed by *Thelymitra* (Thelymitrinae) with 29 species (George 2012). The range of origin and distribution of *Thelymitra* is from South East Asia to Australia and Pacific, while *Pterostylis* is restricted to Australia and the Pacific region (Givnish *et al.* 2016).

Furthermore, over half of New Zealand orchid species are endemic or distinct from those found in other countries, distributed over a wide range of environment from coastal to alpine environments (George 2012). Of 134 species under conservation status, a quarter of New Zealand orchids are regarded as threatened or at risk or data deficient (de Lange *et al.* 2018), identifying that in New Zealand orchids are a priority for conservation.

1.2.2. Orchid conservation: *ex situ* versus *in situ*

Most *in situ* conservation is conducted in natural reserves, forest areas, or protected (forest) areas. All orchid species are becoming increasingly endangered owing to loss of habitat due to human activities (fire, deforestation, illegal logging and illegal harvesting), urbanization, industrialization and economic development, natural disaster and global warming (Barman and Devadas 2013; De and Medhi 2014).

To be precise, there are challenges to *in situ* orchid conservation because human intensive land use neglects the conservation value of the production ecosystem; for example, massive land conversions for agricultural plantation and deforestation consequently trigger environmental changes (Swarts and Dixon, 2009; Seaton *et al.* 2010; Seaton *et al.* 2013). As a consequence, extreme climate change is reflected in global ecosystem and environmental degradation, which negatively affects soil quality causing a reduction or loss of fungi that are important orchid symbionts (Whigham *et al.* 2006; Swarts and Dixon 2009; Cribb 2011; Popova *et al.* 2016) thus *ex situ* conservation will be an important option.

Ex situ conservation by assisted migration enhances plant movement to new suitable climate conditions to maintain biodiversity (Williams and Dumroese 2013) and seed banking (with appropriate knowledge) can also support programmes of assisted migration since seeds are very important as a source of propagation material. Considering orchids' high dependency on the ecosystem, especially pollinators and mycorrhizal fungi, *ex situ* seed conservation becomes the most viable choice to protect orchids from extinction. Furthermore, information from

identification through characterisation and evaluation of germplasm, along with their *in situ* conservation status will contribute to creating guidelines to plan future conservation strategies, to identify gaps in *in situ* collections and set priorities on *ex situ* conservation.

Orchid ex situ conservation was applied as both in the field (living collection) and seed bank (Seaton *et al.* 2010). Living collections, usually in botanic gardens or greenhouses, typically contain large number of species but only few individual specimens. Large diversity of genus or species are held in different collections but genetic diversity within species is much lower than present in their natural environment. There is a high risk of genetic bottlenecks or breeding depression in progeny generated from parent with restricted genetic diversity. Seed banking is the best alternative way to accommodate orchid conservation of wider genetic foundation in storage (Seaton and Pritchard, 2003; Seaton *et al.* 2013).

Most orchid seeds are known to have orthodox behaviour, but their storability in seed bank standard conditions (<5% MC, -20 ºC) is poor with most species experiencing poor seed viability after one year of storage (Seaton *et al.* 2013). More research is required to understand factors that determine variation in seed storability. The presence of oxygen (Groot *et al.* 2012), lipid composition and biothermal properties (Ballesteros and Walter 2011) were suggested as factors influencing seed ageing and deterioration during storage but there has been little research in orchids. Furthermore, optimal storage conditions related to moisture content, relative humidity and temperature may be species specific, thus research in various storage conditions are needed to support orchid seed banking.

1.2.3. Orchid seed characteristics

Seed morphology can provide relevant information on orchid evolution and adaptation. Previous studies have used seed morphology to investigate taxonomic, phylogenetic, and phytogeographic relationships among orchid species (Arditti and

Ghani 2000; Akcin *et al.* 2009; Barthlott *et al.* 2014; Verma *et al.* 2014; Bianco *et al.* 2017; Calevo *et al.* 2017).

Characterisation of orchid seed morphology is related to biological and ecological processes such as seed dormancy and germination (Gallo *et al.* 2016; Prasongsom *et al.* 2017), adaptation to habitat for seed dispersal (Arditti and Ghani 2000; Tsutsumi *et al.* 2007; Gamarra *et al.* 2018) and seed conservation in storage (Neto and Custadio 2005).

The seed structure of orchids is relatively unique and generally consists of only a small globular embryo, with few cells, mainly epidermal and parenchyma cells (Richardson *et al.* 1992). Orchid seeds contain fatty acids as lipid reserves (Hosomi *et al.* 2012; Colville *et al.* 2016), and as an important component of membrane lipids (Meyer and Kinney 2009). Rather than endosperm, the orchid embryo is surrounded by air within the membranous testa. An air sac in orchid seed occupies approximately 0.4-98.7% of the seed volume depending on the species (Arditti and Ghani 2000).

Previous studies show that seed ageing is accelerated under high-pressure oxygen in conventional dry seed banking (Groot *et al.* 2012), while anoxic conditions prolong storage life (Groot *et al.* 2015); this indicates that oxygen plays a vital role in seed deterioration. The close proximity of air to the embryo in the orchid family (Orchidaceae) is suggested to contribute to oxidative stress and induces oxidation of lipid compounds triggered by changes in storage temperature, accelerating ageing during storage. Research in some orchids seed, *Dactylorhiza fuchsia* and *Grammatophyllum speciosum,* showed that fatty acids undergo auto-oxidation of membrane lipids, the primary factor in seed ageing, resulting in the peroxidation of lipids and the production of volatiles (Colville *et al.* 2012).

Even though recent developments with GC-MS techniques now enable both fatty acids and volatiles production analysis in small quantities (mg) of minute orchid seeds (Colville *et al.* 2016), there are a few reports that assess the biochemical changes during ageing in orchid seed. Thus, a better understanding of the

mechanisms associated with seed longevity and viability loss might be explained through changes in fatty acids and volatiles during seed ageing.

Seed ageing occurs with time and deterioration of the seed is indicated by decreasing seed viability. Exposure of the seed to high temperature and high humidity accelerates deterioration thus decreasing seed viability. To understand the factors resulting in loss of viability in orchid seed, many studies had developed accelerated ageing protocols and compared these with natural ageing during storage to predict the seed storage longevity (Ellis and Roberts 1980; Dickie *et al.* 1990; Hay *et al.* 2003; Pritchard and Dickie 2003; Mead and Gray 2007; Balesevic-Tubic *et al.* 2011). Slow ageing is thought to mimic the process occuring during natural ageing at the seed, more closely than artificial ageing.

The proportion of air and embryo volume within orchid seed is also known to relate to their ability to germinate (Prasongsom *et al.* 2017). Prasongsom *et al*. (2017) investigated *ex vitro* germination of nine mature *Dendrobium* seeds and concluded that seeds with a large embryo (based on ratio between air space and embryo volume) showed absence of seed dormancy and were able to germinate *ex vitro*. However, orchid seed germination rates in nature are poor with seedling development less than 1% (Arditti and Ghani 2000; Batty *et al.* 2001), and constrained by dependence on pollinators and mycorrhizae (Seaton *et al.* 2013; Merritt *et al.* 2014).

1.2.4. Orchid seed germination

Orchid is a mycoheterotroph plant and needs pollinators to produce viable seed (Dutra *et al.* 2009; Cribb 2011). Some orchids also need a specific interaction with mycorrhizal fungi to support their seed germination and seedling development (Baskin and Baskin 1998; McKendrick *et al.* 2000; Bustam *et al.* 2014; Fay *et al.* 2015; Khamchatra *et al.* 2016). This is because orchid seed as already described comprises of an unwrapped embryo with no or exiguous food reserves (Gale *et al.* 2010). This symbiotic relationship with fungi results in infection of the seed, which

produces sugar and other nutrients to promote seed germination and growth (McKendrick *et al.* 2000; Bustam *et al.* 2014; Khamchatra *et al.* 2016).

Good germination and propagation methods are very important in seed conservation to ensure the sustainability after storage. The most effective method for overcoming the problem of orchid seed propagation is by using *in vitro* culture. The *in vitro* technique has become the most favoured method in orchid propagation since the first successful *in vitro* orchid seed germination without fungi interaction was obtained by Knudson in 1922 (Yam and Arditti 2009). *In vitro* culture is defined as "cell, organ, or tissue culture are grown under artificial media and sterile condition in tubes, glasses, or dishes" (Schlegel 2003). This technique has been widely investigated to enhance orchid seed germination and growth.

Orchid seed can germinate under artificial media supplemented with vitamins and nutrients in *in vitro* culture, which in nature is supplied by mycorrhizae. *In vitro* protocols can be either symbiotic, in which the culture medium is inoculated with compatible mycorrhiza strain, or asymbiotic, in which seeds are germinated without inoculation of fungi (Zettler and Mclnnis 1994; Zettler and Hofer 1998; Bustam *et al.* 2014; Nikabadi *et al.* 2014). Both symbiotic and asymbiotic of epiphytic and terrestrial orchid seed germination and growth have been widely studied. (Miyoshi and Mii 1995; Rasmussen 1995; da Silva 2013; Zhang *et al.* 2013; Bustam *et al.* 2014).

Despite symbiotic germination, successful asymbiotic *in vitro* orchid seed germination on various culture media has been widely reported. However, these are species specific, both for epiphytic or terrestrial species (Miyoshi and Mii 1995; Nadarajan *et al.* 2011; Dowling and Jusaitis 2012; da Silva 2013; Zhang *et al.* 2013; Bustam *et al.* 2014; da Silva *et al.* 2015; Rasmussen *et al.* 2015; Kartikaningrum *et al.* 2017). Protocols for asymbiotic orchid seed germination typically use media with high soluble vitamins and nutrients, and the choice of medium predominantly affects seed germination and seedling (shoot and root) development (Zeng *et al.* 2011)**.** The *in vitro* medium provides nutrition to enable the unwrapped orchid embryo to germinate and grow.

Investigating the most applicable germination medium for particular species is important in seed propagation to support orchid seed *ex situ* conservation programmes.

1.2.5. Biotechnology to support orchid seed storage: an opportunity for cryopreservation

The storage behaviour of many orchid seeds is regarded as orthodox due to their tolerance to desiccation (Pritchard and Seaton 1993; Seaton *et al.* 2010), thus reducing seed moisture content and storage temperature is expected to increase seed longevity in storage (Pritchard and Seaton 1993). However, some orchid species have a more complex behaviour in which they are tolerant to low moisture content but appear to be short-lived compared with the longevity characteristics (e.g. time for viability to fall to 50% (P50)) of other species in conventional dry storage (Seaton and Pritchard 2003).

Biotechnology offers an option for extending longevity in storage by application of cryopreservation i.e. storage in liquid nitrogen at sub-zero temepratures -150 to -196 ^{o}C (Engelmann 2010). The benefit of cryopreservation for orchid seed in prolonging lifespan (Mweetwa *et al.* 2007; Seaton *et al.* 2013) might not be restricted to protecting the tissues, but also due to the anoxic state induced by immersion in pure liquid nitrogen. Nitrogen rapidly replaces oxygen in the air-space around the embryo improving seed tolerance to detrimental effects of cell freezing (Mweetwa *et al.* 2007).

Feasible cryopreservation methods for orchid seed storage have been developed over a number of decades (Pritchard 1984; Popova *et al.* 2003; Vendrame *et al.* 2007; Thammasiri 2008; Hu *et al.* 2013; Vendrame *et al.* 2014; da Silva *et al.* 2015; Popova *et al.* 2016; Hughes and Kane 2018; Schofield *et al.* 2018). Cryopreserved Taiwanese terrestrial orchid seed, *Bletilla formosana* (Hayata) retained 91% germination following cryopreservation using vitrification technique (Hu *et al.* 2013). Successful cryopreservation of hybrid orchid seed, *Bratonia*, was also

achieved where there was 100% germination following direct immersion of the seed in liquid nitrogen (Popova *et al.* 2003).

However, there is large variation in the response of temperate orchid seeds after cryopreservation, for example, *Platanthera* and *Dactylorhiza* genera, showed variation in germination after fast immersion in liquid nitrogen (Nikishina *et al.* 2007). Success of seed germination after cryopreservation may be varied, depending on species (Nikishina *et al.* 2007). However, Galdiano *et al.* (2014) found that cryopreservation does not affect genetic structure after storage, thus being a promising method to support orchid seed *ex situ* conservation strategies (da Silva 2013; Merritt *et al.* 2014; Popova *et al.* 2016).

From the general review above, it can be concluded that environmental differences, such as living habitats and biogeographical origin, are potential factors that influence seed characteristics, and these traits could determine the success of seed conservation strategies.

1.3. Research aims

The orchid species selected for study are two epiphytic *Dendrobium* species from Indonesia (*D. strebloceras* and *D. lineale*), one epiphytic *Dendrobium* from New Zealand (*D. cunninghamii*), and three terrestrial orchids from different genera from New Zealand (*Gastrodia cunninghamii, Pterostylis banksii, Thelymitra nervosa*).

The New Zealand orchid flora comprises 25 genera and over 100 species, most of which are terrestrial (Lechnebach *et al.* 2013). The New Zealand Plant Conservation Network currently have conservation status records for 120 orchid species with 11 species being classified as 'threatened' and 31 species as 'at risk' (http://nzpcn.org.nz/). In contrast, Indonesia has more than 5,000 orchid species, both epiphytic and terrestrial, and these are found in very diverse habitats (Yahman 2009; Rukmana 2000; Munawaroh and Yuzammi 2019).

Considering the background information and the paucity of comparative studies on orchid seeds to support seed conservation, this thesis aims to compare seed characteristics (qualitative and quantitative morphologies, seed oil contents) of selected New Zealand and Indonesian orchids, specifically comparing epiphytic tropical and temperate *Dendrobium* (Epidendroideae-Dendrobiinae), and selected terrestrial temperate species from different genera (Epidendroideae-Gastrodieae; Orchidoideae-Pterostylidinae; Orchidoideae-Diurideae). Insubsequent experiments, multivariate analyses regarding habit, taxonomy and distribution were not tested because of the limitations of low replication. The main interest was the effects of the treatments imposed on different species.

Presently, orchids are still not well researched. According to The International Union for Conservation of Nature (IUCN), research involving orchids included only 1641 of 27800 species (https://www.iucnredlist.org/search?taxonomies=101295&searchType=species). Of the species used in this study, *Dendrobium Strebloceras* was reported by IUCN as having a data deficient status (Chadburn and Schuiteman, 2020) and *D. lineale* as a species of least concern (Chadburn and Schuiteman, 2018). *Dendrobium cunninghamii*, a New Zealand species, was chosen for comparison because of its similar taxonomic traits to the two Indonesian species (*D. strebloceras* and *D. lineale*). None of the four New Zealand species used in this study have been assessed by IUCN.

Considering New Zealand and Indonesia are countries with a high proportion of threatened species (Bradshaw *et al.* 2010), more research into New Zealand and Indonesian orchids is needed to support their conservation. Even though *P. banksii*, *G. cunninghamii, T. nervosa* and *D. cunninghamii* are not currently threatened (de Lange, 2020; NZPCN 2020), the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) put all orchid species in the threatened list to indicate the urgency of orchid conservation, including all species in this study.

The goal of this study is to support orchid seed conservation by determining the optimal protocols for asymbiotic *in vitro* seed germination and cryopreservation of selected Indonesian and New Zealand orchid species.

The specific aims or objectives of this study were:

1. To determine and compare orchid seed morphology (seed and embryo characteristics, air volume) for different taxa (Orchidoideae, Epidendroideae), growth habits (epiphytic, terrestrial) and biogeographical origin (tropical, temperate)
2. To determine lipid (fatty acid methyl esters) composition and content in orchid seed from a range of species over time, to determine if changes in lipid composition during the natural ageing process are correlated with loss of viability
3. To investigate the best asymbiotic *in vitro* culture media for promoting seed germination and seedling development of the orchid species selected
4. To determine orchid seed cryopreservation protocols that result in high germination and seedling development in the species selected

1.4. Research framework

For all the experiments, the first step was a viability test for each orchid species, to ensure that the seeds are viable and therefore suitable for use in this research. Two seed characteristics were measured in this study. The first is micromorphological measurement on orchid seed (seed and embryo volume, air-space percentage), and the second is seed oil compounds and contents in form of fatty acid methyl esters (FAMEs), either in fresh seed or after natural ageing (60% RH at 20°C).

An asymbiotic *in vitro* germination protocol was developed to identify the best culture media from the following: Murashige and Skoog (MS), Norstog, Vacin and Went (VW), Basic culture medium 1 (BM1), ½ MS, agar only, with combination of 2 or 3% sucrose. Additionally, the best cryopreservation method was determined by comparing seed germination and seedling performance after: (1) vitrification treatment at different application times of the cryoprotectant plant vitrification solution 2 (PVS2) (0, 20, 50, 70 minutes) and at different temperatures (room or on ice), and (2) direct immersion into or above liquid nitrogen vapour, to develop seed storage protocols at -167 to -196°C.

The research scheme of this study and thesis structure is summarised in Figure 1.1.

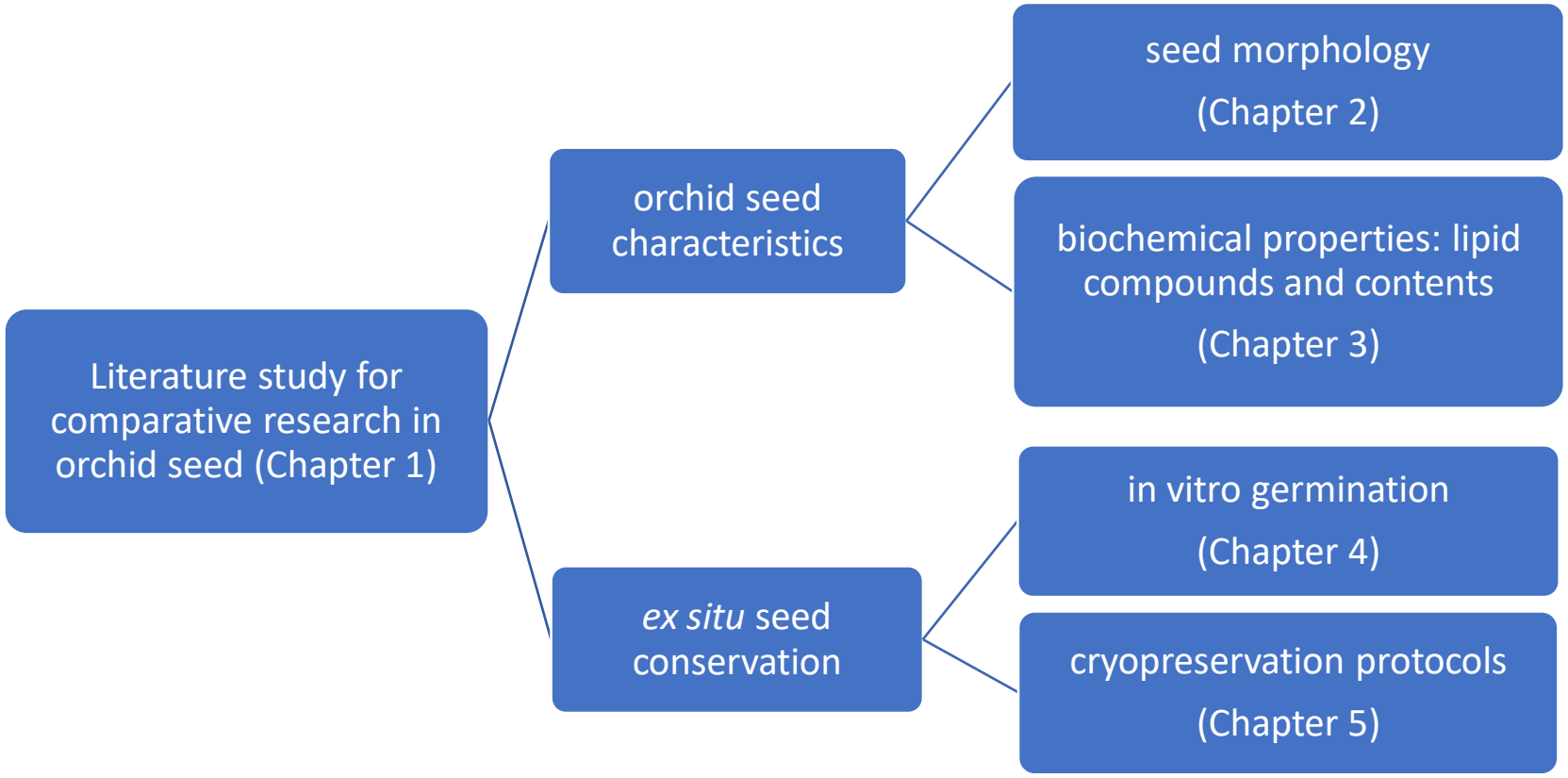

Figure 1.1. Research scheme and thesis structure for investigating seed characteristics and cryopreservation to support *ex situ* conservation strategies for orchid seed

1.5. Structure of the thesis

The structure of this thesis is based on the gaps and aims that have been identified in the previous sections (1.2) of this chapter and the introductions to the other thesis chapters. This research has been developed into four experimental chapters which have been submitted as publications. Each publication forms a chapter of the thesis. This thesis begins with an introduction (Chapter 1) and followed by four experimental chapters (Chapter 2 to Chapter 5).

Each experimental chapter was presented and formatted based on specific journal with some edits and revisions. The thesis ends with a general discussion and conclusions (Chapter 6). A more detail of the structure of this thesis is described as below:

Chapter 1.

Introduction: Background, aims and research framework

This chapter describes the background and review of the literature to gather information and identify gaps in knowledge, describes the aims and structure of the thesis, ends with research framework that describes activities on this research and structure of the thesis in general.

Chapter 2.

Comparative seed morphology of tropical and temperate orchid species with different growth habits

The literature review in Chapter 1 identified the large orchid diversity as well as the relatively unique seed morphology found in Orchids. Chapter 2 therefore, reports on the orchid seed characteristics of the six orchid species studied. This chapter compares qualitative assessments (seed pod colour; seed colour; seed shape) and quantitative morphological traits (seed pod size; seed length, width and volume; embryo length, width and volume; air volume). Here, the aim is to address the gaps

in correlation between orchid seed morphology with taxonomy, biogeographical origin, or growth habit of the studied species.

The results showed that tropical epiphytic *D. strebloceras* have the biggest seeds (1.76 mm in length), as well as the highest air volume percentage (92.4%) compared with seeds from other species studied. The second largest seed is the temperate terrestrial *P. banksii* (1.09 mm) with air occupying about 75.3% of seed volume. From this study, we concluded that there is high variability in the micro-morphological seed characteristics of these orchid species unrelated to their taxonomy, biogeographical origin, or growth habit, suggesting different ecological adaptations possibly reflecting their modes of dispersal.

This work was published in the Journal Plants (Plants 2020, 9 (2), 161; doi:10.3390/plants9020161) in Special issue: Linking Seed Biology to Plant Preservation: New Advances and Perspectives.

Chapter 3.

Variation in seed fatty acid profiles of six orchid species during natural ageing

The literature reported in Chapter 1 identified that orchid seeds contain lipids and that changes in lipid composition during seed storage could be related to seed lifespan. Oxygen was also found to affect seed deterioration due to oxidative stress in storage. The results reported in Chapter 2 showed large variability in air volume surrounding the embryo across the orchid species studied.

Chapter 3 presents a comparative study on lipid compositions and lipid contents in the six orchid species studied, and changes observed in lipids during storage in close to New Zealand natural conditions (20°C room temperature with 60% humidity). Unsaturated linoleic acid (18:2) and oleic acid (18:1) were the most abundant fatty acids in the fresh seeds and found in higher quantities in epiphytic *Dendrobium* compared with terrestrial species, accounting for over 70% of the total lipid content.

Our result suggests that the high abundance of unsaturated fatty acids in the fresh seeds of these orchids, and their consequent degradation, are major contributors to

the seeds' low viability after storage. We postulate that there is a phylogenetic signal to seed lifespan in orchids where taxonomic relatedness rather than distribution range may be a good predictor of seed lipid composition and ageing behaviour in orchids.

This work is under review in the journal Plant Biology.

Chapter 4.

Asymbiotic *in vitro* seed germination, protocorm multiplication and seedling development of tropical and temperate epiphytic and terrestrial orchids

Most orchid seeds have high dependency to mycorrhizal symbionts and require complex environmental conditions to support seed germination and seedling development (Chapter 1). Fast development in tissue culture technqiues has enabled asymbiotic *in vitro* seed germination as practical method in orchid seed propagation, compared with symbiotic germination which requires the associated mycorrhizal fungi. For successful asymbiotic germination, determining suitable germination media is critical. This is further complicated since the media requirement may be species-specific. The ideal media for germination may not necessarily be the same between species or to support seedling growth. Our results found that seed germination for all *Dendrobium* species were better on either on VW or Norstog media, but the best seedling performance for tropical species was found either on full or half-strength MS media. 2% sucrose was found adequate to support seedling development for *D. strebloceras*.

Previous results (Chapter 3), showed that the seed viability test using 2,3,5-Triphenyl tetrazolium chloride (TTC) was ambiguous for the brown-coloured terrestrial *P. banksii* seed. Thus, in this chapter 4, seed germination and seed viability of studied species was compared and tetrazolium staining modified to utilise vacuum degassing to improve staining of brown *P. banksii* seed. Staining at full vacuum (0 atm) condition for 30 minutes improved the clarity of embryo staining.

Terrestrial orchids showed good germination in all media tested however, the best seedling development of *P. banksii* and *T. nervosa* was recorded on either Norstog or BM1 media. *D. cunninghamii* and *G. cunninghamii* seeds were unable to develop beyond embryo swelling stage, possibly due to morpho-physiological dormancy.

This work was published in the Journal of Plant Cell, Tissue and Organ Culture (PCTOC, 2020. 143: 619-633. doi: 10.1007/s11240-020-01947-7).

Chapter 5.

Seed cryopreservation of Indonesian and New Zealand epiphytic and terrestrial orchids

There is evidence in the literature (Chapter 1) that orchid seed conservation at conventional seed banking temperatures of -20°C showed rapid loss of viability compared with other plant species. This has resulted in research into cryopreservation as a good alternative in orchid seed conservation. In this chapter 5, vitrification cryopreservation using plant vitrification solution 2 (PVS2) and direct immersion of dry seeds into liquid nitrogen was evaluated. The results suggested direct immersion may be a fast and effective technique for cryopreservation of orchid seeds of the species studied. Moreover, immersion in liquid nitrogen improved seed germination percentage (Chapter 5) in comparison to the control treatment without immersion in liquid nitrogen (Chapter 4).

This work will be submitted to the Journal of Plant Cell, Tissue and Organ Culture.

Chapter 6.

General discussion, findings and conclusions

This chapter brings together the results and findings from Chapters 2, 3, 4 and 5 and provides recommendation for future work. This chapter also describes the conclusions of this thesis.

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CHAPTER II

Comparative Seed Morphology of Tropical and Temperate Orchid Species with Different Growth Habits

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2.1. Abstract

Seed morphology underpins many critical biological and ecological processes, such as seed dormancy and germination, dispersal, and persistence. It is also a valuable taxonomic trait that can provide information about plant evolution and adaptations to different ecological niches. This study characterised and compared various seed morphological traits, i.e., seed and pod shape, seed colour and size, embryo size, and air volume for six orchid species; and explored whether taxonomy, biogeographical origin, or growth habit are important determinants of seed morphology. Two tropical epiphytic orchid species from Indonesia (*Dendrobium strebloceras* and *D. lineale*), and four temperate species from New Zealand, terrestrial *Gastrodia cunnninghamii*, *Pterostylis banksii* and *Thelymitra nervosa*, and epiphytic *D. cunninghamii* were investigated. Results showed some similarities among related species in their pod shape and colour, and seed colouration. All the species studied have scobiform or fusiform seeds and prolate-spheroid embryos. Specifically, *D. strebloceras*, *G. cunninghamii*, and *P. banksii* have an elongated seed shape, while *T. nervosa* has truncated seeds. Interestingly, high variability in the micro-morphological seed characteristics of these orchid species were observed, unrelated to their taxonomy, biogeographical origin, or growth habit, suggesting different ecological adaptations possibly reflecting their modes of dispersal.

Keywords: air space; epiphytic; micro-morphometric; temperate; terrestrial; tropical

2.2. Introduction

Most orchid species are endangered because of over collection and loss of habitat, so there is an urgent need to develop techniques to conserve them both *in situ* as well as *ex situ* (Rubluo *et al*. 1993). Seed morphology is an important trait related to biological and ecological processes such as seed dormancy and germination (Prasongsom *et al*. 2017; Gallo *et al*. 2016), adaptation to habitat for seed dispersal (Healey *et al*. 1980; Arditti and Ghani 2000; Tsutsumi *et al*. 2007; Gamarra *et al*. 2018), and seed storability (Neto and Custodio 2005). Therefore, investigating seed morphology is relevant to understanding the plant's reproduction under natural conditions and to devise optimal protocols for seed storage and propagation.

Seed morphology can also provide relevant information about orchid evolution and adaptations. This information could be particularly useful in comparative studies, since seed characters are considered inherent traits, being more conservative than other features. Previous studies have used seed morphology to investigate taxonomic, phylogenetic, and phytogeographic relationships among orchid species (Arditti and Ghani 2000; Akcin *et al*. 2009; Barthlott *et al*. 2014; Verma *et al*. 2014; Bianco *et al*. 2016; Calevo *et al*. 2017; Chase *et al*. 2017).

Orchid seeds are extremely light and small, compared with those in other plant families, and are produced in large numbers (reaching up to a million) inside a seed pod (Arditti and Ghani 2000). A unique characteristic of orchid seeds is that instead of an endosperm, there is an 'air space' surrounding a small globular embryo within a membranous testa. The air space volume in orchid seeds varies depending on the species. There is a relationship between seed size and embryo volume that determines the proportion of air space (Arditti and Ghani 2000; Barthlott *et al*. 2014). Moreover, Arditti and Ghani (2000) and Leck *et al*. (2008) suggested that the amount of air trapped influences seed dispersal by affecting floatability (in air and water) and buoyancy, reflecting ecological adaptations to different distribution ranges (which is relevant to *in situ* conservation). For this reason, studies investigating the air space within the seed and its relation to taxonomy and living habitat are important to identify the best conservation strategy.

Historically, orchid species have been classified into five subfamilies by floral morphological characters (Dressler 1979). Among those five subfamilies, Epidendroideae and Orchidoideae are the largest in the orchid family (Orchidaceae), comprising 84% and 14% of the described orchids, respectively (Chase *et al*. 2017). Most orchids belonging to the subfamily Epidendroideae are tropical epiphytic orchids (Lavarack *et al*. 2000). In contrast, most of the Orchidoideae occupy an ecological niche as temperate terrestrial species with mycorrhizal symbionts (Chase *et al*. 2017). Therefore, it is possible that species belonging to these two subfamilies have different adaptations to their ecological niches.

Indonesia, a tropical country, is the habitat of a great diversity of orchids, with roughly 5000 species (Irawati 2013), most of which are epiphytic. In contrast, New Zealand is temperate, having around 160 orchid species in 30 genera. These are predominantly terrestrial and over half are endemic (George 1999; de Lange *et al*. 2009).

This study characterised and compared the morphological seed traits of two tropical epiphytic species, *Dendrobium strebloceras* and *D. lineale* from Indonesia (subfamily Epidendroideae) and four temperate species from New Zealand, the endemic epiphytic orchid *D. cunninghamii* (Epidendroideae) and terrestrial orchids *Gastrodia cunnninghamii* (Epidendroideae), *Pterostylis banksii* (Orchidoideae), *Thelymitra nervosa* (Orchidoideae). The aim of the study was to determine whether taxonomy, biogeographical origin, or growth habit of the species are important determinants of orchid seed morphology.

2.3. Materials and methods

2.3.1. Seed materials

Seeds of six orchid species from Indonesia and New Zealand were collected from mature and naturally dehiscing capsules. Two orchid species from tropical Indonesia, an epiphytic-lithopytic *Dendrobium lineale* (morebe shower/kui blue)

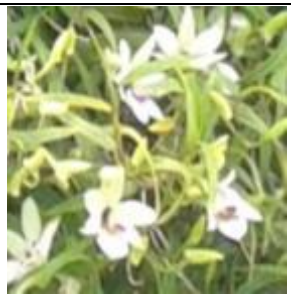
and epiphytic *Dendrobium strebloceras* (twist-horn *Dendrobium*), were obtained from the IAARD (Indonesia Agency for Agricultural Research and Development) collection. They were hand-pollinated and grown in glasshouse conditions at Cipanas Experimental Field, West Java (tropical rainforest climate, 19 ± 6 °C, ~ 1100 m a.s.l.). Two mature capsules from *D. lineale* and one mature capsule from *D. strebloceras* from were obtained from one plant at about 5 months post pollination. One epiphytic-lithophytic temperate orchid, *D. cunninghamii* (winika/pekapeka) seeds were collected from several plants Pukeiti Forest, North Island, New Zealand (temperate rainforest climate, 8 ± 5 °C, ~ 490 m a.s.l.).

Seed pods of three terrestrial New Zealand orchids, *Gastrodia cunninghamii* (potato orchid/huperei), *Pterostylis banksii* (greenhood orchid/tutukiwi) and *Thelymitra nervosa* (spotted sun orchid), were collected from Iwitahi Native Orchid Heritage Protection Area where they were growing under a *Pinus nigra* plantation at Kaingaroa forest, Taupo Napier Road, North Island (marine west coast climate, 11 ± 5 °C, ~544 m a.s.l.). Seed pods from New Zealand species were collected from several plants and bulked. The plant taxonomy, morphology, biogeographical origin, and ecological habits for each species are described in Table 2.1.

Table 2.1. Plant taxonomy, morphology, biogeographical origin and ecological habits of the orchid species used in this study

Species (Common name)	**Sub family/ tribe/ Genus/ Section**	**Distribution/ Conservation status**	**Habitat & Morphology**	**Flowering time/ Flower duration/ Capsule maturity time**	**Flower**	**Reference(s)**
D. strebloceras (twisted horn *Dendrobium*)	Epidendroideae/ Dendrobiinae/ *Dendrobium*/ *Spatulata* Lindl.	Indonesia. The Moluccas, Halmahera Island / Data deficient	Habitat: tropical epiphytic, rain forests, low altitudes, warm conditions, high humidity, bright-filtered light Morphology: - Large-sized plant - Pseudobulbs: up to 1.5 m long, 1.5 cm in diameter - Leaves: fleshy, ovate-oblong to lanceolate along the upper half, up to 16 cm long, 5 cm wide - Inflorescence: up to 40 cm long, 6-8 flowers, each up to 5 cm long, fragrant - Petal and sepal: twisted, pale yellow or green suffused with brown to dark brown, darkens with age	Throughout the year/ up to 2 months/ 150 days	 (Photo: Suskandari)	Lavarack *et al.* 2000; Chadburn and Schuiteman, 2020

Species (Common name)	Sub family/ tribe/ Genus/ Section	Distribution/ Conservation status	Habitat & Morphology	Flowering time/ Flower duration/ Capsule maturity time	Flower	Reference(s)
D. lineale (morobe shower orchid, kui blue)	Epidendroideae/ Dendrobiinae/ *Dendrobium/ Spatulata* Lindl.	Indonesia, Irian Jaya border to Milne Bay (North-eastern coast of New Guinea) / Least concern	Habitat: Tropical epiphytic/lithophytic, rain forests, low altitudes (coastal or water streams at sea level to above 800 m), warm conditions, high humidity, direct sunlight Morphology: - Large-sized plant - Pseudobulbs: up to 2 m long and 2-3 cm in diameter - Leaves: fleshy, leathery oblong to lanceolate along the upper two-thirds of stem, 8-15 cm long - Inflorescence: up to 75 cm long, arching, flowers up to 5 cm across - Petal and sepal: white, pale yellow–purplish vein predominating lip, dark brown at edge of lower petals	Throughout the year/ up to 2-3 months/ 120 days	 (Photo: Suskandari)	Lavarack *et al.* 2000; Chadburn and Schuiteman, 2018

Species (Common name)	Sub family/ tribe/ Genus/ Section	Distribution/ Conservation status	Habitat & Morphology	Flowering time/ Flower duration/ Capsule maturity time	Flower	Reference(s)
D. cunninghamii (christmas orchid, bamboo orchid, winika, pekapeka)	Epidendroideae/ Dendrobiinae/ *Winika*/ -	New Zealand (endemic). North and South Islands, the Chatham & Stewart Island / Not threatened	Habitat: Temperate epiphytic/lithophytic, moist rain forests, low altitudes below 500 m), intermediate-cool temperature, semi-shade Morphology: - Pseudobulbs: long, slender, yellowish canes up to 2 m long, branched, pendulous, clustering in large clump - Leaves: leaf sheath throughout stem, lateral, borne on the upper half of final branches of the stem, 2-3 cm long, 3-3.5 mm wide - Inflorescence: lateral, 3-6 flowers, each up to 2.5 cm across - Petal and sepal: white-yellow base with purple column wings, 4 yellow-green purple topped ribs in the mid-lamina, membranous segments, 3-lobed lip, broad labellum	December-January/ up to 10 days/ 1 month	 (Photo: Paul Topp)	Lavarack *et al*. 2000; New Zealand Native Orchid Group 2015; de Lange 2020

Species (Common name)	Sub family/ tribe/ Genus/ Section	Distribution/ Conservation status	Habitat & Morphology	Flowering time/ Flower duration/ Capsule maturity time	Flower	Reference(s)
G. cunninghamii (potato orchid)	Epidendroideae/ Gastrodieae/ *Gastrodia/* -	New Zealand. East coast of North and South Island, Middle and lower part of South Island, the Chatham & Stewart Island / Not threatened	Habitat: temperate, terrestrial, saprophytic, lowland to montane forests, common in mature pine plantations Morphology: - Tall plant (up to 1 m height) - Tuber: Large and extensively branched - Leaves: no leaves, no chlorophyll, plant is brown, black or greenish with tiny scales on the stem as residual leaves - Inflorescence: up to 70 flowers per stem, knobbly and tubular flowers - Petal and sepal: petals and sepals are merged and separated only at their tips, labellum is attached at the inner base of the tube, only black tipped yellow end shows	November-February/ up to 2 months/ information not available	 (Photo: Surya Diantina)	George 1999; New Zealand Native Orchid Group 2015; de Lange 2020

Species (Common name)	Sub family/ tribe/ Genus/ Section	Distribution/ Conservation status	Habitat & Morphology	Flowering time/ Flower duration/ Capsule maturity time	Flower	Reference(s)
P. banksii (greenhood orchid)	Orchidoideae/ Pterostylidinae/ *Pterostylis*/ -	New Zealand. North and South Islands (except some part of east-coast and middle of South Island), the Chatham & Stewart Island / Not threatened	Habitat: Temperate, terrestrial, lowland to montane and subalpine forests, scrub Morphology: - Tuber: ovoid, form dense colonies, plant height up to 50 cm (one of New Zealand's largest orchids) - Leaves: thin grass-like leaves, usually 4-6, up to 25 cm in length, long and smooth, internodes very short near the base, distributed up the stem - Petal and sepal: single flower, 3-5 cm high, green with translucent white stripes, tips of petals range in colour from white to orange-red, dorsal sepal very long, often slightly upturned at the tip. Petals much shorter than the dorsal sepal. Lateral sepals diverge at a narrow angle, tips very long, erect or swept backward. Labellum tip is flat or arched, top reddish. Stigma long and narrow. (Notes: Size, structure, and colouring can be quite variable)	October - December/ 1 month (*P. patens*)/ 2 months (*P. patens*)	 (Photo: Surya Diantina)	George 1999; Lechnebach 2005; New Zealand Native Orchid Group 2015; de Lange 2020

Species (Common name)	Sub family/ tribe/ Genus/ Section	Distribution/ Conservation status	Habitat & Morphology	Flowering time/ Flower duration/ Capsule maturity time	Flower	Reference(s)
T. nervosa (sun orchid)	Orchidoideae/ Diurideae/ *Thelymitra*/ -	New Zealand. Several parts of central and lower North Island, upper part and few areas on the east coast of South Island, the Chatham Island /Not threatened	Habitat: Temperate, terrestrial, open areas, low vegetation often on clay, wet, peaty areas Morphology: - Tuber: ovoid (oval) - Leaf: single, long, narrow and thin leaf, quite fleshy, channelled and keeled, sheathering the stem at its base - Inflorescence: bright colour flower, blooms in warm sunny days, regular shape, short column, grows up to 30 cm, up to 6 flowers of 1.5 cm in diameter - Petal and sepal: dark lavender-blue or pink, unstriped, petal spotted, column blue with thick erect white hairs on the horizontal arms, dark brown slightly hooded midlobe, warty with dark tubercles and yellow toothed rim	October - January/ 5 days (*T. longifolia*)/ information not available	 (Photo: Surya Diantina)	George 1999; Lechnebach 2005; New Zealand Native Orchid Group 2015; NZPCN 2020

2.3.2. Evaluation of seed morphological variability

Seed pod shape and seed colour were visually assessed and recorded. They were classified based on the general orchid seed colour proposed by Barthlott *et al.* (2014). Seed pod length was measured with a ruler and pictures of the seeds were taken using a Nikon DSLR camera on a copy stand with 1 cm grid (Kaiser RS1, Germany). To improve visibility, seeds were stained with a reagent, 2,3,5-Tetrazolium Chloride, following the procedure described by Hosomi *et al*. (2011). Both seed and embryo shapes and measurements were taken using a light microscope (microscope: Olympus SX7; light: Olympus DF PLAPO $1X_{-4}$, Olympus Optical, Tokyo, Japan) and photographed using a digital colour camera (Olympus SC 100, Olympus, Tokyo, Japan).

Seeds were categorised by size (seed length) as follows (Barthlott *et al*. 2014): very small (100–200 µm), small (200–500 µm), medium (500–900 µm), large (900–2000 µm), and very large (2000–6000 µm). Seed pods were also treated as categorical to correlate with seed size, and were classified according to size as follows: very small (10–20 mm), small (20–30 mm), medium (30–40 mm), large (40–50 mm), and very large (>50 mm).

2.3.3. Assessment on seed micro-morphometric

Seeds treated with 2,3,5-Tetrazolium Chloride (TTC) were measured under the microscope to determine seed size (length and width) and embryo size (length and width). Seeds with a length/width (SL/SW) above 5 were classified as elongated and those with an SL/SW below 5, as truncated. For each species, 50 seeds were measured, and the approximate seed, embryo and air space volume were calculated following Prasongsom *et al*. (2017), as below:

Embryo volume *:

$$EV = \left(\frac{4}{3}\right) X \left(\frac{22}{7}\right) \text{X} \left(\frac{EL}{2}\right) \text{X} \left(\frac{EW}{2}\right)^2 \quad (1)$$

Seed volume:

$$SV = 2\,X \left(\left(\frac{SW}{2}\right)^2 X \left(\frac{SL}{2}\right) X\,(1.047) \right) \quad (2)$$

Air space volume:

$$ASV = \frac{(\text{SV} - \text{EV x } 100)}{SV} \quad (3)$$

(SW = Seed width, SL = Seed length, EW = Embryo width, EL = Embryo length).

* For the measurement of embryo volume, we used the equation of Prasongsom *et al*. (2017) for prolate-spheroid-shaped embryos, which all seeds in this study had (Arditti and Ghani 2000). The equation may change for oblate-spheroid embryos.

2.3.4. Statistical analysis

To evaluate micromorphological differences between the seeds, a Multivariate General Linear Model (GLM) was used to explore the effect of taxonomy (subfamily), distribution range (temperate or tropical), habit (epiphytic or terrestrial) as independent variables on seed morphology traits (dependent variable). The MANOVA model is appropriate giving the categorical nature of the variables (IV) and large sample sizes.

To fulfil the assumptions of the model, univariate and multivariate outliers were removed, and data were transformed using a natural logarithm (Ln) to meet (or approximate) normality.

Since the model requires a moderate linear relationship, a correlation matrix was generated (Figure 2.1), and two variables, seed volume (SV) and embryo volume

(EV), were removed from the model since they had a strong positive correlation (R^2 > 90) with seed weight (SW) and embryo weight (EW), respectively. Pairwise post-hoc tests (LSD) were used to identify differences between species for each individual trait. Calculations were performed using IBM SPSS Statistics for Macintosh, Version 24.0. (IBM Corporation, Armonk, NY).

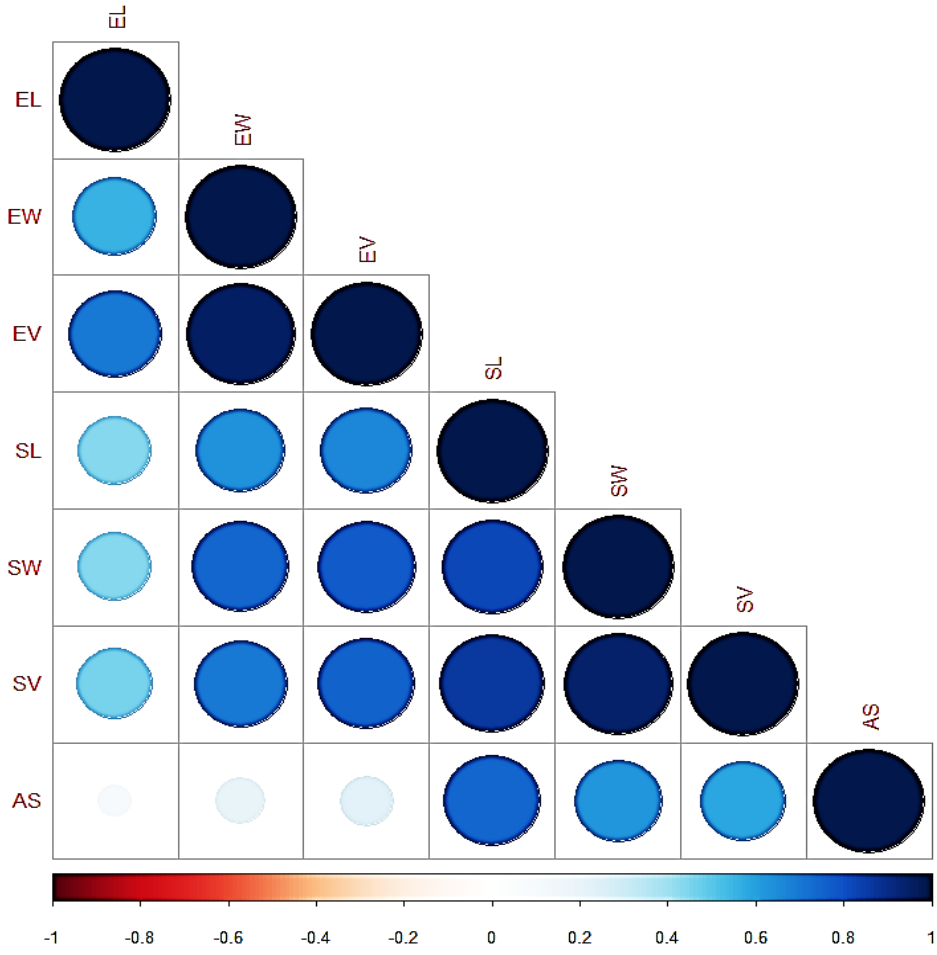


Figure 2.1. Correlation matrix between the studied morphological aspects. AS = air space, EW = embryo width, EV = embryo volume, SL = seed length, SW = seed width, and SV = seed volume

The relationship (ratio) between the different parameters investigated, i.e., EL/EW, SL/SW, and SV/EV was also calculated then analysed in the same way as described

above. There was little correlation found among these variables (Figure 2.2), so they were all included in the final model.

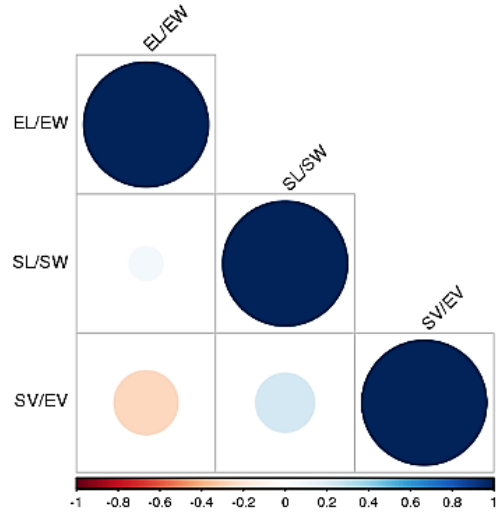


Figure 2.2. Correlation matrix between measurement ratios. EL = embryo length, EW = embryo width, SL = seed length, SW = seed width, SV = seed volume, and EV = embryo volume

2.4. Results

2.4.1. Pod and seed morphology

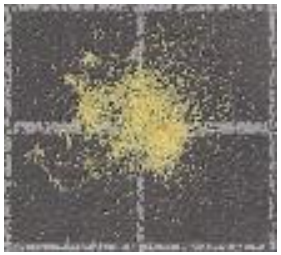
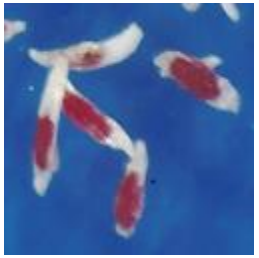
Dendrobium species produced yellow-green seed pods when mature (Figure 2.3), while the other three genera; *Gastrodia*, *Pterostylis*, *Thelymitra*, produced brown pods with small differences in hue at maturity. In the subfamily of Epidendroideae, a large variation in pod length was found in the tribe *Dendrobieae* (Table 2.2). *D. strebloceras* produced the largest seed pods, followed by *D. lineale*, while *D. cunninghamii* was found to produce the smallest pods. Terrestrial temperate species, *G. cunninghamii* (Epidendroideae), *P. banksii and T. nervosa* (Orchidoideae) produced intermediate-sized pods. All pods split after seeds inside the pod reached maturity.

Table 2.2. Variation in colour and size of seed pods and seeds in two tropical and four temperate orchid species

Species	Seed Pod Length (cm)	Mature Pod Colour	Seed Colour
Tropical epiphytic			
D. strebloceras	6–7 (very large)	Yellowish green	Whitish yellow
D. lineale	3.5–4 (medium)	Yellowish green	Yellowish golden
Temperate epiphytic			
D. cunninghamii	1–1.5 (very small)	Yellowish green	Brownish yellow
Temperate terrestrial			
G. cunninghamii	2–2.5 (small)	(Dark) brown	Brownish tan (light brown)
P. banksii	2–2.5 (small)	(Whitish) brown	Ochre (deep-orange brown)
T. nervosa	1.5–2 (very small)	(Purplish) brown	Dark brown

Seeds in the *Dendrobium* species were generally yellow with slight differences in intensity; the remaining genera produced brown seeds that also varied in tone intensity, from light to dark brown. Moreover, during seed morphology assessment, seeds of *D. strebloceras* and *P. banksii* were found to have a sticky or adhesive surface.

Irrespective of their taxonomy or ecological habitat, most species in this study had a fusiform or scobiform seed shape and prolate-spheroid embryos (Figure 2.3). Specifically, *D. strebloceras*, *G. cunninghamii*, and *P. banksii* had an elongated-balloon shape and *T. nervosa* had a truncated seed shape, tapered at one end and blunt at the other end.

Species	Seed pod	Seed colour and size	Seed shape (light microscope image)
D. strebloceras			
D. lineale			
D. cunninghamii			

Species	Seed pod	Seed colour and size	Seed shape (light microscope image)
G. cunninghamii			
P. banksii			
T. nervosa			

Figure 2.3. Morphological characteristics of orchid pods and seeds. White squares represent one centimetre square.

2.4.2. Seed micromorphology assessment

A general linear model (GLM) revealed no effect of taxonomy (Epidendroideae or Orchidoideae), geographical origin (tropical or temperate) or habit (epiphytic or terrestrial) on seed traits. However, all micro-morphological traits varied significantly among species (Pillai's Trace F = 173.439, df = 10, $p < 0.001$). The micro-morphological data for the six species is shown in Table 2.3.

The relationship (ratio) between some of the parameters investigated (Table 2.3) was calculated, i.e., embryo length (EL) and embryo width (EW), seed length (SL) and seed width (SW), seed volume (SV) and embryo volume (EV) to further explore the occurrence of morphological trends related to taxonomy, origin, and habit, with a similar outcome (i.e., only species has a significant effect F = 96.686, df = 15, $p < 0.001$).

T. nervosa and *G. cunninghamii* did not differ in their seed volume, but *T. nervosa* had a larger EV, and consequently, a smaller air space than *G. cunninghamii*. On the other hand, although *P. banksii* has a smaller embryo size than *T. nervosa*, a comparison of seed volume showed that *P. banksii* had the biggest SV, which was 2.25-fold higher than either *T. nervosa* or *G. cunninghamii*, thus the largest airspace among the temperate species in this study (Table 2.3).

Table 2.3. A comparative measurement of seed micromorphological characteristics of six selected orchid species and post-hoc comparisons after a GLM

Embryo Traits			
Species	**Length (mm)**	**Width (mm)**	**Volume *(mm^3)**
D. strebloceras	0.25 ± 0.007 a	0.16 ± 0.005 a	0.004 ± 0.0003 a
D. lineale	0.20 ± 0.004 d	0.097 ± 0.001 d	0.001 ± 0.00003 d
D. cunninghamii	0.21 ± 0.004 c	0.099 ± 0.002 d	0.001 ± 0.00004 d
G. cunninghamii	0.23 ± 0.005 b	0.097 ± 0.001 d	0.001 ± 0.00004 d
P. banksii	0.21 ± 0.004 c	0.13 ± 0.002 c	0.002 ± 0.0001 c
T. nervosa	0.24 ± 0.003 a, b	0.15 ± 0.002 b	0.003 ± 0.0001 b

Seed Traits				
	Length (mm)	**Width (mm)**	**Volume *(mm^3)**	**Air space (%)**
D. strebloceras	1.76 ± 0.04 a	0.32 ± 0.009 a	0.052 ± 0.003 a	92.4 ± 0.4 a
D. lineale	0.42 ± 0.006 f	0.12 ± 0.002 d	0.001 ± 0.00006 d	32.2 ± 2 d
D. cunninghamii	0.48 ± 0.008 e	0.16 ± 0.003 b	0.003 ± 0.0002 c	66.2 ± 1.7 c
G. cunninghamii	0.85 ± 0.02 c	0.13 ± 0.002 c	0.004 ± 0.002 c	66.7 ± 1.2 c
P. banksii	1.09 ± 0.03 b	0.17 ± 0.003 b	0.009 ± 0.0004 b	75.3 ± 1.2 b
T. nervosa	0.54 ± 0.006 d	0.17 ± 0.003 b	0.004 ± 0.0001 c	29.3 ± 1.5 d

* A separate ANOVA followed by LSD for embryo and seed volume (Log transformed) was undertaken, since these variables were excluded from the original model. Different letters indicate significant differences ($p < 0.05$) between species for each individual trait (mean ± SE) after a pairwise (LSD) post-hoc test

Although specific traits related to orchid taxonomy, origin, or habit were not found; most Epidendroideae observed in this study (*D. lineale*, *D. cunninghamii* and *G. cunninghamii*) had a high EL/EW ratio (>2), and low embryo volume. In contrast, the Orchidoideae had relatively low EL/EW ratio and high EV. *D. strebloceras* had the lowest EL/EW ratio and highest EV of all species. Similar trends were not found for other traits; however, it was consistently observed that *D. strebloceras* had different morphological traits than the other species, even those in the same genus (Tables 2.3 and 2.4).

Table 2.4. Calculated ratios for embryo length to embryo width (EL/EW), seed length to seed width (SL/SW), and seed volume to embryo volume (SV/EV) for six orchid species and post-hoc comparisons after a GLM

Species	**EL/EW**	**SL/SW**	**SV/EV**
D. strebloceras	1.55 ± 0.3 d	5.6 ± 0.1 c	15.7 ± 1.2 a
D. lineale	2.02 ± 0.4 c	3.7 ± 0.8 d	1.6 ± 0.08 c
D. cunninghamii	2.13 ± 0.4 b	2.9 ± 0.7 f	3.4 ± 0.2 b
G. cunninghamii	2.36 ± 0.5 a	6.7 ± 0.2 a	3.2 ± 0.1 b
P. banksii	1.61 ± 0.3 d	6.4 ± 0.2 b	4.9 ± 0.5 b
T. nervosa	1.64 ± 0.3 d	3.29 ± 0.5 e	1.4 ±0.03 c

Different letters indicate significant differences ($p < 0.05$) between species for each trait (mean± SE) after a pairwise (LSD) post-hoc test

Elongated seeds (SL/SW ratio > 5) were found in *G. cunninghamii*, *P. banksii* and *D. strebloceras*, respectively (Table 3). While *D. lineale*, *D. cunninghamii* and *T. nervosa* had truncated seeds (SL/SW ratio < 5). Following the classification in Barthlott *et al.* (2014), *D. strebloceras* and *Pterostylis banksii* produced large seeds. *G. cunninghamii* had medium-sized, but elongated seeds, with the biggest SL/SW ratio. On the other hand, *T. nervosa* was also classified as a medium seed (Table 2.4).

T. nervosa had a bigger seed and embryo volume than *D. lineale* but a lower SV/EV ratio, explained by the high occupancy of the embryo, resulting in a low air volume within the seed. Seeds of *D. cunninghamii*, *G. cunnninghamii*, and *P. banksii* had intermediate SV/EV ratios with higher airspace percentages. *D. strebloceras* had the highest SV/EV ratio and the largest airspace of the six studied species (Tables 2.3 and 2.4).

When comparing *Dendrobium* species (Tables 2.3 and 2.4), *D. lineale*, a tropical epiphytic-lithophytic, had a similar embryo size as that of *D. cunninghamii*, a temperate epiphytic-lithophytic, but *D. lineale* had a significantly smaller air space compared with *D. cunninghamii*. In contrast, *D. strebloceras*, which has a similar habitat as *D. lineale* had significantly higher values for most parameters measured than the other *Dendrobium* species.

2.5. Discussion

Pod and seed morphological traits of six orchid species were characterised and compared. The results show that pod appearance and colour can be good taxonomic indicators, while there is high variability in seed measurements (especially at the micromorphological level), suggesting that these traits are species-specific, possibly reflecting different modes of seed dispersal, and might not be suitable to identify taxonomical relationships. However, micromorphological traits can be useful to prioritise species for conservation and select appropriate *in situ* and *ex situ* conservation strategies.

Similarities within the genus *Dendrobium* in their pod qualitative morphology were found; in particular, their pod appearance and colour. Many other *Dendrobium* species share similar traits (Irawati 2013), suggesting that these features are useful taxonomic criteria to identify *Dendrobium* species. Similarity in shape of testa and seed pigmentation (yellow colour) have also been reported in most *Dendrobium* species (Wang and Xiao 2010), thus may be important characters for taxonomic markers, reflecting their close phylogenetic relationship. However, Barthlott *et al*. (2014) suggest that seed colour is not a reliable taxonomic trait, as inconsistencies

can be found within clades or genera, except in *Diuris* clade (a terrestrial orchid genus restricted to Australia, which has a very characteristic seed appearance with dark brown colour).

This study provides evidence of significant variability between species at the micro-morphometric level, independently of their genus, distribution range, and habit. Differences in seed morphology (coating, shape, weight and air volume) may reflect variations in their dispersal mechanisms or adaptations to different environments (Howe and Smallwood 1982; Arditti and Ghani 2000; Brzosko *et al.* 2017). For instance, *P. banksii* and *D. strebloceras* belong to different genera, and have different growth habits and distribution ranges; but share several seed traits that facilitate dispersion. Both have a large air volume, allowing longer seed floatation time in the air (Arditti and Ghani 2000; Brzosko *et al*. 2017), elongated seeds that disperse further than truncated seeds (Eriksson and Kainulainen 2011) and sticky or adhesive surfaces can assist animal-mediated seed dispersal (Russel and Musil 1961).

Observations of seed qualitative morphology showed that species in this study had a fusiform testa or prolate-spheroid embryo, which are the most common shapes in the Orchidaceae family. The elongated seed shape in *D. strebloceras*, *G. cunninghamii*, and *P. banksii*, is not predominant among orchids but has been reported for other orchid species of unrelated taxonomic groups, which suggests that it may be a result of different adaptations to their habitats (Verma *et al*. 2014).

The findings of this study support the results of Wang and Xiao (2010), who suggest that seed morphometric has no relationship with division of section in *Dendrobium*. However, Lavarack *et al*. (2000) proposed that *Dendrobium* species are characterised by having very small seeds (<5 mm long), with some exceptions in the *Spatulata* section (which includes *D. strebloceras*). Therefore, more research is needed to confirm an association between seed morphometry and taxonomy in *Dendrobium* and the other groups.

According to Arditti and Ghani (2000), the relationship between orchid seed dimension and air space percentage is closely connected with their ecological

adaptations. Moreover, association of airspace with climatic condition has been reported in some *Dendrobium* species (Chaudhury et al. 2014; Prasongsom et al. 2017). Chaudhary *et al*. (2014) suggest that *Dendrobium* species from temperate regions with relatively low atmospheric pressure require a higher air space to facilitate buoyancy for optimal seed dispersal than species from sub-tropical or tropical regions. This study found that *D. cunninghamii* (temperate) had a bigger (two-fold) air space percentage than *D. lineale* (tropical). Prasongsom *et al*. (2017) also reported the air space percentage of nine tropical *Dendrobium* species from Thailand fell in a range of 12.8%–36.3%. However, seed morphometric characters of *D. strebloceras* and *D. lineale* were significantly different, albeit they shared the same ecological habitat and taxonomic traits; both being epiphytic, large plants, with large flower size, present in low-altitude tropical rain forests. It is possible that, *D. strebloceras* is an exception to the rule together with other members of the same clade (Lavarack *et al*. 2000). Therefore, more research is required to understand the ecological or evolutionary conditions that led this species (and probably others in the *Spatula* section) to develop larger seed sizes and air volumes.

Despite its minuscule size, the embryo is a crucial element that determines airspace proportion within the seed, thus seed buoyancy, floatation time and dispersal. There is evidence that bigger embryo volume is positively correlated to seed weight (Dangat and Gurav 2016), determining seed ability to float on air and distribution (Arditti and Ghani 2000). EL/EW ratios above 1 show that all orchid species in this study have prolate-spheroid shaped embryos. Orchids from the subfamily Epidendroideae had higher EL/EW ratios (except *D. strebloceras*) than those in the subfamily Orchidoideae. Observations of seed volume showed that the Epidendroideae group has smaller seed volumes than Orchidoideae (except *D. strebloceras*), further supporting the hypothesis that *D. strebloceras* has atypical morphological traits (Lavarack *et al*. 2000).

According to Verma *et al*. (2014), terrestrial species have bigger air spaces because of their bigger seed volume, thus bigger SV/EV ratio than epiphytic species. Moreover, seeds with SV/EV ratios above 2.2 were suggested to be more buoyant than those with lower ratios, thus enabling wider plant distribution ranges.

Calculation of SV/EV from this study (Table 2.4) support Verma *et al.* (2014); SV/EV ratio of the temperate terrestrial species *D. cunninghamii*, *G. cunninghamii* and *P. banksii* was bigger than tropical epiphytic *D. lineale*. Nonetheless, the terrestrial orchid *T. nervosa* did not follow the same pattern. This species has a comparatively big embryo volume, thus low SV/EV ratio and low air volume. A similar pattern as *T. nervosa* was found in *Paphiopedillum* sp., which has limited seed dispersal and shorter buoyancy periods than other terrestrial species, consequently having a restricted distribution area (Arditti and Ghani 2000).

Plant adaptations to specific or limited geographic distribution ranges stimulate the evolution of a wide variety of morphological characters, including seed dispersal mechanisms (Emadzade *et al*. 2010). This study reflects such variability, indicating that even closely related species may have different seed morphologies associated to their optimal dispersal strategies. Based on the seed dispersal characteristics proposed by Howe and Smallwood (1982), it is possible to speculate that *D. lineale*, being epiphytic and having small seeds with low air percentages, is better suited for water dispersal (hydrochory) along the coastal of New Guinea. The wax-coated testa in *Dendrobiinae* (Gamarra *et al*. 2018) may be an advantage for seed distribution along the coastal stream. In contrast, bigger *D. strebloceras* seeds with a large air space and high SV/EV ratio may rely on wind dispersal or take advantage of their adhesive surface to better attach them to the bark of the trees or use animals as dispersers (zoochory); which is suggested to be the most effective seed dispersal below closed canopies (Croat 1978).

Regarding terrestrial species, Lechnebach and Robertson (2004) suggested wind-dispersal as the main vector in *G. cunninghamii* and related the ability of seeds to easily be carried by wind with the widespread distribution of this species in the North and South Islands of New Zealand, and on the Stewart and Chatham Islands (New Zealand Native Orchid Group 2015).

Overall, the results from this study show similarities in seed pod colour and shape between orchid genera, but a high diversity at the micro morphological level, where ecological traits (e.g., seed dispersal) rather than taxonomy, biogeographical origin,

or growth habit are likely to determine seed morphology. Further studies including more orchid species to validate these results are strongly encouraged.

2.6. Conclusions

The results of this study show some similarities in qualitative traits (pod colour and size and seed colour) among three *Dendrobium* orchid species. However, there was high variation in orchid seed morphometrics and *D. strebloceras* has atypical morphological traits. Information on orchid seed morphometrics may be useful related to *in situ* conservation strategies. Further research including additional orchid species especially in *Spatulata* section and more species within the same genus in terrestrial orchids are needed to validate these results.

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CHAPTER III

Variation in Seed Fatty Acid Profiles of Six Orchid Species during Natural Ageing

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3.1. Abstract

Changes in lipid composition are associated with seed viability loss. However, little is known about the potential changes in seed lipid content and composition during ageing in orchids. To address this knowledge gap, the fatty acid profiles of seeds of six orchid species during slow ageing were characterised and compared. Two tropical epiphytic orchid species from Indonesia (*Dendrobium strebloceras* and *D. lineale*), and four temperate species from New Zealand, epiphytic *D. cunninghamii*, and terrestrial *Gastrodia cunninghamii*, *Pterostylis banksii* and *Thelymitra nervosa* were used for the study. Direct transesterification was applied to extract fatty acid methyl esters (FAMEs), which were then analysed using a gas chromatograph-mass spectrometer. Unsaturated fatty acids linoleic acid (18:2) and oleic acid (18:1) were the most abundant compounds in the fresh seeds of all studied species, accounting for > 70% of the total fatty acid content. Whilst palmitic acid (16:0) was the most abundant saturated fatty acid accounting for 8 - 16% of the total lipids. Fresh seeds from epiphytic orchids had similar fatty acid composition, while terrestrial orchids were characterised by the absence of erucic and palmitoleic acid. Unsaturated fatty acids are less stable and more prone to degradation than saturated fatty acids, showed by strong correlation of changes in oleic acid and linolenic acid with seed viability loss. The proportion of polyunsaturated fatty acids decreased with age in most species, but the extent of this reduction was variable. *D. strebloceras* showed the highest reduction in polyunsaturated fatty acids after ageing, linked to a significant increase in the saturated to unsaturated fatty acid ratio (S:U), thus indicating that it may be more susceptible to seed lipid degradation than other species in this study.

Keywords: epiphytic *Dendrobium*, fatty acids methyl esters (FAMEs), gas chromatography (GC), seed lipids, seed storage, terrestrial orchids.

3.2. Introduction

Seed lipids are important structural components of membranes and energy reserves (Meyer and Kinney 2009; Murphy 2016; Colville *et al.* 2016). Their degradation can have important physiological and ecological consequences for seeds. Membrane lipids mostly consist of glycerolipids including phospholipids and galactolipids; while storage lipids are mainly oils, almost always triacylglycerols, i.e., esters of glycerol and fatty acids (De Man 1999; Srivastava 2002; Yu *et al*. 2015). Amongst seed lipid components, fatty acids are the most commonly studied due to their biological and economic relevance (e.g., in human consumption and biofuel production). The fatty acid profiles of seeds can be characterised using gas chromatography (GC). This often requires transesterification of fatty acids to produce fatty acid methyl esters (FAMEs) (Liu 1994; Aluyor *et al*. 2009).

Fatty acids vary in chain length and degree of saturation. Fatty acids typically contain an even number of carbon atoms (between 12 and 24) and vary in the number of double bonds joining them (saturation), being classified into saturated fatty acids (SAFAs – having no double bonds), monounsaturated fatty acids (MUFAs – having one double bond) and polyunsaturated fatty acids (PUFAs – having two or more double bonds) (Berg *et al*. 2002). For example, stearic acid (18:0) is a saturated fatty acid with an 18 single carbon chain (no double bonds), while oleic acid (18:1) is considered as a monounsaturated fatty acid of 18 carbon atoms with one double bond and linoleic acid (18:2) is a polyunsaturated fatty acid with 18 carbon atoms and two double bonds.

The properties of fatty acids and of lipids derived from them depend on the chain length and degree of saturation. For instance, unsaturated fatty acids have lower melting points than saturated fatty acids of the same length; and those with shorter chains have lower melting points than those having longer chains with similar saturation (Berg *et al.* 2002).

According to Linder (2000), ratio of lipid saturation (saturated: unsaturated) was related to biogeographical origin. Greater saturated oil content was found in tropical than temperate species, consequently a higher ratio of S:U fatty acids was expected

in tropical species. Furthermore, Colville *et al.* (2015) report tropical epiphytic species have more fatty acids than temperate terrestrial orchids. According to these authors (Colville *et al.* 2015), the most important fatty acids in both epiphytic *Grammatophyllum speciosum* and terrestrial species *Dactylorhiza fuchsii* were: palmitic acid, oleic acid, and linoleic acid.

Oleic (18:1), linoleic (18:2) and alpha-linolenic (18:3) acid are the three acids most commonly found in plants (He *et al.* 2020; He & Ding 2020). These authors mentioned that C18 acids are important as antioxidants, precursors of various bioactive molecules (e.g. stress hormones, and extracellular barriers components such as cutin and suberin), as well as in plant defence, e.g. the pathways associated with pathogen invasion.

Lipids act as seed reserves for orchid seeds and provide required nutrients for seeds during germination. Degradation of lipid is consequential for seed membranes (Hartman and Mattick 1976; Shaban 2013) and generally means the seeds will not be able to germinate, produce normal seedlings and adapt to the ecological environment. Degradation of fatty acids also affects the general defense system of seeds against biotic and abiotic stresses (He and Ding 2020; He *et al.* 2020), as well as response to phatogen invasion (Chen *et al*. 2007).

Variation in fatty acid composition is thought to be a significant factor determining seed ageing and lifespan due to lipid degradation through oxidation or enzymatic hydrolysis both *in situ* and under *ex situ* storage conditions (Pritchard 1984; Hulbert 2005; Wiebach *et al*. 2020). Various studies have investigated the relationship between seed ageing and fatty acid composition for other plant species but not orchids (e.g., Ponquett *et al*. 1992; Wiebach *et al*. 2020).

Since the rate of physiological ageing depends on water activity and thermal input, most of these studies involve high moisture environments and elevated temperature, inducing rapid ageing (Bonner 2008). However, there are fewer studies investigating fatty acid degradation under natural ageing conditions.

Some studies report changes in seed fatty acid composition during slow or natural ageing. Zacheo *et al*. (1998) found a decrease in linoleic acid (C18:2) and linolenic acid (18:3) in almond seeds after 40 days at 80% relative humidity (RH) and 20 °C storage. A decrease in saturated (myristic (14:0) and stearic acid (18:0)) and unsaturated (linoleic and linolenic acid) was also found in different rice varieties after six months storage at room temperature (15-20°C) at 85% RH (Yasumatsu and Moritaka 1964). Likewise, Pichardo-Gonzales *et al*. (2014) observed a decrease in both saturated (stearic acid) and unsaturated (linoleic acid) fatty acids in natural ageing of tomatillo seeds during storage under ambient conditions (18.2 ± 5 °C and 41 ± 10% RH).

Most orchid species are endangered because of over collection and loss of habitat, so it is necessary to preserve them both *in situ* as well as *ex situ* (Rubluo *et al*. 1993). Seed storage is a relatively cheap and viable alternative to conservation *in situ* for securing germplasm but given the complex storage behaviour of different seed species, it is important to have a good understanding of the seed ageing process under different conditions (Bonner 2008).

Orchid seeds are known to have complex storage performance, tending to be relatively short-lived under seed bank conditions (-20 °C, 15% RH), and germinability can be loss after a few years storage in some species (Pritchard *et al.* 1999; Hay *et al*. 2010; Seaton *et al.* 2013). Therefore, investigating the lipid composition of orchid seeds and the changes occurring during ageing could contribute to orchid conservation efforts. However, investigating the lipid composition of orchid seeds is challenging due to their minuscule size; only relatively recently has a methodological approach has been developed for this purpose (Colville *et al.* 2016).

Besides their small size, orchid seeds are unusual in that, instead of an endosperm, the very small globular embryo is surrounded by air within a membranous testa that generally consists of few cells, mainly, epidermal and parenchymal cells (Richardson *et al.* 1991). While the airspace content in orchid seeds may facilitate seed dispersal by enhancing buoyancy in air (Arditti and Ghani 2000; Leck *et al*. 2008; Diantina *et al.* 2020a), its relationship to seed deterioration remains unknown.

For this study six orchid species were selected, two tropical epiphytic species, *Dendrobium strebloceras* and *D. lineale* from Indonesia (subfamily Epidendroideae) and four temperate species from New Zealand, the endemic epiphytic orchid *D. cunninghamii* (Epidendroideae) and terrestrial orchids *Gastrodia cunninghamii* (Epidendroideae), *Pterostylis banksii* (Orchidoideae) and *Thelymitra nervosa* (Orchidoideae). The aims of the study were to characterise the fatty acid profile of these species and to investigate its changes after natural ageing.

3.3. Materials and Methods

3.3.1. Biological materials

Two tropical epiphytic Indonesian orchids, *Dendrobium strebloceras* and *D. lineale*, were obtained from the Indonesian Agency for Agricultural Research and Development (IAARD) collection. They were hand-pollinated and grown in glasshouse conditions at Cipanas Experimental Field, West Java (tropical rainforest climate, 19 ± 6 °C, ~ 1100 m a.s.l.). Seed pods were mature at 4-5 months after pollination and harvested in either 2017 or 2018 for *D. lineale* or *D. strebloceras*, respectively. Fresh seed pods were courier posted to New Zealand for the research.

The temperate epiphytic orchid *D. cunninghamii* was obtained in 2017 from Pukeiti Forest, North Island, New Zealand (temperate rainforest climate, 8 ± 5 °C, ~ 490 m a.s.l.). Seed pods of three temperate terrestrial orchids; *G. cunninghamii*, *P. banksii* and *T. nervosa*, were collected in 2018 from Iwitahi Native Orchid Heritage Protection Area where they are growing under a *Pinus nigra* plantation at Kaingaroa forest, Taupo Napier road, North Island (marine west coast climate, 11 ± 5 °C, ~ 544 m a.s.l.).

Seed viability and initial fatty acid status of the fresh seeds were assessed immediately after seed arrival. A portion of the seeds were then equilibrated to 15% RH in a drying room at 15 ± 2 °C until used for further experiments at the Margot Forde Forage Germplasm Centre, AgResearch, Palmerston North, New Zealand. Later, natural ageing experiment was conducted altogether.

3.3.2. Seed viability assessment

Seed viability was tested before investigation of the fatty acid profile. Germination of orchid seeds is complex, involving the presence of dormancy, the requirement for fungal symbionts, and the need for species-specific culture media for *ex situ* germination (Rasmussen *et al.* 2015). Therefore, the tetrazolium chloride staining technique (TTC) was applied to test viability, as described in Hosomi *et al.* (2012), rather than a germination test. Due to limited seed availability, either four (*D. lineale* and *D. cunninghamii*) or three (the rest of species) replicates were used to test seed viability. Due to their very small size, approximately similar number of seeds were taken with the tip of a paintbrush as a replicate and transferred them into vials for viability assessment. Viability was assessed for both fresh seeds and after ageing treatment. The average number of seeds per replicate was c. 150, determined by counting under a binocular microscope.

Seeds were initially incubated in a 10% sucrose solution overnight at room temperature. The next day, the sucrose solution was replaced with 1% TTC and allowed to incubate overnight under dark conditions at 40 °C. Then, the number of viable seeds and non-viable seeds (shown by a red-stained embryo) was observed under a light microscope (microscope: Olympus SX7; light: Olympus DF PLAPO $1X_{-4}$, Olympus Optical, Tokyo, Japan).

3.3.3. Natural ageing experiment

For the natural ageing treatment, seeds were packed in envelope-shaped filter paper and equilibrated over 60% LiCl solution in an air-tight closed container (electrical enclosure box, IP67) at 20 $^{\circ}$C incubation room at Massey University. A relative humidity of 60% was selected to resemble the environmental conditions that seeds experience in their geographical ranges (60-70% RH in New Zealand and up to 85% in Indonesia - Weather Atlas 2020), while avoiding very high humidity levels that have been associated with seed rotting (Pritchard and Dickie 2003; Walters 2004). The humidity of the LiCl solution was measured with a hygrometer (Rotronics

HygroPalm, AW-KHS) to ensure similar conditions for all species. Seeds were stored for 3 months under these conditions; and then tested for viability and lipid status.

3.3.4. Seed lipids (fatty acids)

3.3.4.1. Preparation of FAMEs: Direct transesterification of fatty acids

Seed fatty acid content was determined from the methyl ester form (FAMEs), obtained by direct transesterification of the fatty acids as described by Colville *et al*. (2016). Three replicates of each sample (10 mg each) were placed into a 20 ml glass vials with 1 ml of toluene (containing 50 mg/l BHT as antioxidant), and 10 µl of 10 mg/ml solution of pentadecanoic acid as an internal standard. Samples were then incubated for four hours at room temperature.

Direct transesterification using 2 ml of 1% (v/v) sulphuric acid in methanol (prepared on ice) as a catalyst, were used for fatty acid methylation. Samples were incubated overnight in a water bath at 50°C with constant shaking at 150 rpm.

The fatty acid methyl esters (FAMEs) were isolated by partitioning with 5 ml of hexane and 5 ml of NaCl (5% w/v). The solid phase was transferred to a clean vial with a Pasteur pipette and the aqueous phase was washed with another 5 ml of hexane. The solid phases then were combined and dried at 35°C under a vacuum concentrator. The residue was then dissolved in 1 ml of hexane and transferred to 2 ml vials for GC-MS analysis.

3.3.4.2. Identification of FAMEs using GC-MS analysis

A Gas Chromatography-Mass Spectrometry (GC-MS) machine (GC2010, Shimidzu, Japan) was used for FAME analyses. FAMEs were separated by GC using a Zebron^{TM} ZB-FAME w/5m Guardian (High cyano phase, capillary column 30 m length x 0.25 mm internal diameter x 0.2 µm film thickness (df)). The initial

temperature was 70 ℃ and increased at 20 ℃/min until 19 5℃, 5 ℃/min until 240 ℃, and held for 10 min at 240 ℃, with helium at a constant flow rate of 1 ml/min as carrier gas. A solvent delay of six minutes was allowed.

The FAMEs were identified by comparison with the NIST mass spectral database and analytical standards (FAME Mix C4-C24). Quantification of fatty acid methyl esters was performed using standard curves as a reference.

3.3.5. Statistical analyses

Statistical analysis was performed using R (R Core Team). All data were tested for normality (Shapiro-Wilk test) and homogeneity of variables (Levene's test) and transformed when required. Variables were treated as fixed effect to focus on fatty acid composition on each species and the effect of ageing on fatty acid composition. The effect of species, ageing and their interaction on seed viability, SAFA, MUFA and PUFA content, as well as on the saturated/unsaturated fatty acid ratios (S:U), was tested using a two-way ANOVA, followed by post-hoc tests to explore differences within species*treatment combinations, or by one-way ANOVA, followed by Tukey's test, to describe simple main effects when no significant effect of the interaction or ageing treatment was found.

3.4. Results

3.4.1. Seed viability before and after natural ageing

A two-way ANOVA showed that seed viability was significantly influenced by species ($F= 126.47$, $DF= 5$, $P< 0.001$), ageing ($F= 526.68$, $DF= 1$, $P< 0.001$) and their interaction ($F= 17.73$, $DF= 5$, $P< 0.001$). Most of the species had very good initial viability (above 85%) except *D. lineale*. All species showed significant loss of viability after storage, except for *G. cunninghamii* (although there was an observable viability loss). Viability losses ranged from 18.1% (*G. cunninghamii*) to 62.4% (*P. banksii*) in 3 months (Table 3.1).

Table 3.1. Orchid seed viability before and after ageing treatment (three months storage at 60% RH, 20 °C)

Species	Fresh seed viability (%)	Seed viability (%) after storage	The difference between viability before and after storage (%)
D. strebloceras	88.5 ± 2.6 d,e	59.6 ± 0.9 b,c	28.9
D. lineale	56.1 ± 2.3 c	9.9 ± 0.7 a	46.2
D. cunninghamii	93.4 ± 0.7 f	56.5 ± 2.0 b,c	36.9
G. cunninghamii	97.4 ± 1.0 f	79.3 ± 1.1 e,f	18.1
P. banksii	86.3 ± 5.1 d,e	23.9 ± 0.6 b	62.4
T. nervosa	93.1 ± 1.7 f	71.3 ± 1.7 d,e	21.8

Different letters indicate significant differences after two-way ANOVA (mean ± SE) followed by Tukey post-hoc tests ($p < 0.05$) for each species*treatment (fresh or aged) combination

3.4.2. Seed fatty acid composition before and after natural ageing

From the five saturated fatty acid (SAFA) identified, palmitic acid was the most abundant in all species, ranging from 8.6% to 19.4% in fresh seeds, followed by small amounts (1-2%) of stearic acid. Longer carbon chains (arachidic acid, behenic acid and lignoceric acid) were also present as minor components (lignoceric acid was not found in *G. cunninghamii* and *T. nervosa*) (Table 3.2). There is significant two-way ANOVA interaction effect of species on SAFA seed content. *D. cunninghamii* has the lowest SAFA among studied species and significant increasing of SAFA following ageing was found only on *D. strebloceras*.

For monounsaturated fatty acids (MUFA), oleic acid was found to be the most abundant in all species, ranging from 10.2% to 32.9%. Palmitoleic and erucic acid, were detected in small amounts in all fresh seeds of epiphytic *Dendrobium*, but not in terrestrial species (Table 3.2). A significant effect was observed for species, but not with ageing. Therefore, only the simple main effects on fresh seed for species are reported (Table 3.2). *D. strebloceras* had the lowest MUFA values compared

with all other species, while both *G. cunninghamii* and *P. banksii* had significantly higher proportions.

Linoleic acid was the most abundant polyunsaturated fatty acid (PUFA) in fresh seed of all species, and the most abundant fatty acid methyl ester (FAME), with values ranging from 44% to 71.8%. Linolenic acid was found in small quantities in all species while some fatty acids, such as lignoceric acid, palmitoleic acid, erucic acid, and eicosadienoic acid were not detected in some species in this experiment. This may be because they were not present or present in small quantities below the detection limits of the testing method used (Table 3.2). After a two-way ANOVA a significant interaction effect of species on the PUFA seed content was found. *G. cunninghamii* had a significantly lower PUFA content in fresh seeds in contrast with other species, while *D. strebloceras*, *G. cunninghamii* and *T. nervosa* had significantly higher proportions, with a similar pattern in aged seeds. The effect of ageing was significant for these three species as well.

A significant interaction effect of the species on the ratio of saturated to unsaturated fatty acids (S: U) was found. In this case, the only significant difference among treatments observed was between fresh and aged *D. strebloceras* (Figure 3.1), and only the S:U ratio of aged *D. strebloceras* differed markedly from the remaining species (Figure 3.1).

Table 3.2. Fatty Acid Methyl Ester (FAME) composition of fresh and aged seed of six orchid species

Lipid	Chemical name (methyl ester)	Treatment	*D. strebloceras*	*D. lineale*	*D. cunninghamii*	*G. cunninghamii*	*P. banksii*	*T. nervosa*	Pearson correlation (R^2)
Palmitic acid	Hexadecanoic acid (16:0)	Fresh	16.04 ± 0.13	15.38 ± 0.17	8.57 ± 0.60	19.40 ± 0.30	14.55 ± 1.24	14.77 ± 0.64	R^2 = -0.129
		Aged	23.76 ± 1.61	18.45 ± 1.52	11.13 ± 0.68	19.87 ± 1.93	16.69 ± 8.51	18.94 ± 1.42	P = 0.806
Stearic acid	Octadecanoic acid (18:0)	Fresh	1.46 ± 0.24	1.40 ± 0.44	2.23 ± 0.19	2.33 ± 0.22	1.23 ± 0.17	1.53 ± 0.28	R^2 = 0.544
		Aged	4.41 ± 1.05	1.19 ± 0.43	2.49 ± 0.46	3.84 ± 1.18	1.05 ± 0.96	1.73 ± 1.19	P = 0.265
Arachidic acid	Eicosanoic acid (20:0)	Fresh	0.21 ± 0.02	0.30 ±0.05	0.15 ± 0.03	0.49 ± 0.02	0.33 ± 0.08	0.93 ± 1.14	R^2 = -0.127
		Aged	0.38 ± 0.01	0.35 ±0.02	0.17 ± 0.02	0.62 ± 0.03	0.22 ± 0.11	0.28 ± 0.04	P = 0.811
Behenic acid	Docosanoic acid (22:0)	Fresh	0.41 ± 0.22	0.42 ± 0.05	0.23 ± 0.04	0.33 ± 0.15	0.30 ± 0.06	0.18 ± 0.05	R^2 = 0.316
		Aged	0.38 ± 0.04	0.37 ± 0.04	0.26 ± 0.02	0.39 ± 0.06	0.30 ± 0.16	0.16 ± 0.09	P = 0.542
Lignoceric acid	Tetracosanoic acid (24:0)	Fresh	0.26 ± 0.13	0.61 ± 0.17	0.27 ± 0.22	n.d.	0.59 ± 0.44	n.d.	n.c.
		Aged	1.08 ± 0.23	0.96 ± 0.1	1.27 ± 0.5	n.d.	0.90 ± 0.50	n.d.	
Total SAFA		**Fresh**	**18.39 ± 0.23 b,c,d**	**18.11 ± 0.29 b,c,d**	**11.39 ± 0.38 a**	**22.56 ± 0.12 d,e**	**17.00 ± 0.97 b,c**	**17.39 ± 0.84 b,c**	
		Aged	**30.00 ± 0.57 f**	**21.31 ± 1.18 c,d,e**	**15.33 ± 0.69 a,b**	**24.73 ± 0.97 e**	**19.15 ± 1.83 b,c,d**	**21.11 ± 1.55 c,d,e**	

Lipid	Chemical name (methyl ester)	Treatment	*D. strebloceras*	*D. lineale*	*D. cunninghamii*	*G. cunninghamii*	*P. banksii*	*T. nervosa*	Pearson correlation (R^2)
Palmitoleic acid	9-Hexadecenoic acid (16:1)	Fresh	0.04 ± 0.01	0.41 ± 0.12	0.18 ± 0.16	n.d.	n.d.	n.d.	n.c.
		Aged	n.d.	n.d.	0.002 ± 0.0002	n.d.	n.d.	n.d.	
Oleic acid	9-Octadecenoic acid (18:1)	Fresh	10.21 ± 0.23	15.90 ± 0.39	15.90 ± 0.44	32.92 ± 0.28	23.27 ± 0.28	15.22 ± 0.95	R^2 = 0.857
		Aged	13.89 ± 2.72	16.37 ± 2.03	17.89 ± 0.44	38.07 ± 3.41	24.26 ± 12.2	18.26 ± 3.55	P = 0.03
Eicosenoic acid	11-Eicosenoic acid (20:1)	Fresh	0.33 ± 0.04	0.27 ± 0.08	0.06 ± 0.01	0.33 ±0.06	0.43 ±0.07	0.09 ± 0.02	R^2 = 0.063
		Aged	0.17 ± 0.07	0.3 ± 0.07	0.07 ± 0.03	0.16 ±0.04	0.26 ± 0.2	0.12 ± 0.07	P = 0.906
Erucic acid	13-Docosenoic acid (22:1)	Fresh	0.04 ± 0.02	0.04 ± 0.01	0.04 ±0.04	n.d.	n.d.	n.d.	n.c.
		Aged	0.03 ± 0.03	0.06 ± 0.01	0.03 ± 0.01	n.d.	n.d.	n.d.	
Total MUFA		**Fresh**	**10.62 ± 0.19 a**	**16.63 ± 0.58 b**	**16.23 ± 0.34 b**	**33.25 ± 0.16 c**	**23.69 ± 0.14 c**	**15.31 ± 0.53 b**	
		Aged	**14.10 ± 1.59**	**16.73 ± 1.14**	**18.00 ± 0.33**	**38.23 ± 1.99**	**24.52 ± 1.15**	**18.39 ± 2.01**	

Lipid	Chemical name (methyl ester)	Treatment	*D. strebloceras*	*D. lineale*	*D. cunninghamii*	*G. cunninghamii*	*P. banksii*	*T. nervosa*	Pearson correlation (R^2)
Linoleic acid	9,12-Octadecadienoic acid (18:2)	Fresh	70.4 ± 0.3	64.62 ± 0.4	71.78 ± 1.06	44.01 ± 0.05	58.66 ± 1.76	67.01 ± 0.57	R^2= -0.565
		Aged	55.06 ± 2.84	61.38 ± 0.73	66.03 ± 1.77	36.7 ± 3.28	55.81 ± 27.9	60.13 ± 2.45	P= 0.244
Linolenic acid	9,12,15-octadecatrienoic acid (18:3)	Fresh	0.6 ± 0.04	0.61 ± 0.01	0.53 ± 0.07	0.18 ± 0.06	0.52 ± 0.21	0.29 ± 0.06	R^2= 0.914
		Aged	0.77 ± 0.01	0.53 ± 0.06	0.59 ± 0.05	0.34 ± 0.02	0.38 ± 0.19	0.37 ± 0.17	P= 0.011
Eicosadienoic acid	11,14-Eicosadienoic acid (20:2)	Fresh	0.04 ± 0.02	0.03 ± 0.02	0.05 ± 0.005	n.d.	0.13 ± 0.01	n.d.	n.c.
		Aged	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	n.d.	0.14 ± 0.07	n.d.	
Total PUFA		**Fresh**	**70.99 ± 0.17 g,h**	**65.36 ± 0.23 e,f,g**	**72.38 ± 0.65 h**	**44.19 ± 0.06 b**	**59.31 ± 1.06 c,d**	**67.29 ± 0.30 f,g,h**	
		Aged	**55.89 ± 1.65 c**	**61.95 ± 0.44 d,e,f**	**66.68 ± 1.01 f,g,h**	**37.04 ± 1.91 a**	**56.33 ± 0.87 c**	**60.5 ± 1.35 c,d,e**	

Values represent the mean ± SEM (n=3) abundance of each fatty acid as a percentage of the total FAME molar concentration. SAFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids. Letters indicate significant differences between species*treatment combinations after a Tukey test for SAFA and PUFA, and between species only for MUFA. The last column shows the Pearson correlation coefficient (R^2) between the changes in individual FAMEs (fresh-aged) for those compounds present in all orchid species, alongside the corresponding p-value (P). n.d= not detected, n.c=not calculated

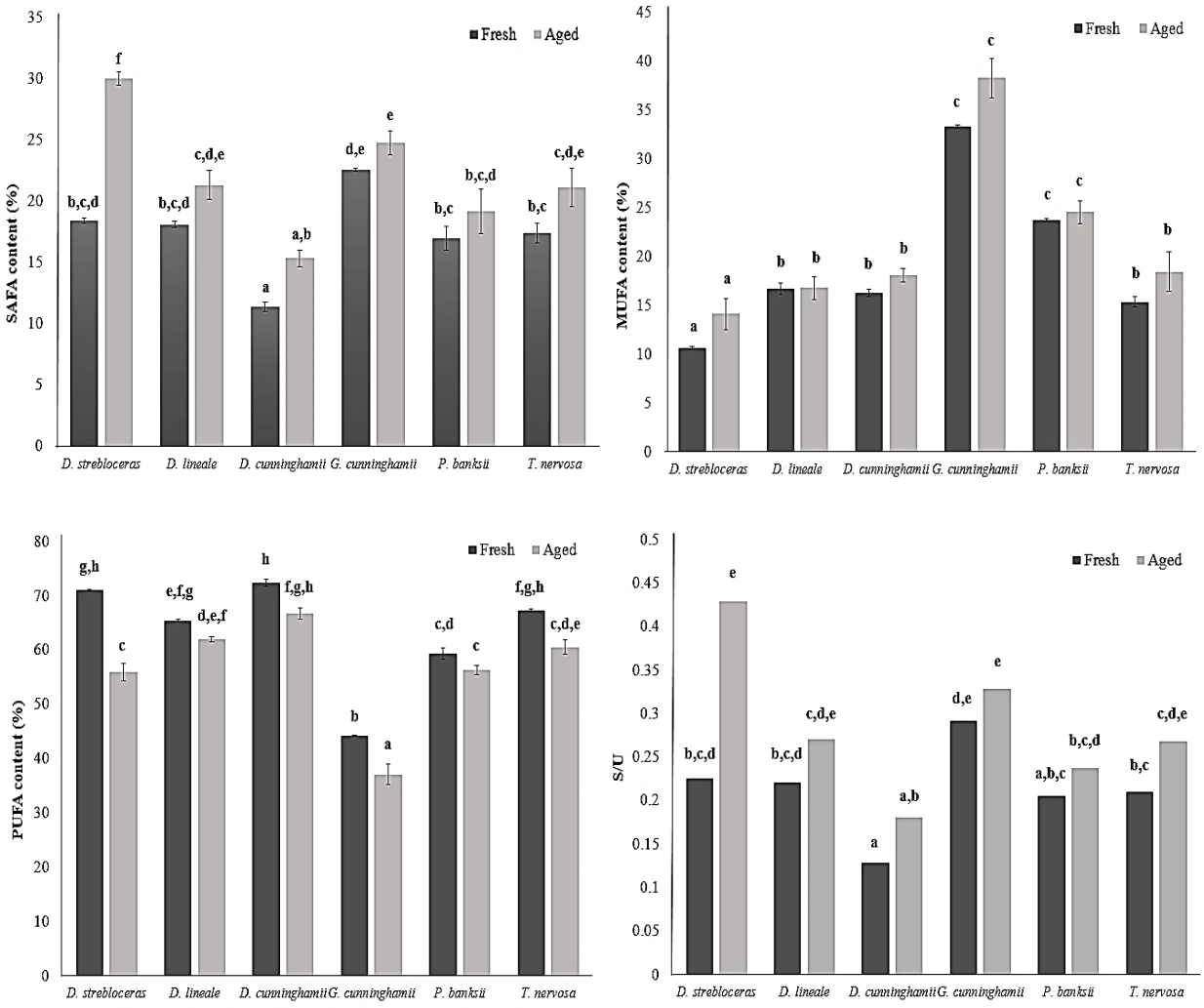

Figure 3.1. Proportion of SAFA, MUFA, PUFA and ratio of S/U (as a percentage), before and after ageing treatment. Bars represent mean ± SE and differences between treatments (fresh vs. ageing) are based on two-way ANOVAs followed by Tukey's tests

3.5. Discussion

This study revealed commonalities and differences in the fatty acid profiles of six orchid seeds and their responses to ageing. Of particular interest are the species *P. banksii and D. lineale* that showed a very pronounced reduction in viability but little change in their fatty acid profile. Considering high lipid composition in *D. lineale*, lipid peroxidation might already have happened before the experiment

started thus decrease initial seed viability (Table 3.1). *G. cunninghamii* had less unsaturated lipids and may have retained viability until favourable conditions were achieved. Colville *et al.* (2015) found that tropical epiphytic *G. speciosum* was more susceptible to oxidative damage and lost seed germination faster than temperate terrestrial *D. fuchsii* due to higher unsaturated lipid. Moreover, seed initial viability and seed viability during storage may be varied due to harvest time, postharvest handling, equilibrated temperature and humidity before seed storage. There are many factors contributing to viability loss during storage. Lipid degradation due to oxidation or enzymatic hydrolosis (Pritchard 1984, Hulbert 2005; Wiebach et al. 2020), temperature, nature of the seeds, initial seed viability, seed moisture content, relative humidity, accumulation of genetic damage (Pradhan and Badola, 2012).

Seed fatty acids are major storage components (Colville *et al.* 2015). Seeds containing lipids may be shorter-lived than seeds with mainly protein or carbohydrate reserves (Nagel and Borner, 2010) while others have found no association between seed storage reserves and longevity (Walters et al., 2005; Probert et al., 2009). However, seed storability may be related to lipid composition rather than lipid content, particularly in relation to lipid stability (Ponquett et al., 1992) and thermal properties (Crane et al., 2003, 2006), including that of orchids (Pritchard and Seaton, 1993).

According to He *et al.* (2020) and He & Ding (2020), oleic (18:1), linoleic (18:2) and alpha-linolenic (18:3) acids are the three acids most commonly found in plants. Palmitic and oleic acid in horse chesnuts are varied with geographical site (Cukanovic *et al*. 2020). Mayworm and Salatino (2002) also found correlation of these fatty acids with taxonomic trait of tropical trees and shrubs species.

Unsaturated fatty acid species are derived by the sequential unsaturation of fully saturated species. This chemical reaction is catalyzed by the activity of various fatty acid desaturase enzymes that convert single bonds at specific positions in the fatty acyl chains to double bonds by the removal of two hydrogen atoms, or by specific polyunsaturated fatty acid elongase via desaturation biosynthetic pathways (Yuan *et al*. 2014).

The presence of saturated lignoceric acid was confirmed in all epiphytic and one terrestrial orchid in this study, indicating the existence of hydrophobic lignin, which is thought to contribute to membrane permeation and germination (Barsberg *et al.* 2018; Pierce *et al*. 2019). Polyunsaturated eicosadienoic acid was also found in some species in small amount in this study (Table 3.2). According to Yuan *et al*. (2014), elongated fatty acids, including eicosadienoic acid, mitigate drought resistance in *Arabidopsis* through the action of ABA.

Various fatty acid compositions within plants were related to the cumulative melting temperature (CMT), which small changes in seed storage lipid composition have potential impact in seed germination (Thompson *et al.* 2019). Furthermore, combination of palmitic, stearic, oleic and linoleic acid compositions determined cotton seed germination on certain soil temperature needed for establishment (CMT) which lower palmitic acid effective to increase cotton seed germination at lower temperature (Thompson *et al.* 2019). This study found the highest palmitic acid composition in *G. cunninghamii* while *D. cunninghamii* was the lowest (Table 3.2). Thus suggests *G. cunninghamii* may require higher germination temperature than *D. cunninghamii*.

A change in fatty acid contents after ageing, mainly a decrease in the proportion of polyunsaturated acids, was found in all species (Table 3.2, Figure 3.1). Some species showed highly significant changes, whereas others did not. *D. strebloceras* seeds had the greatest change in fatty acid profile with ageing, having a significant decrease in PUFA, alongside a proportional increase in SAFA and S:U fatty acid ratios. *D. cunnighamii*, *G. cunninghamii* and *T. nervosa* seeds also evidenced significant decreases in their PUFA proportion, while aged seeds of *D. linneale* and *P. banskii* had lower PUFA but not significantly so.

Monounsaturated palmitoleic and erucic acid were detected on fresh seeds of all epiphytic *Dendrobium* species, but not in terrestrial ones. All species in this study share a high abundance of polyunsaturated oils, particularly linoleic acid (44 to 72%), and a lesser proportion of unsaturated or monounsaturated fatty acids. A similar occurrence has been reported for other orchid species (e.g.,

Grammatophyllum speciosum and *Dactylorhiza fuchsii* [67-78% linoleic acid respectively] (Colville *et al*. 2016). Vanilla orchid also has linoleic, oleic, and palmitic acid as the mayor fatty acids, respectively, with variation depend on species and origin (Brunschwig *et al.* 2009).

According to Linder (2000), higher latitude plants have comparatively more unsaturated fatty acids (smaller S:U ratio) than plants at lower latitude. Consequently, differences in the seed fatty acid composition and proportion of the species in this study was expected (Table 3.2). Differences in lipid composition was suggested as an adaptive mechanism to selection pressures imposed by germination temperature (Shanzes *et al.* 2019).

The two tropical epiphytic orchids from the genus *Dendrobium* had completely different responses to ageing in terms of their fatty acid profiles, with *D. strebloceras* showing multiple significant changes to its lipid profile while *D. lineale* did not. However, some MUFAs (palmitoleic and erucic acid) were only detected in fresh *Dendrobium* orchids and absent in all temperate terrestrial species. Furthermore, the overall fresh fatty acid profiles of *Dendrobium* were also similar, suggesting that seed fatty acid composition may be taxonomically determined, but ageing responses are not.

The high proportion of polyunsaturated fatty acids in orchid seeds could be a contributing factor to their relatively rapid viability loss during storage (*ex situ* and *in situ*). A reduction of oleic, linoleic and linolenic acid contents (PUFAs) has been reported in conjunction with a reduction of seed germination, electrolyte leakage, and an increase in malondialdehyde content for sweet pepper (Kaewnaree *et al.* 2011). Linoleic acid content was also found to be highly correlated with a reduction in germination, vigour and respiration of tomatillo (Pichardo-Gonzales *et al.* 2014). This suggests that a reduction in polyunsaturated fatty acids is a common response to ageing in seeds.

Similarly, in animals, the total number of double bonds in membrane fatty acids is also negatively correlated with longevity (Pamplona *et al.* 1998; Hulbert 2005). This is not surprising, as polyunsaturated fatty acids are prone to peroxidation and

enzymatic hydrolysis as result of low melting points and low energy required for catabolism, whereas monounsaturated and saturated fatty acids are more stable (Linder 2000; Shahidi and Zhong 2010; Repetto *et al*. 2012; Sanyal and Decocq 2016; Wiebach *et al*. 2020).

Regarding the role of the internal seed airspace in the deterioration process, a Pearson correlation between the air volumes (%) of these seed species as calculated in a previous study (Diantina *et al*. 2020), and the percentages of seed viability loss showed a poor and non-significant correlation (R^2=0.163, p=0.758) between these two variables (Supplementary figure 3.1). This suggests that the relative volume of air contained within the seeds is not a good predictor of the rate of seed viability loss. However, more studies using a larger number of orchid species may be needed to confirm this observation.

Further research with more replications and additional ageing points to further exploration of responses to ageing and their relationship to fatty acid degradation in orchid seeds is warranted. There are differences between seed germination and viability data assessed using the tetrazolium test (Bradbeer 1988), therefore further studies to establish the relationship between viability data and real germination values are suggested.

3.6. Conclusions

Linoleic acid (polyunsaturated fatty acid) is the most abundant seed fatty acid in all species in this study. Similarities in lipid composition was found in epiphytic *Dendrobium* orchids, while terrestrial species were characterised by an absence of palmitoleic and erucic acid. However, all orchid species varied significantly in their responses to ageing. A reduction in polyunsaturated acids after ageing was found in most species, presumably due to lipid peroxidation or enzymatic hydrolysis. *D. strebloceras* showed the strongest change in fatty acid composition after ageing, indicating that this species is more susceptible to lipid deterioration than the other species studied. Two species, *P. banksii and D. lineale*, showed no significant variation in their lipid profiles despite a marked viability loss, suggesting other

factors are associated with viability loss in these species. There was no correlation between air volume and viability loss during slow ageing, and the relationship between seed fatty acids and ageing in orchid seeds requires further elucidation.

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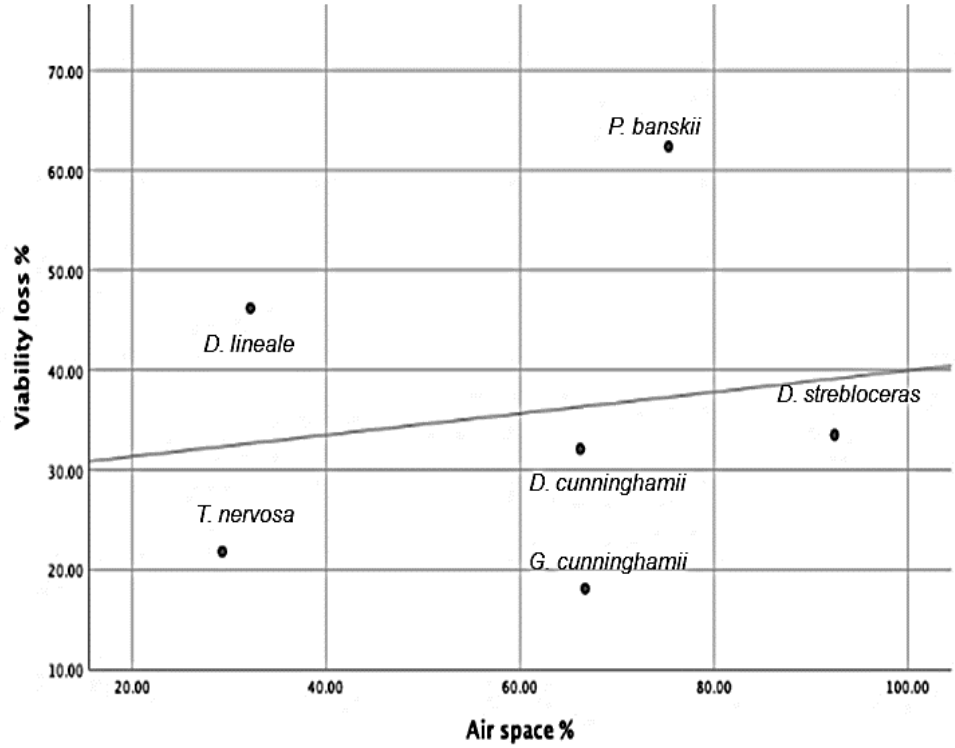
Supplementary figure 3.1.

Pearson correlation between airspace percentage and seed viability loss for the six orchid species used in this study (R^2=0.163, p=0.758)



CHAPTER IV

Comparative *in Vitro* Seed Germination and Seedling Development in Tropical and Temperate Epiphytic and Temperate Terrestrial Orchids

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4.1. Abstract

Ex situ conservation of orchid seeds requires the application of modern biotechnology to determine seed quality and to optimise *in vitro* regeneration. Crucially, the nutrient requirements for germination and seedling growth can be species-specific and can be influenced by life traits and habitats. In this study, *in vitro* seed germination and subsequent seedling development of selected tropical and temperate epiphytic and temperate terrestrial orchids were investigated. Seeds of three epiphytic orchids [*Dendrobium strebloceras, D. lineale* (tropical) and *D. cunninghamii* (temperate)] were sown on four different media supplemented with 2% sucrose: (1) Murashige and Skoog (MS); (2) Vacin and Went (VW); (3) Norstog; and (4) water agar. In addition, *D. strebloceras* seed were sown on the media above supplemented with 3% sucrose and half-strength MS supplemented with either 2% or 3% sucrose. Temperate terrestrial orchid (*Gastrodia cunninghamii, Pterostylis banksii* and *Thelymitra nervosa*) seed germination was assessed on media (1-4) but with VW replaced by terrestrial orchid medium-BM1. Whilst all epiphytic orchids germinated on all media tested, the best seedling development for the two tropical species was on full or half-strength MS media. The temperate epiphyte *D. cunninghamii* germinated best on Norstog medium but did not develop further. Norstog and BM1 media supported germination and seedling development better in *P. banksii* and *T. nervosa*. This study concludes that the temperate terrestrial *P. banksii* and *T. nervosa* needs excating media (Norstog or BM1 media) for in vitro seed germination and subsequent seedling development compared to tropical epiphytic species *D. strebloceras* and *D. lineale*. Optimising these nutrient requirements *in vitro* will underpin successful *ex situ* conservation of orchid species. Soil nutrients and availability of specific mycorhizal fungi in nature is critical, especially for terrestrial orchid. Poor soil nutrient content and loss of specific fungi could lead to species extinction.

Keywords: culture media, epiphytic *Dendrobium*, *in vitro* regeneration, protocorm, seedling development, terrestrial orchid

4.2. Introduction

Orchidaceae, the second-largest plant family with an estimated 25,000 species (Willis 2017), is at imminent risk of genetic loss. This is due to many factors, including over-harvesting and illegal trade, despite all species are protected under the Convention on International Trade in Endangered Species (CITES 2017) of the Wild Fauna and Flora Treaty. Consequently, *in situ* and *ex situ* conservation strategies are being developed constantly to safeguard orchid germplasm.

Most terrestrial orchid seeds will only germinate in nature following infection by mycorrhizal fungi, an interaction that can be species-specific (Kauth *et al*. 2008). A decrease of favourable soil conditions and reduction or loss of fungi from the habitat may therefore become major constraints for orchid plant regeneration and negatively affect *in situ* conservation efforts (Arditti and Ghani 2000; Batty *et al*. 2001). This means development of the *ex situ* conservation of orchids is even more urgent, as part of an integrated strategy of conservation that links the storage of germplasm with the reintroduction of plants to the natural habitat.

Kew garden has approximately 4500 orchid accessions in their collection, with 608 records in a seed information database but no information on how many species or accession in seedbank (https://www.kew.org/kew-gardens/plants/orchids-collection). Orchid Seed Stores for Sustainable Use (OSSSU), a Darwin Initiative Project, aimed to store ca. 250 orchid species over a period of three years (Seaton and Pritchard 2011).

Seed banking underpins long-term *ex situ* conservation of orchids and other flowering plants (Li and Pritchard 2009). Preferably, seeds of high initial quality are used, but the means of assessing seed quality in orchids is still challenging. Estimation of the maximum number of seeds able to grow under optimum conditions can be used as a measure of seed viability (Bradbeer 1988), assuming any dormancy in the seeds has been removed. Alternatively, viability per se can be assessed in orchids by using the triphenyl tetrazolium chloride (TTC) test (Hosomi *et al*. 2012). Whilst the test works well for many epiphytic species, in terrestrial species where seeds may be pigmented owing to the presence of lignins,

visualisation of the red formazan produced from TTC in viable seeds can be difficult to interpret. Chemical scarification in some terrestrial orchids by either prolonged seed soaking in NaClO (Dowling and Jusaitis 2012; Magrini *et al*. 2019) or acid fuchsin (Vujanovic *et al*. 2000) has been reported to improve the reliablity of the test. However the use of vacuum degassing has not been investigated for orchid seed TTC. Hence, in this study, the efficacy of vacuum degassing to facilitate permeation of TTC solution into the seeds was investigated as this method was proven successful for viability testing in pine species (Daws *et al*. 2006).

Determining a suitable germination medium for a particular orchid species is an essential prerequisite for seed propagation within *ex situ* conservation programmes. Whilst *in vitro* seed germination is a common practice among orchid breeders (Vendrame *et al*. 2007), the environment and climatic factors required to stimulate seed germination and seedling growth is known to vary greatly amongst orchids (Baskin and Baskin 1998; Fay *et al*. 2015). To increase the predictability of success, some have investigated the relationship between nutritional requirements and life-history traits and habitat (Nadarajan *et al*. 2011). Direct comparative studies are lacking on this subject, however, and based on a few reports available, it seems that seeds of temperate, terrestrial orchids need a more complex medium for germination than tropical epiphytic orchids (Rasmussen 1995; Nadarajan *et al*. 2011). This could be due to epiphytic orchids having a wider compatibility with general mycorrhiza and a simpler requirement for nutrition (da Silva 2013; Rasmussen *et al*. 2015); whatever the reason, asymbiotic germination has a higher success rate with epiphytic species (da Silva *et al*. 2015). In contrast, terrestrial orchid species seeds have a greater dependency on a particular fungal strain as the nutrient provider for germination, and generally have a lower success rate in an asymbiotic germination test (Baskin and Baskin 1998; Bustam *et al*. 2014a,b; Miyoshi and Mii 1995; Zhang *et al*. 2013).

Symbiotic mycorrhizal fungi are also known to support post-germination growth, i.e. protocorm and seedling development. In some species, this support is not critical for early germination but is necessary to ensure further development of protocorms and seedlings (Rasmussen 1995; Weston *et al*. 2005). Hence, it is likely

that the optimum composition of nutrients required for *in vitro* seed germination is different from that needed for seedling development. For example, a different form of nitrogen source may be needed as the germination progresses (Kauth *et al*. 2008). The combination of these factors add to the complexity of species-specific nutritional requirements for *in vitro* orchid seed germination and seedling development (Nadarajan *et al*. 2011; da Silva *et al*. 2015; Kartikaningrum *et al*. 2017).

This study investigated different nutrient media requirements for optimization of orchid seed germination and subsequent seedling development. Orchid species with different life-history traits (i.e., epiphytic or terrestrial) and growth environments (i.e., tropical or temperate) were investigated. For this, three epiphytic *Dendrobium* species (two tropical species from Indonesia and one temperate species from New Zealand) and three terrestrial species from New Zealand were selected.

The main hypothesis tested in this comparative study is, that epiphytic orchids have a less stringent media formulation for germination and growth than terrestrial orchids and similarly, that seeds of tropical species germinate more easily than those of temperate species.

4.3. Materials and methods

4.3.1. Seed materials

Two epiphytic tropical orchids, *D. lineale* and *D. strebloceras*, were obtained from the glasshouse collection of the Indonesia Agency for Agricultural Research and Development (IAARD), Indonesia. Flowers were hand-pollinated and seed pods harvested at maturity, indicated by a change in colour from green to greenish-yellow; *D. lineale* was harvested four months after pollination (November 2016 – March 2017) and *D. strebloceras* at five months (March 2018 – August 2018). Seed pods were then couriered to New Zealand. The open-pollinated seed of epiphytic temperate orchid *D. cunninghamii* was harvested in early autumn (March 2017) from Pukeiti Forest, North Island, New Zealand. Seed germination of *D. lineale* and

D. cunninghamii was test together while *D. strebloceras* was tested at the following year. Plants of three species of terrestrial orchid species (*Gastrodia cunninghamii*, *Pterostylis banksii* and *Thelymitra nervosa*), growing in the Iwitahi Native Orchid Heritage Protection Area, North Island, New Zealand, were monitored and mature pods were collected in February 2018 and seed germination of these three species was tested simultaneously.

4.3.2. Seed cleaning and handling before experiment

Seeds were removed from their pods and temporarily stored (~1-4 weeks) in filter paper envelopes at 15°C and 15% relative humidity at the Margot Forde Forage Germplasm Centre, AgResearch, Palmerston North, New Zealand until the commencement of the experiment.

4.3.3. Seed viability assessment using Triphenyl Tetrazolium Chloride (TTC) test

A TTC viability test was conducted according to the method of Hosomi *et al*. (2012) to confirm seed viability prior to *in vitro* germination experiments. The seeds were incubated in 10% (w/v) sucrose solution overnight at room temperature then the sucrose solution was replaced with 1% (w/v) Tetrazolium Chloride (TTC) followed by incubation overnight in the dark at 40°C. Either four (*D. lineale* and *D. cunninghamii*) or three (the rest of species) replicates were used to test seed viability. The average number of seeds per replicate was c. 150. Number of viable seeds (indicated by red-stained embryo) were counted from the total numbers of full seeds observed under a binocular microscope.

The potential of using a vacuum to facilitate the permeation of TTC into the embryos was investigated for *Pterostylis banksii,* which has brown-coloured testa. For this experiment, seeds that had been stored at 15% RH and 15°C for one year prior to assessment were used. The seeds were pre-conditioned in 10% (w/v)

sucrose solution overnight at room temperature, then the sucrose solution was replaced with 1% (w/v) TTC solution, incubated under vacuum infiltration with 50% vacuum (50% atm, 15 in Hg), and 100% vacuum (0 atm, 0 in Hg) for 0, 1, 10 and 30 min. Room atmospheric pressure was used as control. This was followed by incubation of the seeds overnight in the dark at 40°C. Three replicates of c. 100 seeds were used and viable seeds were counted as described above.

4.3.4. *In vitro* germination media composition

Seeds of three epiphytic orchid species*; Dendrobium strebloceras*, *D. lineale* (tropical) and *D. cunninghamii* (temperate) were sown on four different media (Supplementary Table 4.1), all supplemented with 2% sucrose: 1) Murashige and Skoog (MS) (Murashige and Skoog 1962); 2) Vacin and Went (VW) (Vacin and Went 1949); 3) Norstog (Norstog 1973); and 4) water agar (control). A further comparative study was conducted on *D. strebloceras* where seed was tested for in vitro germination on the four media above supplemented with 3% sucrose, and half-strength MS supplemented with either 2% or 3% sucrose. Due to limitation in seed availability for other species, this comparison was only possible for *D. strebloceras.*

Germination of three temperate terrestrial orchid (*Gastrodia cunninghamii*, *Pterostylis banksii* and *Thelymitra nervosa*) was assessed on media 1), 3) and 4) above, and with 5) BM1 (van Waes and Debergh 1986) with all five media supplemented with 2% (w/v) sucrose. B5 vitamins (Gamborg *et al.* 1968) were added to both MS and VW media and all media were supplemented with 7.5 g/L Davis minimal agar (Sigma Aldrich, USA). BM1 was previously known for its efficacy in promoting temperate terrestrial orchid seed germination, thus it is important to investigate this media in this study.

4.3.5. Seed disinfection and germination

Prior to germination, seeds were wrapped in filter paper envelopes and surface disinfected by immersion in 10% (v/v) bleach solution (0.42 g/L NaClO) for 5 min, followed by rinsing in sterile distilled water twice and immersion in 1% (v/v) antibiotic-antimycotic solution (Sigma-Aldrich) for 1 min. Seeds were then air-dried in the laminar flow hood for about 3 min before sowing on germination media. Four replicates of *Dendrobium* species (epiphytic), approximately 100 seeds were sown onto each plate and incubated under a 12 h photoperiod (60 µmol/m2/s) provided by cool white fluorescent tubes at 25 ± 2°C in a germination chamber.

Epiphytic and terrestrial orchids have different abiotic requirements to induce seed germination. Most tropical orchid species germinate readily in nature while temperate species required more specific requirement to grow (dark/light, photo period, temperature, or inhibited by requirement of dormancy breaking (da Silva 2013; Kauth *et al*. 2008; Rasmussen 1995). It was also widely known that most terrestrial orchids require dark incubation and light inhibitory effect was found in some terrestrial orchid species (Baskin and Baskin 2014; Kauth *et al*. 2008; Rasmussen 1995). For this reason, seeds of terrestrial species were incubated in darkness at 25 ± 2°C for 2 months. Following that, the seeds were transferred to a germination chamber with 12-h photoperiod (60 µmol/m2/s) at 25 ± 2°C to promote shoot development.

4.3.6. Seed germination and seedling development

The germination process begins with seed imbibition, a physical response to water uptake, followed by a physiological process indicated by the swollen green embryos. The criteria by Kauth *et al*. (2008) and Batty *et al*. (2001) for epiphytic and terrestrial orchids respectively was used to identify seed germination stages (Supplementary Table 4.2). It was difficult at the initial stage to distinguish between imbibed-dead seeds and imbibed seeds with viable embryos. To avoid misleading results, the first stage of germination (embryo swollen stage) was scored after either two months (for epiphytic orchid seed) or five months (for terrestrial orchid seed)

following sowing. This was because after this period, the imbibed dead seeds had not increased any further in size, whereas the embryo size of viable seed had increased as their embryos developed into the next embryo developmental stages (Supplementary Figure 4.1).

Further quantitative measurements on *D. strebloceras* were also made under a microscope at 6 months after sowing. Embryo diameter and area measurement were done under a light microscope. Quantitative measurement of plantlets was made for height, leaf and root length, and the number of roots, leaves and shoots. Plantlets were removed from the media and measurement was made with a ruler (mm), with 30 replications for each treatment.

Total seed germination and seedling development assessment were conducted using a light microscope (microscope: Olympus SX7; light: Olympus DF PLAPO $1X_{-4}$, Olympus Optical, Tokyo, Japan).

4.3.7. Data analysis

Each species was analysed separately to identify media composition effect on seed germination. Randomization in allocating plates or experimental units was applied by randomly placing plates across the growth cabinet and rotating every week. For *D. strebloceras*, a factorial design testing media composition and sucrose concentration was conducted. Statistical analysis was performed using IBM SPSS statistic 24. Data were log-transformed to meet normality assumptions (if needed) before analysis of variance (ANOVA), and significant results were statistically compared with Duncan's New Multiple Range Test (DMRT, $\alpha = 0.05$).

4.4. Results

4.4.1. Seed viability by triphenyl tetrazolium chloride (TTC) staining

Estimation of viable embryos of three epiphytic species was determined by stained bright red embryo with TTC, confirming the high quality of the seed lots (Figure 4.1a-c). For the terrestrial species *G. cunninghamii* and *P. banksii*, the seeds had a light brown (tan coloured) testa and viable seeds had cream-brown/pinkish coloured embryos (Figure 4.1d-e). Seed of *T. nervosa* is covered by a brown testa and viable embryos showed up as light to dark brown in colour (Figure 4.1f). For terrestrial orchids it was difficult to achieve a good red-stained embryo, making the quantification of viability challenging. However, any white embryo was considered non-viable. All three terrestrial species showed c. 90% or more initial viability (Supplementary Table 4.3).

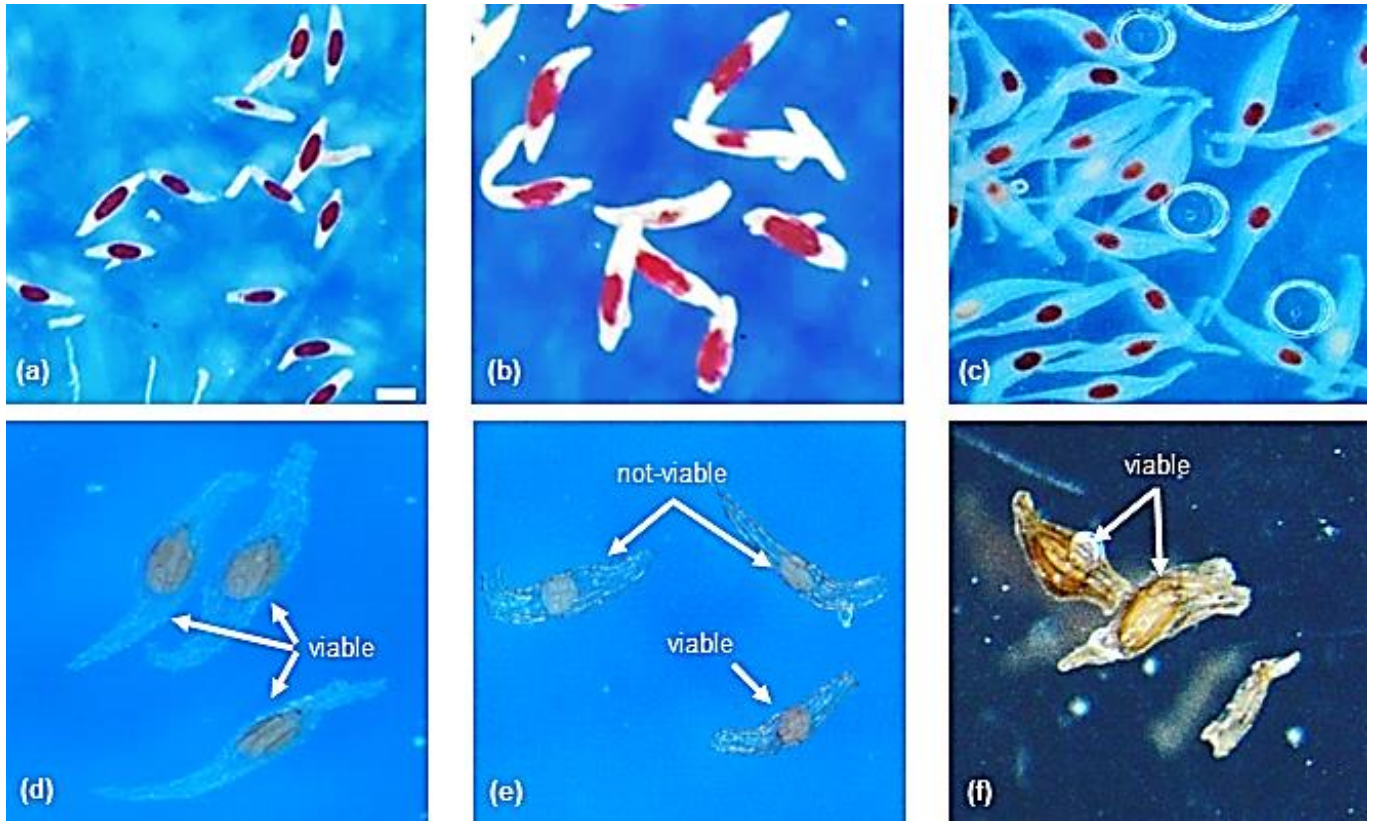


Figure 4.1. Epiphytic and terrestrial orchid seeds viability assessment using triphenyl tetrazolium chloride (TTC) staining: (a) *Dendrobium cunninghamii*, (b) *D. lineale*, (c) *D. strebloceras* (Average seed length ± SE: (a) 0.48 ± 0.008 mm; (b) 0.42 ± 0.006 mm; (c) 1.76 ± 0.04 mm respectively); (d) *Gastrodia cunninghamii*, (e) *Pterostylis banksii*, (f) *Thelymitra nervosa* (Average seed length ± SE: (a) 0.85 ± 0.02 mm; (b) 1.09 ± 0.03 mm; (c) 0.54 ± 0.006 mm, respectively [Diantina, *et al*. 2020a])

4.4.2. TTC Seed viability using vacuum infiltration

The TTC test for *P. banksii* seed showed that seed viability declined from around 86% (when they were fresh) to about 23% after one-year storage at 15% RH at 15°C. Treating seeds with TTC solution at 50% and 100% vacuum for 1 and 10 min did not show any difference in the viability, however, increasing the treatment time to 30 min did increase the viability recorded. In particular, treating the seeds at 100% vacuum for 30 min showed a significant difference in viability which showed an increase of 15% compared with the control treatment (Figure 4.2).

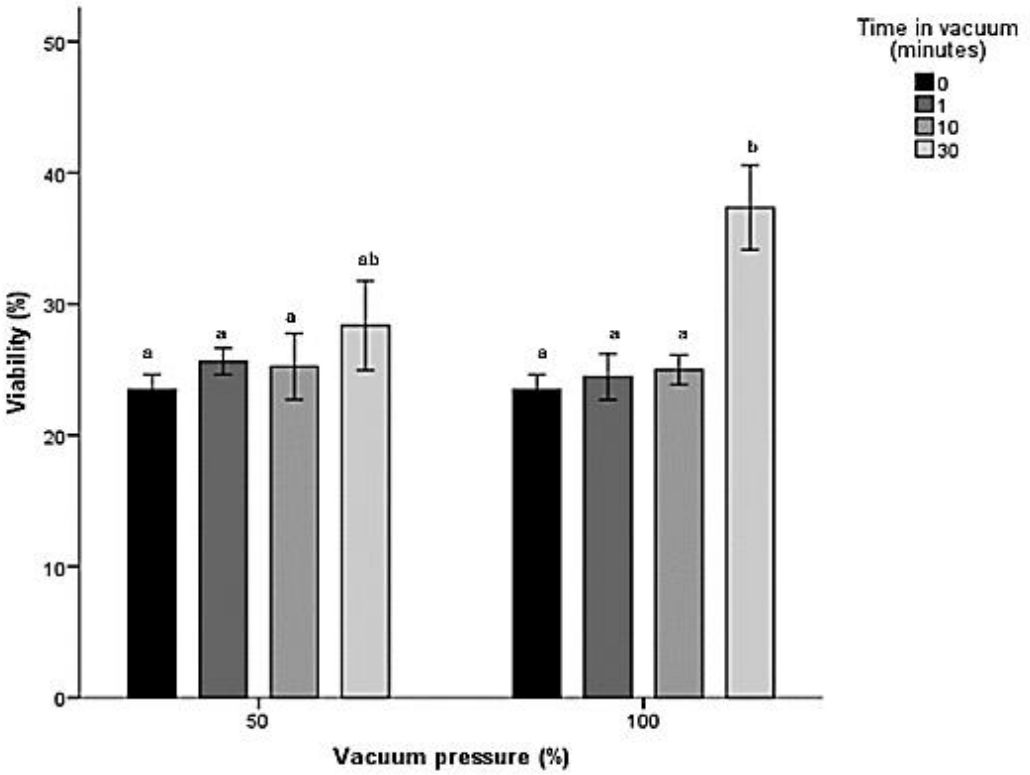


Figure 4.2. Comparative seed viability assessment following one year storage at 15% RH and 15°C using a triphenyl tetrazolium chloride (TTC) test of *Pterostylis banksii*, a terrestrial orchid at different atmospheric pressures. Initial test on fresh seed showed c. 86% viability. Bars represent mean ± standard error. Different letters indicate significant differences using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$) for each vacuum pressure treatment)

4.4.3. Seed germination of epiphytic orchids

The earliest epiphytic orchid seed germination stage is indicated as an imbibed seed with swollen embryo while the seed still covered by testa (Supplementary Figure 4.2a.1). *D. lineale* which is a tropical epiphyte, showed significantly better germination on VW medium, whereas the temperate epiphyte *D. cunninghamii* showed significantly higher germination on Norstog and VW media (Figure 4.3a).

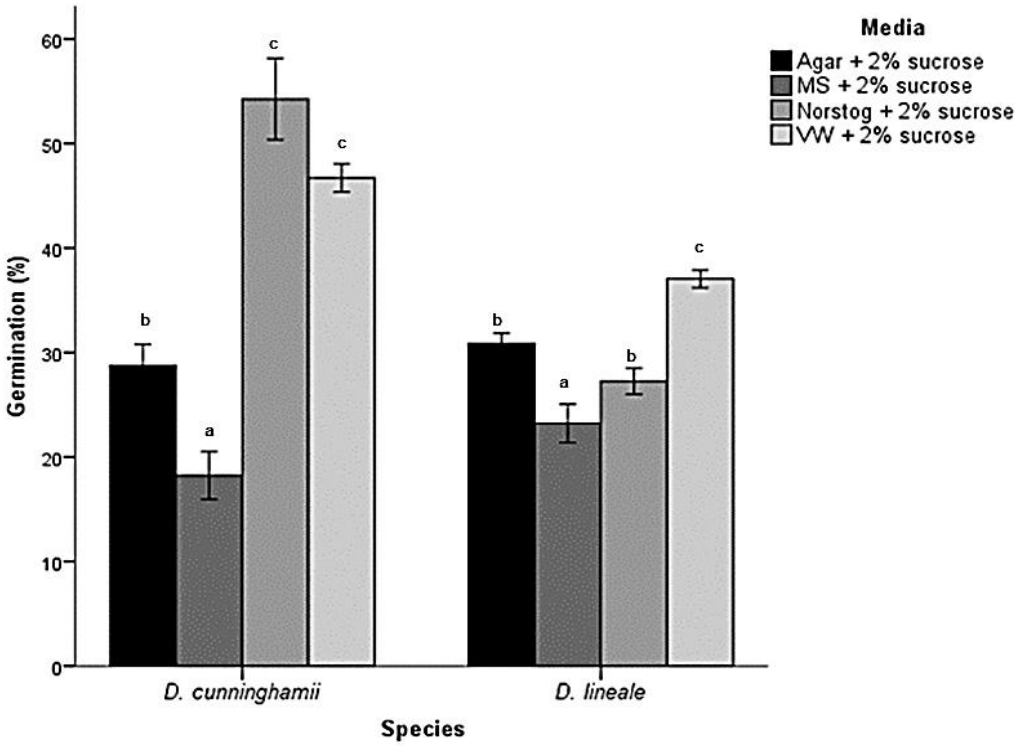


Figure 4.3.a. Percentage of seed germination up to embryo swollen stage of two epiphytic orchid seeds; *Dendrobium cunninghamii* and *D. lineale,* on four different media added with 2% (w/v) sucrose two months after sowing. Bars represent mean ± standard error. Different letters indicate significant differences of each media tested tested for each species using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$)

Increasing sucrose up to 3% in MS medium increased seed germination and when MS medium with 2% sucrose was reduced to half-strength, *D. strebloceras* seeds

showed the highest seed germination (~56%; Figure 4.3b). However, increasing sucrose concentration to 3% in half-strength MS significantly reduced germination (~35%; Figure 4.3b). This was also true for Norstog medium but not in VW medium where the germination remained at c. 43-44% for both 2% and 3% sucrose concentrations (Figures 4.3b).

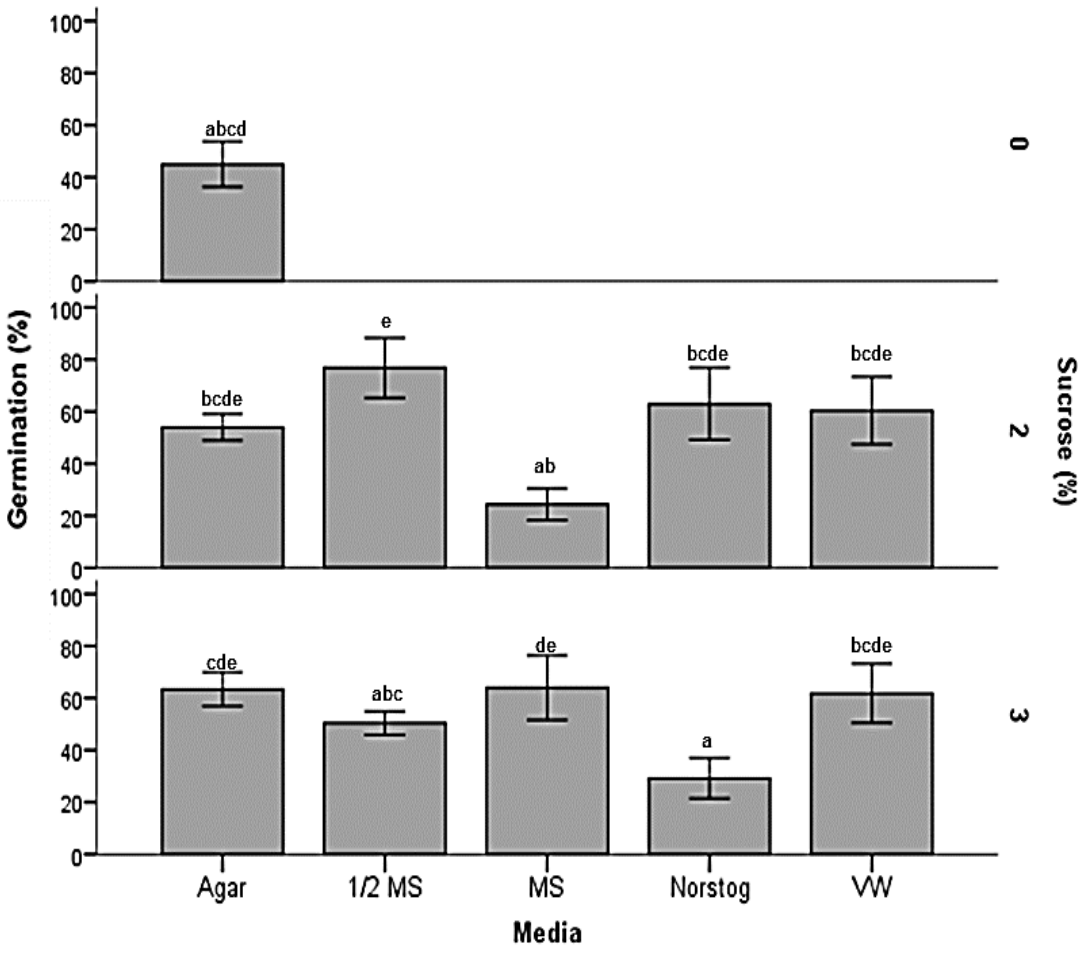


Figure 4.3.b. Percentage of seed germination up to embryo swollen stage of *D. strebloceras* on four different media added with either 2% or 3% (w/v) sucrose, and agar only without sucrose as control. Bars represent mean ± standard error. Different letters indicate significant differences of each media tested on *D. strebloceras* using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$)

On the other hand, *D. strebloceras* seed was able to germinate on water agar alone without any sucrose supplement (Figure 4.3b). Addition of either 2% or 3% sucrose for *D. strebloceras,* induced better protocorm development with the largest protocorms (diameter and area) were recorded with the addition of 2% sucrose (Table 4.1).

Table 4.1. Embryo growth of *Dendrobium strebloceras* after six months of germination on water agar media supplemented with three different concentrations of sucrose, in comparison to control seeds

Treatment	Embryo diameter (µm)	Embryo area ($\times$1000 µm^2)
Seeds sown on water agar supplemented with 0% (w/v) sucrose	561.8 ± 110.7a	232.1 ± 105.0a
Seeds sown on water agar supplemented with 2% (w/v) sucrose	790.0 ± 174.2b	505.3 ± 142.0c
Seeds sown on water agar supplemented with 3% (w/v) sucrose	719.3 ± 240.9b	374.1 ± 172.8b

The control (non-imbibed seeds) had embryo length c. 0.25 ± 0.007 mm, width c. 0.16 ± 0.005 mm and volume c. 0.004 ± 0.0003 mm^3 (Diantina *et al*. 2020a)

All epiphytic orchids were able to germinate up to embryo swollen stage (Stage one) on all *in vitro* media tested, including water agar medium (Figure 4.4). In both tropical epiphytes, *D. lineale* and *D. strebloceras,* germination process progressed beyond testa rupture (stage two) with protocorm production and rhizoids (stage three) after 2 months of sowing on MS, Norstog and VW media. These both species reached leaf emergence (stage four) faster on MS medium supplemented with 2% sucrose compared to other media tested (Figure 4.4b.4 & c.4). Interestingly, for *D. cunninghamii,* a temperate epiphyte, although embryos became swollen on all media tested, further germination to testa rupture and appearance of rhizoid was noted only on Norstog medium (Figure 4.4a.2), however, no seedling development was observed on this medium.

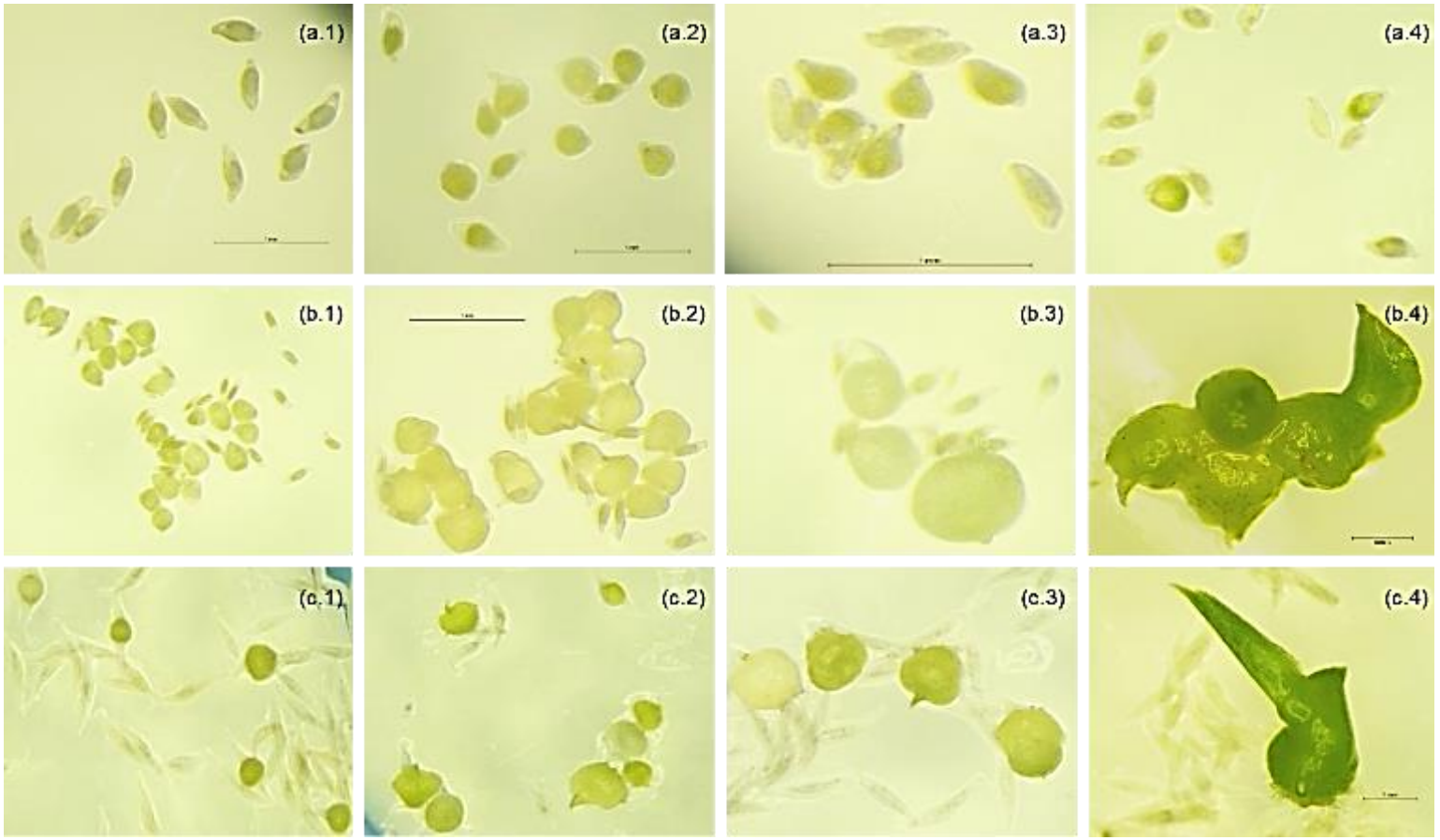


Figure 4.4. Epiphytic orchid seeds of (a) *Dendrobium cunninghamii* (temperate), (b) *D. lineale* (tropical), (c) *D. strebloceras* (tropical), showing different stages of germination three months after sowing on different media: (1) water agar, (2) Norstog, (3) VW, (4) MS media, supplemented with 2% sucrose

4.4.4. Seed germination of terrestrial orchids

For the temperate terrestrial orchid species, early seed germination stage has been described as an imbibed seed, and swollen embryo followed by testa rupture and appearance of trichomes (hairy roots) on protocorm surface (Supplementary Figure 4.2b & c). All three species showed significantly lower germination on water agar supplemented with 2% sucrose (Figure 4.5a) than on nutrient-supplemented media. *G. cunninghamii,* the temperate epiphyte seeds showed no significant difference in germination on all other media tested. However, germination was very low (~20%). Norstog and BM1 were found to be the best media for *P. banksii* and *T. nervosa* seed germination (Figure 4.5a).

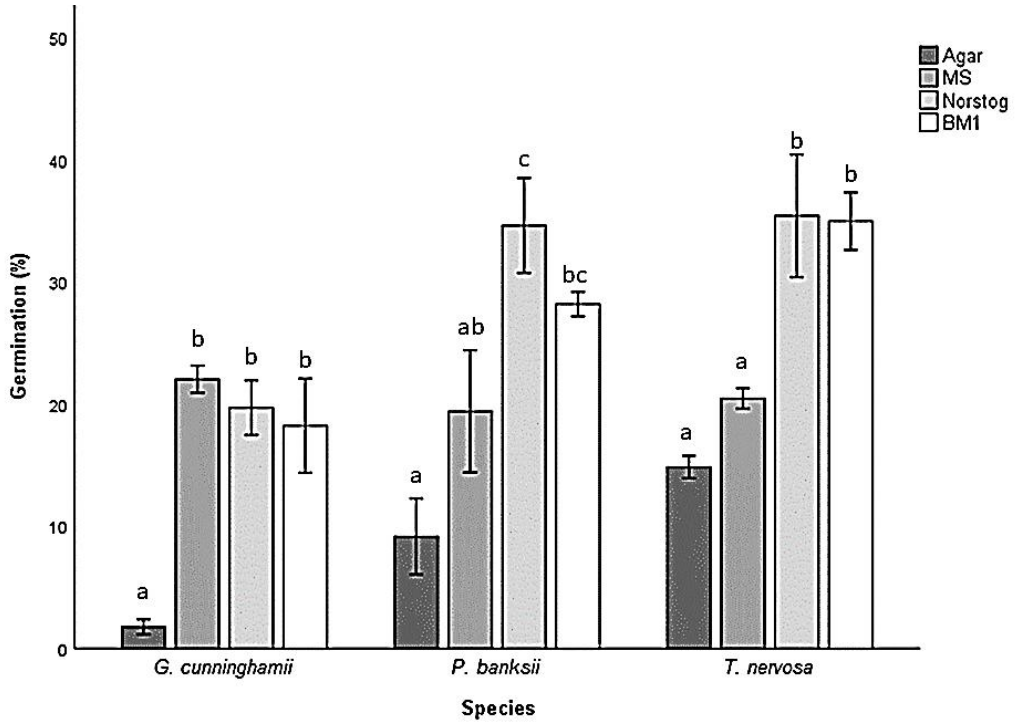


Figure 4.5.a. Comparative germination (embryo swollen stage observed at five months after sowing) of temperate terrestrial orchid seeds *Gastrodia cunninghamii, Pterostylis banksii* and *Thelymitra nervosa*, on four different media. Bars represent the mean ± standard error. Different letters indicate significant differences of each treatment media for each species tested using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$) for each species

Further observation of embryo growth and shoot development after six months showed that the germination process continued in *P. banksii* with the appearance of protomeristems (initiation of shoots and roots) on both Norstog and BM1 media (Supplementary Figure 4.2b & c). For *T. nervosa,* a protomeristem developed only on Norstog medium. However, *G. cunninghamii* seeds showed germination up to stage one only (Figure 4.5b).

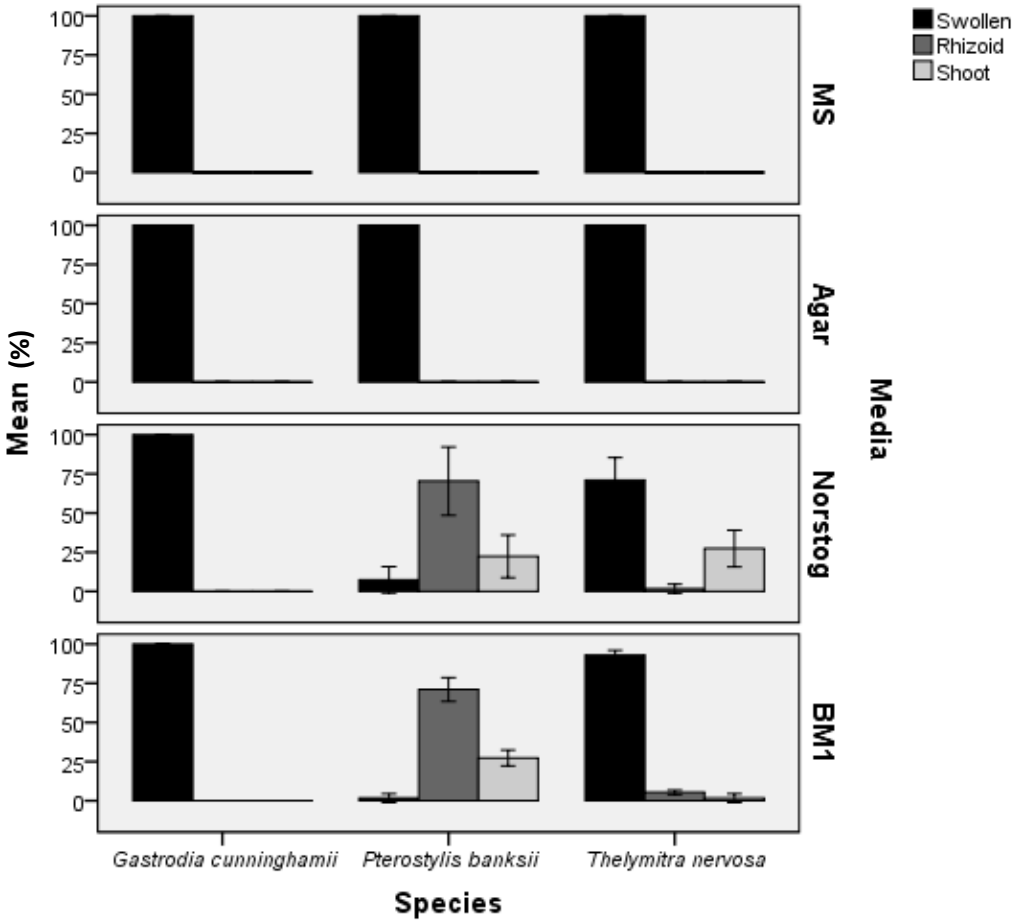
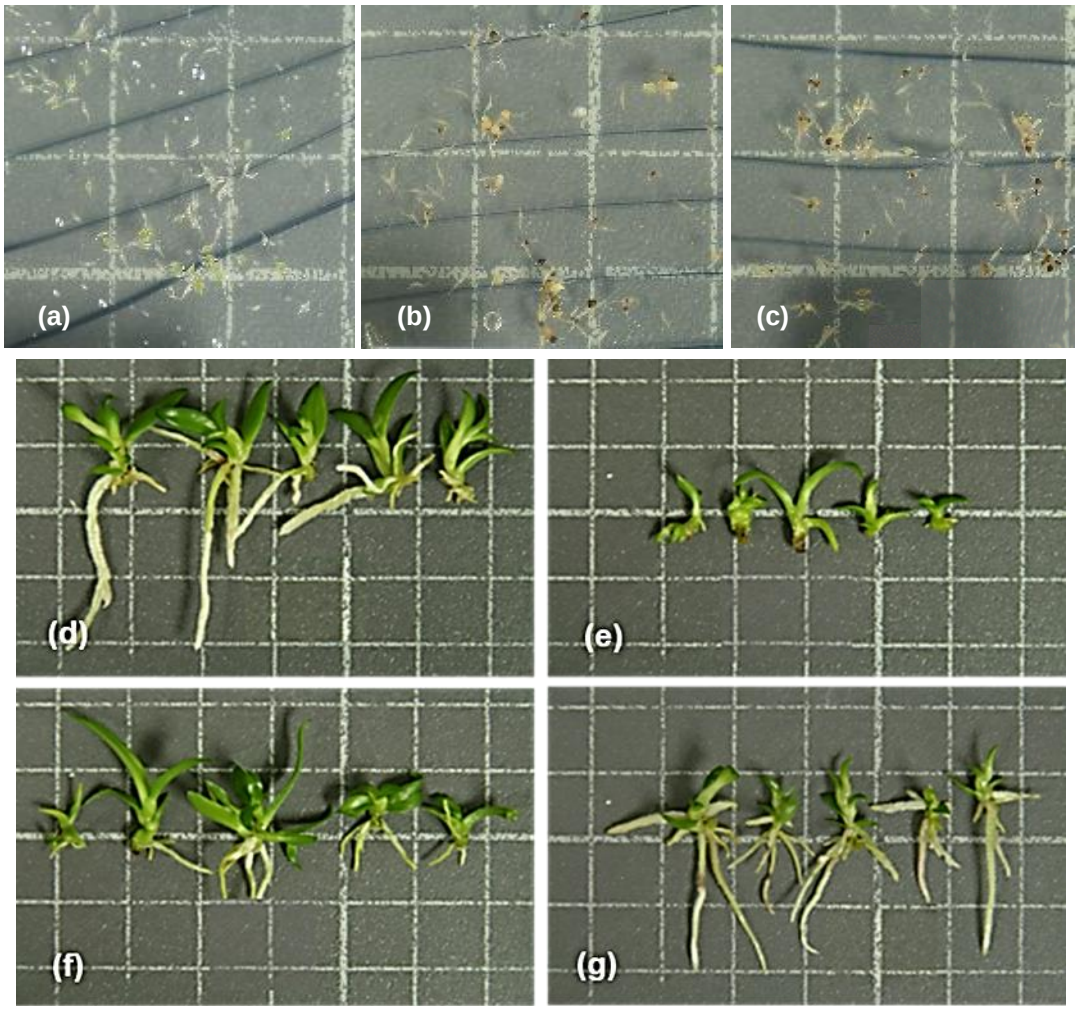


Figure 4.5.b. Further embryo growth and development (observed at six months after sowing) of temperate terrestrial orchid seeds *Gastrodia cunninghamii, Pterostylis banksii* and *Thelymitra nervosa,* on four different media. Bars represent the mean ± standard error. Different letters indicate significant differences of each treatment media for each species tested using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$) for each species

4.4.5. Seedling development and vigour assessment in *Dendrobium strebloceras*

Further observations were carried out on *D. strebloceras* on the growth of embryos into protocorms and the development of complete plantlets (with leaves and roots) on all nutrient media supplemented with either 2% or 3% sucrose. In the presence of sucrose at up to 3%, root growth was promoted on MS, Norstog and VW media, but not on half-strength MS. However, plantlets on half-MS with 2% sucrose (Figure 4.6d) produced the best leaf and root growth compared with those on other media combinations (Figure 4.6).



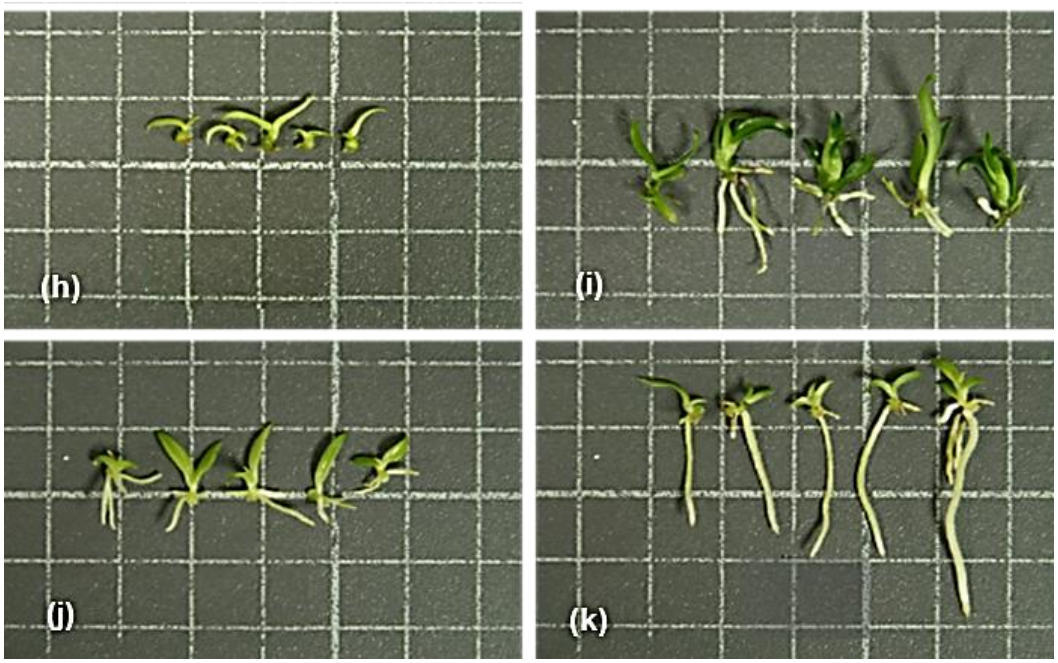


Figure 4.6. Seed germination and seedling development for the tropical epiphytic orchid, *Dendrobium strebloceras* after six months on: (a) water agar only (control), (b) water agar 2% (w/v) sucrose, (c) water agar 3% (w/v) sucrose, (d) ½MS + 2% (w/v) sucrose, (e) ½MS + 3% (w/v) sucrose, (f) MS + 2% (w/v) sucrose, (g) MS + 3% (w/v) sucrose, (h) Norstog + 2% (w/v) sucrose, (i) Norstog + 3% (w/v) sucrose, (j) VW + 2% (w/v) sucrose, (k) VW + 3% (w/v) sucrose. Grids represent 1 centimetre square

Vigour assessment by counting the numbers of leaves, roots and shoots produced on each medium was carried out for *D. strebloceras*. Norstog media supplemented with 2% sucrose consistently produced the lowest number of leaves (not significant with VW media), number of roots (not significant with ½ MS + 3% sucrose) and plantlets height (not significant with VW + 3% sucrose) (Figure 4.7). Half-strength MS supplemented with 2% sucrose increased number of leaves, and plantlets' height produced, whilst 3% sucrose suppressed seedling performance. Interestingly, on full MS media, sucrose concentration of 3% improved the numbers of roots produced (Figure 4.7b).

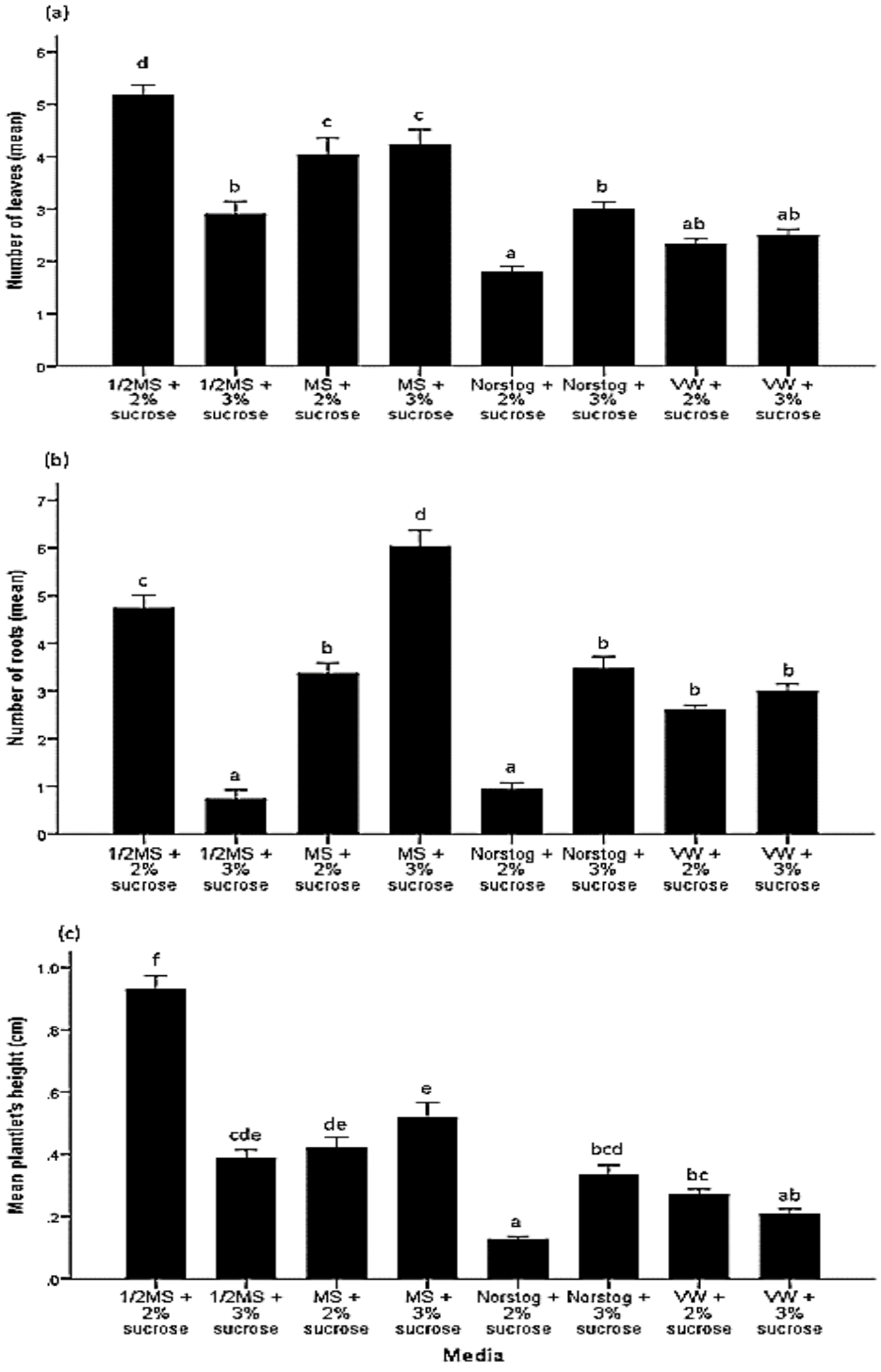


Figure 4.7. Number of leaves (a), number of roots (b) and plantlet's height (c) of *Dendrobium strebloceras* explants on different media at five months after sowing. Bars represent mean ± se. Different letters indicate significant differences using ANOVA followed by DMRT ($p<0.05$) for each medium tested

Also, there was no statistical difference in the numbers of shoots produced on half- or full-strength MS media with varying sucrose concentrations. Further quantitative assessment of seedling growth was made at six months after sowing. The combination of half-strength MS media with the addition of 2% sucrose consistently promoted leaf and root growth (Table 4.2).

Table 4.2. Leaf and root length, and plant height of *Dendrobium strebloceras* plantlets on different media with combinations different concentration of sucrose

Plantlet root and leaf measurement (mean ± standard error)					
Media	Sucrose (%)	Longest root (cm)	Longest leaf (cm)	Average leaves length (cm)	Average root length (cm)
½ MS	2	1.6 ± 0.8d	1.1 ± 0.2c	0.9 ± 0.2d	1.03 ± 0.5d
	3	0.07 ± 0.09a	0.5 ± 0.3ab	0.4 ± 0.2ab	0.27 ± 0.5a
MS	2	0.8 ± 0.8bc	1.2 ± 0.4c	0.9 ± 0.3d	0.5 ± 0.4b
	3	1.2 ± 0.5c	0.7 ± 0.3b	0.6 ± 0.2c	0.9 ± 0.4cd
Norstog	2	0.1 ± 0.06a	0.4 ± 0.39a	0.3 ± 0.2a	0.03 ± 0.04a
	3	0.7 ± 0.3b	1.1 ± 0.3c	0.9 ± 0.3d	0.5 ± 0.2b
VW	2	0.9 ± 0.3bc	0.7 ± 0.2ab	0.5 ± 0.2bc	0.6 ± 0.2b
	3	1.2 ± 0.6cd	0.5 ± 0.2ab	0.4 ± 0.1ab	0.7 ± 0.3bc

Different letters indicate significant differences using ANOVA followed by Duncan's New Multiple Range Test ($p<0.05$) for each growth measurement assessed

4.5. Discussion

In vitro orchid seed germination is a prerequisite if a large number of plants for conservation, reintroduction and breeding programmes are to be provided. However, the nutrient requirements for *in vitro* germination can differ significantly between species, and even within species sourced from different habitats from within the same geographic regions, as these habitats may have resulted in locally adapted ecotypes (Hufford and Mazer 2003).

Assessment of orchid seed viability using the TTC staining test provides crucial information on seed quality and has been successfully used on epiphytic orchids and to some extent on terrestrial orchids (Magrini *et al*. 2019). However, this approach needs to be optimized on a species by species basis, particularly for terrestrial species. This is because of their hydrophobic testa, characterized by cells with walls containing a wide range of lipids, suberins and polyphenolics such as lignin and tannins (Thompson *et al*. 2001; Barsberg *et al*. 2018; Pierce *et al*. 2019). Moreover, previous study found the presence of lignoceric acid in three *Dendrobium* species and *P. banksii* (Diantina *et al*. 2020b, Chapter 3).

TTC viability testing for terrestrial orchids often requires chemical scarification to allow testa permeation. Even with scarification treatments, testa permeability varies and this could lead to false-negative results. In this study, the use of a vacuum infiltration TTC viability test for a terrestrial orchid, *P. banksii,* was explored. In agreement with Daws *et al*. (2006), vacuum infiltration of TTC did improve the viability recording. Increasing the time under full vacuum to 30 minutes improved red coloration of the viable embryos for this species and showed an increase of c. 15% in viability recorded compared to the conventional method. Air from the inter-cellular spaces was removed by vacuum environment and thus eliminated any pressure differential in the tissue, and enabled the TTC staining solution to permeate uniformly through the seed coat into the embryo.

As orchid seeds have limited carbohydrate reserves, exogenous carbohydrate sources in the form of sugar and gelling agent are often required for *in vitro* germination. The use of sucrose as the carbon source in culture media has been

demonstrated to be very important in orchid seed germination and protocorm development (Rasmussen *et al*. 2015; Gupta 2016), for both mature and immature seeds (Arditti 1967; Rasmussen 1995; Prasongsom *et al*. 2017). Nevertheless, all epiphytic *Dendrobium* species studied were able to germinate on water agar without added sucrose up to embryo swollen stage. This finding is in agreement with that of Prasongsom *et al*. (2017), that some epiphytic orchids can complete the early stages of germination without external nutrient supplement.

Though adding sucrose to the water agar showed increased germination for *D. strebloceras* seeds from c. 35% (water agar alone) to c. 45% following 2% and 3% sucrose supplement respectively, and production of largest protocorms with the addition of 2% sucrose, the embryos did not develop further for germination. After six months, swollen seeds of *D. strebloceras* on water agar (without sucrose) remained green, while seeds on water agar with sucrose turned brown. Despite showing early signs of germination, seeds of this species were unable to develop into seedlings on water agar alone or with sucrose supplement. Even though the existence of sucrose is important as a source of carbon and energy in tissue culture environment, George (2008) reported that sucrose may inhibit chlorophyll formation whilst increase the respiration rate. Thus, for efficient germination of orchid seed that lack an endosperm, there is a need to balance both macro and micronutrients (Vaz and Kerbauy 2000; Yeung 2017).

Subsequent observations on seedling performance indicated that MS supplemented with 2% sucrose is optimal for the epiphytic orchids (Figure 4.4), but not for the terrestrial *P. banksii* and *T. nervosa* (Figure 4.5b). MS medium is widely used in *in vitro* germination of *Dendrobium* species (da Silva *et al*. 2015), and contains higher (in general) and more complex macro-micronutrients than Norstog and VW media. Reducing MS to half-strength supplemented with 2% sucrose was found to increase seed germination. Meanwhile, increasing sucrose concentration to 3% (w/v) in all nutrient-containing media promoted root development, except on half-strength MS media. The findings in this study broadly support those of Yates and Curtis (1949), who reported that sucrose as a carbon source correlates strongly with the shoot and root growth in orchid seeds and that the precise sucrose concentration required

could be species-specific. Some of the inter-species differences in optimum sucrose concentration and plant growth probably relate to osmotic effects on the seedlings' shoot and root development (George 2008).

For the temperate terrestrial orchids, seed germination and seedling development of *P. banksii* and *T. nervosa* were higher on Norstog and BM1 media, reflecting a possible species-specific preference for nitrogen source in the form of L-Glutamine amino acid as previously reported in other terrestrial orchids (Kauth *et al*. 2008; Nadarajan *et al*. 2011). It is generally reported that temperate orchid prefers organic nitrogen (Popova *et al*. 2003). Likewise, nitrogen source particularly in the ratio of ammonium to nitrate that presence in the media was considered to play important role at various stages of terrestrial orchid seed germination and seedling development (Kauth *et al*. 2008). It was postulated nitrogen in the form of amino acids may be more readily available to germinating seeds or developing protocorms than inorganic nitrogen sources (van Waes and Deberg 1986; Stewart and Kane 2006). When ammonium is utilized by the germinating seeds, the nitrogen is converted to amino acids which could be beneficial for further seedling development. It is therefore important to investigate the effects of concentration and the combination of amino acids that presence in the media on different stages of terrestrial orchid seed germination.

For the two temperate species (the epiphyte *D. cunninghamii* and the terrestrial *G. cunninghamii*), the embryos were unable to develop beyond the embryo swollen stage, indicating that the seeds do not have physical dormancy (Thompson *et al*. 2001). However, since the swollen embryos were unable to develop further into a protocorm, *D. cunninghamii* and *G. cunninghamii* may have morpho-physiological dormancy (MPD) which is common in temperate species including orchids (Rasmussen 1995; Miyoshi and Mii 1995; Poff *et al*. 2016). Therefore, dormancy breaking treatments need to be determined for these species before optimizing their germination on in vitro culture media.

Gastrodia is the most primitive genus in orchid, unique due to a lack of chlorophyll with a high dependency on specific mycorrhiza for seed germination (*Mycena*) and plant growth (*Armillaria mellea*), while urea metabolism might be an important

source of nitrogen for saprophytic *G. elata* (Yuan *et al.* 2018). Therefore, further research is needed to understand what specific nutrients and factors are required for germination and growth.

4.6. Conclusions

This study concludes that epiphytic and terrestrial orchids have different requirements for seed germination and subsequent seedling development. Tropical *D. lineale* and *D. strebloceras* orchids germinated well and able to produce seedling development in all media tested. Temperate terrestrial orchids *T. nervosa* and *P. banksii* not only required a more stringent media (only produced seedling on Norstog and BM1 media), but also required longer incubation in dark for germination. Temperate orchids, *D. cunninghamii* and *G. cunninghamii* may also require suitable dormancy breaking treatments for their germination. Understanding the differences in nutrient requirements for seed germination and subsequent seedling development, and optimising these conditions, will underpin successful *ex situ* conservation of orchid species with different life traits and habitats. The advancement of such studies on threatened orchids remains an urgent priority.

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Supplementary Table 4.1.

Comparative nutrient composition of orchid seed germination media tested: MS, VW, Norstog, BM-1

	MS	VW	Norstog	BM-1
Macronutrients (mM)				
Ammonium	20.61	7.57	-	-
Calcium	3	1.93	5.03	-
Chlorine	3	1.5	15.1	0.0021
Magnesium	1.5	1.01	3	0.83
Nitrate	39.4	5.19	-	-
Potassium	20.05	7.03	16.75	2.2
Phosphate	1.25	3.77	6.69	2.2
Sulfate	1.84	8.71	3	1.1
Sodium	0.1	0.20		0.2
Micronutrients (µM)				
Boron	100.26	50	8.1	161.7
Cobalt	0.11	0.053	0.105	0.105
Copper	0.1	0.5	0.1	0.1
Iron	183	50	40.83	100.2
Iodine	5	2.5	-	-
Manganase	89.3	50	17.75	147.9
Molybdenum	1.03	0.52	0.1	1.03
Zinc	29.9	14.95	1.74	34.8
Organics (mg/l)				
D-biotin	-	-	-	0.05
Casein hydrolysate	-	-	-	500
Calcium Panthotenate	-	-	0.25	-
Folic acid	-	-	-	0.5
L-Glutamine	-	-	400	100
Alanine	-	-	50	-
Cysteine	-	-	20	-
Arginine	-	-	10	-
Leucine	-	-	10	-
Phenylalanine	-	-	10	-
Glycine	-	-	-	2
Myo-inositol	100	100	50	100
Nicotinic acid	1	1	0.25	5
Peptone	-	-	-	-
Pyridoxyne. HCl (vit B6)	1	1	0.25	0.5
Thiamine. HCl (vit B1)	10	10	1	0.5

Supplementary Table 4.2.

Seed germination stages, protocorm development and seedling development for epiphytic* and terrestrial orchid**

Seed germination stage	Epiphytic orchid	Terrestrial orchid
0	Small embryo inside seed, testa covered surrounding embryo	Small embryo inside seed, testa intact covered surrounding embryo
1	Embryo swollen, testa intact (embryo still covered by testa)	Embryo swollen (imbibed seed)
2	Testa rupture, appearance of protocorm and rhizoids	Enlargement of embryo followed by testa rupture
3	Initiation of protomeristem (initial shoot and root)	Appearance trichomes (root hairs) and hairy tuber-like protocorm
4	Emergence of the first leaf	Initiation of protomeristem (initial shoot and root)
5	NA	Emergence of the first leaf

Modified and simplified from *Kauth *et al*. (2008) and **Batty *et al*. (2001)

Supplementary Table 4.3.

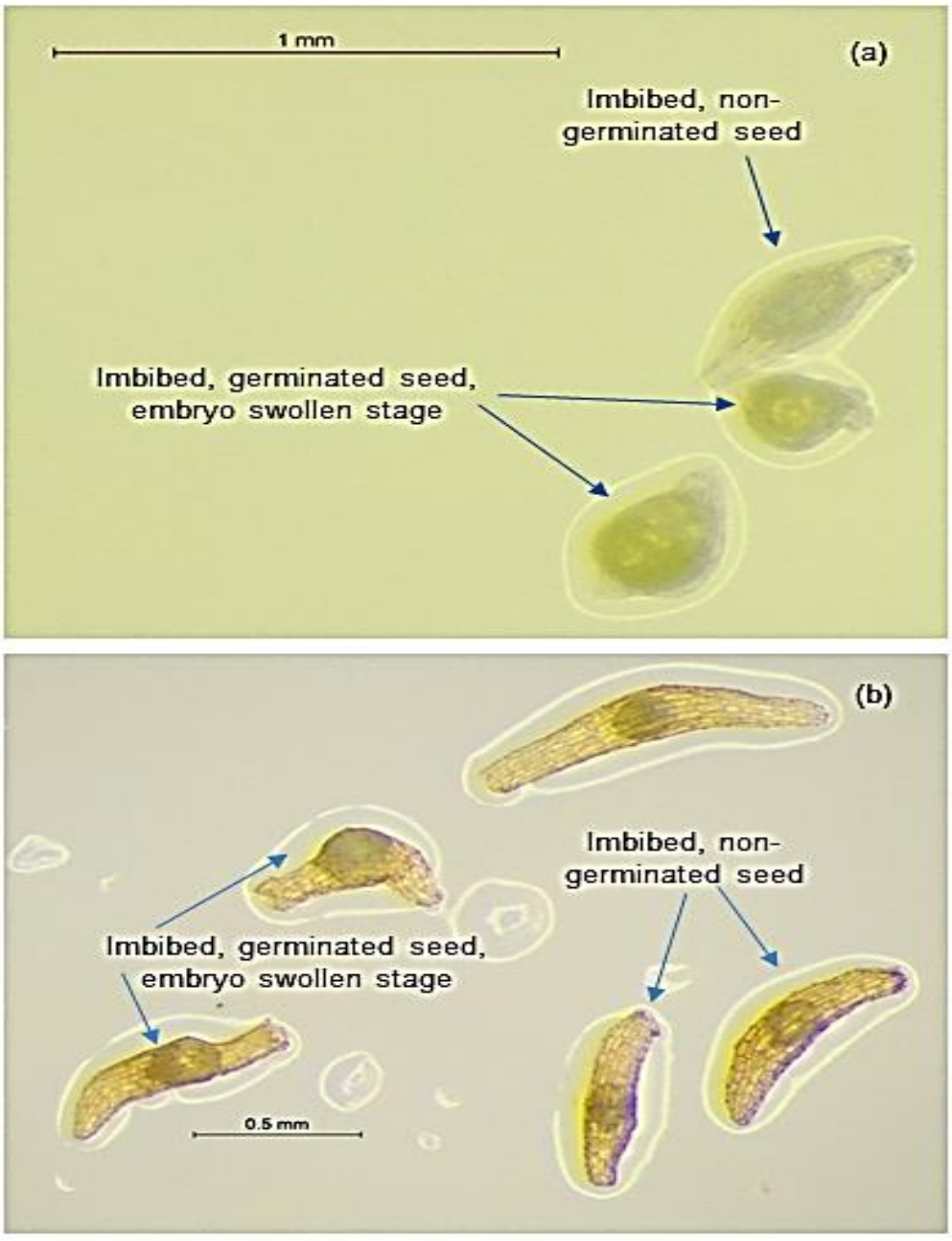
Comparative initial viability and germination assessment on epiphytic and terrestrial orchids

Species	Viability (%) (Mean ± SD)	Germination (%) (Mean ± SD)
Dendrobium lineale	56.06 ± 4.67	37.05 ± 1.69
Dendrobium cunninghamii	93.37 ± 1.30	54.25 ± 7.80
Dendrobium strebloceras	88.54 ± 4.73	53.86 ± 4.53
Pterostylis banksii	86.25 ± 8.90	34.59 ± 6.71
Gastrodia cunninghamii	97.37 ± 1.72	22.02 ± 1.92
Thelymitra nervosa	93.11 ± 3.00	35.38 ± 8.68

*Germination was up to embryo swollen stage only

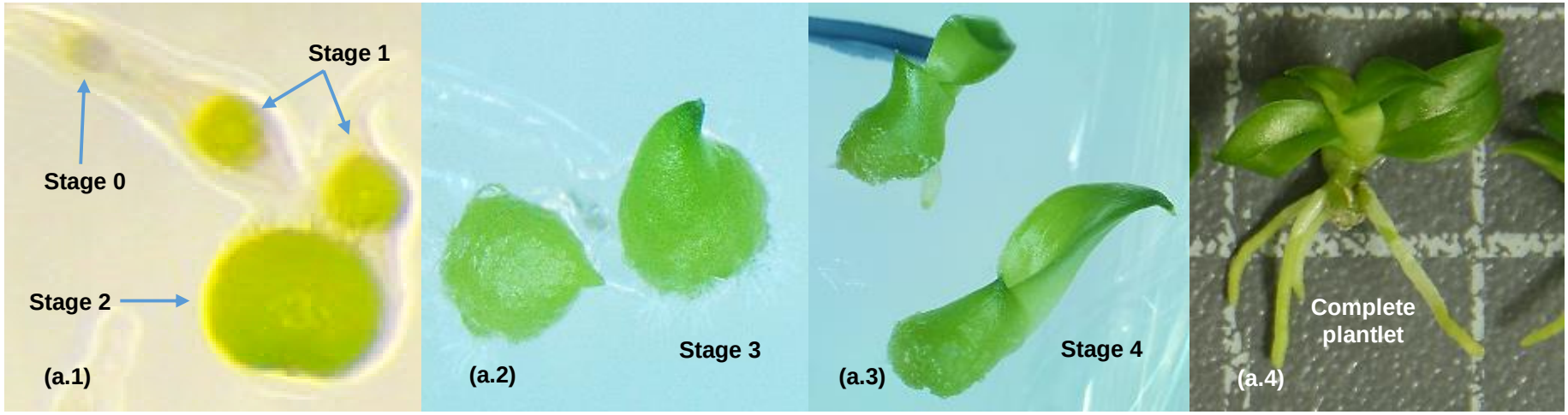
Supplementary figure 4.1.

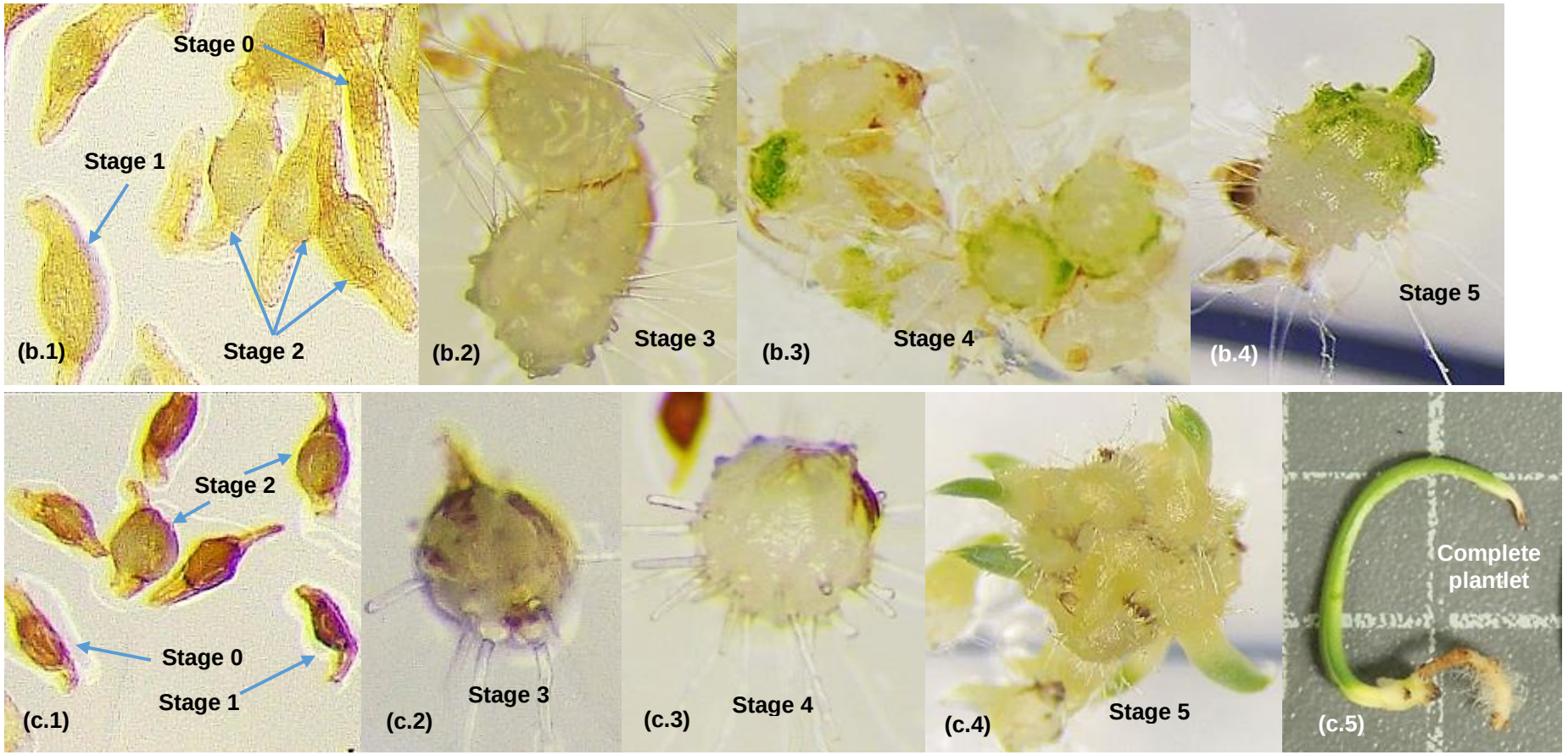
Differentiation of imbibed, non-germinated seeds with imbibed, germinated seeds at the early stage of seed germination for a) epiphytic orchid, *Dendrobium cunninghamii* (3 months after sowing) and b) terrestrial orchid, *Pterostylis banksii* (5 months after sowing).



Supplementary figure 4.2.

Various germination stages of a) *Dendrobium strebloceras* (epiphyte), b) *Pterostylis banksii* (terrestrial) and c) *Thelymitra nervosa* (terrestrial)




Stage 0
Stage 1
Stage 2
(b.1)
(b.2)
Stage 3
(b.3)
Stage 4
(b.4)
Stage 5
Stage 2
Stage 0
Stage 1
(c.1)
(c.2)
Stage 3
(c.3)
Stage 4
(c.4)
Stage 5
Complete plantlet
(c.5)

CHAPTER V

Seed Cryopreservation of Indonesian and New Zealand Epiphytic and Terrestrial Orchids

5.1. Abstract

Worldwide, orchids face an imminent risk of genetic loss, thus requiring for the urgent development of *ex situ* conservation strategies. This is particularly true for New Zealand which has many terrestrial endemic orchids and Indonesia which has approximately one-fifth of the world's orchid species that are mostly epiphytic. The atypical seed storage behaviour reported in several orchid species, favours cryopreservation as a complementary conservation strategy to conventional seed banking. In this study, seeds of five orchid species; *Dendrobium strebloceras*, *D. lineale* (tropical epiphytic, Indonesia), *D. cunninghamii*, (temperate epiphytic, New Zealand), *Pterostylis banksii* (temperate terrestrial, New Zealand) and *Thelymitra nervosa* (temperate terrestrial, New Zealand) were cryopreserved following direct immersion in liquid nitrogen and plant vitrification solution 2 (PVS2). PVS2 was tested for 0, 20, 50, and 70 min exposure, at either room temperature or on ice. All the studied species germinated well following direct freezing into liquid nitrogen. No difference was noted in the seedling development between cryopreserved and non-cryopreserved seeds in both the tropical epiphytic species. Cryopreservation with direct immersion in liquid nitrogen also noted to enhanced seed germination and shoot formation in both temperate terrestrial *T. nervosa* and *P. banksii*. PVS2 vitrification cryopreservation is less efficient for all species studied except for *D. cunninghamii*. This study shows successful cryopreservation of all the five species tested using both direct freezing and PVS2 vitrification cryopreservation, with direct freezing proving to be more effective. Cryopreservation is therefore recommended as feasible option for long-term conservation of epiphytic or terrestrial orchids from tropical and temperate regions.

Keywords: orchid seed conservation, liquid nitrogen, PVS2 vitrification, seed banking.

5.2. Introduction

Orchidaceae (orchids) are one of the most highly diversified plant groups among flowering plant families (Roberts and Dixon 2008; Chase *et al*. 2015; Givnish *et al*. 2015). Unfortunately, the existence of orchids in nature is currently threatened. Orchids are endangered due to illegal trading, intensive land-use, and climate change (Cribb 2011; Popova *et al.* 2016). These threats limit *in situ* conservation efforts for orchids.

More than half of New Zealand orchids are endemic and the large majority are terrestrial (George 2012). Approximately over quarter of 134 New Zealand orchids listed as threatened or likely to be threatened/extinct, while the other quarter are restricted to specific geographical regions. Less than half of New Zealand's orchids are considered to be in safe populations, and the number of threatened species is increasing (de Lange *et al*. 2018).

According to Bradshaw *et al*. (2010), New Zealand is listed as the country that has the highest loss proportion of threatened species. This highlights the importance of their conservation. New Zealand orchid conservation is mostly focused on protecting *in situ* populations (Lechnebach 2013; New Zealand Native Orchid 2020) or as *ex situ* collections held largely at the Iwitahi Native Orchid Heritage Protection Area in Taupo (New Zealand Native Orchid 2020; Orchid Council 2020). Other *ex situ* collections including *in vitro* collections are almost non-existent (Frericks *et al*. 2018).

Tropical Indonesia possesses approximately one-fifth of the world's orchids. These are mostly epiphytic species that are threatened with extinction due to natural habitat loss including as a result of deforestation (Bradshaw *et al*. 2010; Irawati 2013; Givnish *et al*. 2015). Several studies have aimed at increasing the availability of Indonesian orchid species and rescuing them from extinction, using approaches such as *in vitro* seed propagation (Dwiyani *et al*. 2012; Handini *et al*. 2016; Kartikaningrum *et al*. 2017) and production of desiccated synthetic seeds (Muliawati *et al*. 2016). Conventional seed banking at -20°C after drying the seed has also been reported for several species and genera. However, viability loss

occurred within 3 – 48 months of storage (Puspitaningtyas and Dwiarum 2012; Puspitaningtyas and Handini 2016).

Orchid seed storage behaviour is atypical, with seeds of some species showing tolerance to desiccation and low moisture contents (in equilibrium with c. 5 – 23% RH), but being short-lived under conventional seed bank storage temperature at c. -20°C (Hay *et al*. 2010; Seaton *et al*. 2013; Merritt *et al*. 2014). Storing seeds at cryogenic temperatures using either the liquid or vapour phase of nitrogen has been recommended as a good alternative to conventional seed banking (Engelmann 2011).

Cryopreservation is acknowledged as a better solution for non-orthodox seed or vegetatively propagated plants (Pritchard 2004; Li and Pritchard 2009; Walters *et al*. 2004), as well as for species having low number of populations, and seeds that are inherently short-lived which also includes many orchid species (Wade *et al*. 2016).

An early seed cryopreservation study on both epiphytic and terrestrial orchids was reported by Pritchard (1984). This study utilized direct immersion of dry seed into liquid nitrogen. Since then, many cryopreservation methods have been applied to support orchid conservation with direct immersion into liquid nitrogen (LN) and vitrification being the most investigated methods (Merritt *et al*. 2014; Vendrame *et al*. 2014; Popova *et al*. 2016). Neither of these techniques result in genetic changes being observed after storage (Galdiano *et al*. 2014). Furthermore, low germination due to seed dormancy in orchids (Baskin and Baskin 1998) may be diminished through liquid nitrogen exposure (Acharya *et al*. 1999).

In this study, the effectiveness of two different cryopreservation procedures (direct LN immersion and PVS2 vitrification) on five orchid species; *Dendrobium strebloceras, D. lineale* (tropical epiphytic, Indonesia), *D. cunninghamii*, (temperate epiphytic, New Zealand), *Pterostylis banksii* (temperate terrestrial, New Zealand) and *Thelymitra nervosa* (temperate terrestrial, New Zealand) was compared with the aim of optimising storage of seed to enable the long term conservation of these species.

5.3. Materials and methods

5.3.1. Seed materials

Seeds of two tropical epiphytic orchids, *D. lineale* and *D. strebloceras*, were received from the glasshouse collection of the Indonesian Agency for Agricultural Research and Development (IAARD), Indonesia. Flowers of those two *Dendrobium* were hand-pollination and the seed pods were harvested at maturity, as indicated by their greenish-yellow colour. The seed pods were then couriered to New Zealand. Open-pollinated seeds of epiphytic temperate orchid *D. cunninghamii* were harvested in early autumn from the Pukeiti Forest, North Island, New Zealand. Dendrobium seeds were tested as they became available. Two species of terrestrial orchids from different genera, *Pterostylis banksii* and *Thelymitra nervosa*, were open-pollinated and mature seeds were collected from the Iwitahi Native Orchid Heritage Protection Area, North Island, New Zealand. Seeds were removed from their pods and temporarily stored (~1 – 4 weeks) in filter paper envelopes at 15 ˚C and 15% relative humidity at the Margot Forde Forage Germplasm Centre, AgResearch, Palmerston North, New Zealand prior to experimental use and tested simultaneously.

5.3.2. Cryopreservation

5.3.2.1. Seed cryopreservation by direct immersion into liquid nitrogen

Approximate 100 pre-packed seeds in filter paper envelopes of *Dendrobium strebloceras*, *D. lineale*, *D. cunninghamii*, *Pterostylis banksii*, and *Thelymitra nervosa* were transferred into cryovials and cryopreserved by rapid freezing in liquid nitrogen vapour (LN) at around -150 to -160 ºC. The seeds were stored in LN for 1 h and then thawed in a water bath at 40 ± 2 °C for 3 min. Following thawing, the seeds were surface sterilised as described below and tested for germination *in vitro* as described below. Non-cryopreserved seeds of each species were used as a control. The experiment was replicated three times.

5.3.2.2. Seed cryopreservation by PVS2 vitrification

Only four species (*Dendrobium strebloceras, D. lineale, D. cunninghamii* and *Pterostylis banksii*) were tested for this method of cryopreservation as *Thelymitra nervosa* did not produce enough seeds. Seeds wrapped in filter paper envelopes were placed into cryo-vials before being treated with vitrification solution. The vitrification procedures consisted of eight steps: (1) dehydrating seeds in 1 ml loading solution containing 2 M glycerol and 0.4 M sucrose for 20 min at 25 °C; (2) followed by dehydration with 1 ml cryoprotectant PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose, pH 5.8]. Four PVS2 treatments consist of vitrification procedures without PVS2 immersion (0 min); or soaking the seeds for 20, 50, 70 min in PVS2 solution at either room temperature or on ice; (3) PVS2 solution (after 20, 50, 70 min treatments) was then replaced with fresh PVS2 solution at the same temperature; (4) cryovial sealed with the lid and plunged into liquid nitrogen; (5) seeds were stored in liquid nitrogen for 1 h; (6) thereafter, seeds were removed from liquid nitrogen; (7) and were rapidly warmed by placing the cryovials in a water bath at 40 ± 2 °C for 3 min; (8) and the last step was rehydration in 1 ml liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1.2 M sucrose for 20 min at room temperature. Step (3) was skipped for 0 min dehydration in PVS2 (0 min treatment). Following thawing and rehydration, seeds were surface sterilised and tested for germination *in vitro* as described below. Seeds that were non-cryopreserved (-LN) but treated with vitrification procedures with 0, 20, 50, 70 min immersion in PVS2 at either room temperature or on ice were used as a control. Three replicates of 100 seeds were used for each treatment.

5.3.3. Seed sterilisation and germination

Following cryopreservation, seeds were surface disinfected by immersion in 10% (v/v) bleach solution (0.42 g/L NaClO) for 5 min, followed by rinsing in sterile distilled water twice and immersion in 1% (v/v) antibacterial-antimycotin solution

(Sigma Aldrich, New Zealand) for 1 min. Seeds were air-dried under a laminar flow hood for about 3 min before sowing on germination media.

Based on findings from a previous study on the optimum media for *in vitro* germination of orchids (Diantina *et al*. 2020a), all the epiphytic *Dendrobium* species were germinated on MS media (Murashige and Skoog 1962), whereas Norstog (Norstog 1973) media was used for germination of temperate terrestrial species, *P. banksii* and *T. nervosa*. Both media were supplemented with 3% (w/v) sucrose and pH adjusted to 5.8. Seeds were initially incubated in the dark at 25 ± 2°C for 24 hours, then all *Dendrobium* species were transferred to a germination chamber with a 12 h photoperiod (60 µmol/m^2/s) provided by cool fluorescent tubes with temperature kept at 25 ± 2 °C. A previous study (Diantina *et al*. 2020a) showed that terrestrial *P. banksii* and *T. nervosa* seeds require dark environment for the first 2 months of germination. Therefore these two species were kept in the dark at 25 ± 2 °C for two months before being transferred back to the same germination conditions as for the *Dendrobium* species to optimise their germination.

5.3.4. Seed germination assessment

Approximately 100 seeds were sown onto each plate containing the germination medium. Based on the previous observation and results of in vitro germination (Chapter four), three replicates were used for each treatment and seed germination was recorded after either two months (epiphytic) or five months (terrestrial orchids). Seed germination was observed under a light microscope (microscope: Olympus SX7; light: Olympus DF PLAPO 1X-4, Olympus Optical, Tokyo, Japan). Seeds were considered germinated when they produced complete seedlings or plantlets except for *D. cunninghamii* which germinated only up to the first stage of germination (indicated by the swollen green embryos).

5.3.5. Further seedling development assessment

For two of the terrestrial species *P. banksii* and *T. nervosa*, seedling development from initial germination right up to full plantlet formation i.e., embryo swollen stage, rhizoid production, and shoot formation was recorded for both PVS2 vitrification cryopreservation and direct LN immersion cryopreservation methods used.

5.3.6. Data analysis

Statistical analysis was performed using IBM SPSS statistic 24. Data were log-transformed to meet normality assumptions if needed (based on results of either Shapiro Wilk normality test or Levene's test) before statistical testing. T-tests were used to compare the effect of direct immersion into liquid nitrogen on seed germination in each species. A two way ANOVA was used to analyse the effect of storage treatments on species. Interactions betweenstorage treatments were explored. Taxonomic group, origin and niche were not included in the model because the main interest in this experiment was the treatment effect on species. Differences between treatments were established using a post-hoc Tukey Test ($\alpha = 0.05$).

5.4. Results

5.4.1. Cryopreservation using direct LN immersion

All five species studied survived direct immersion in LN with *D. streblocerας, D. lineale* and *D. cunninghamii* showing no significant difference in post-cryopreservation germination compared with non-cryopreserved seeds. Though the post-cryopreservation germination for *D. lineale* and *D. cunninghamii* was quite low ~ c. 20%, this was higher than the germination of non-cryopreserved seeds. Direct freezing into LN had a positive effect on the post-cryopreservation germination of both the terrestrial species, *P. banksii* and *T. nervosa* which showed a significant increase in germination c. 60% and 90% respectively (Figure 5.1).

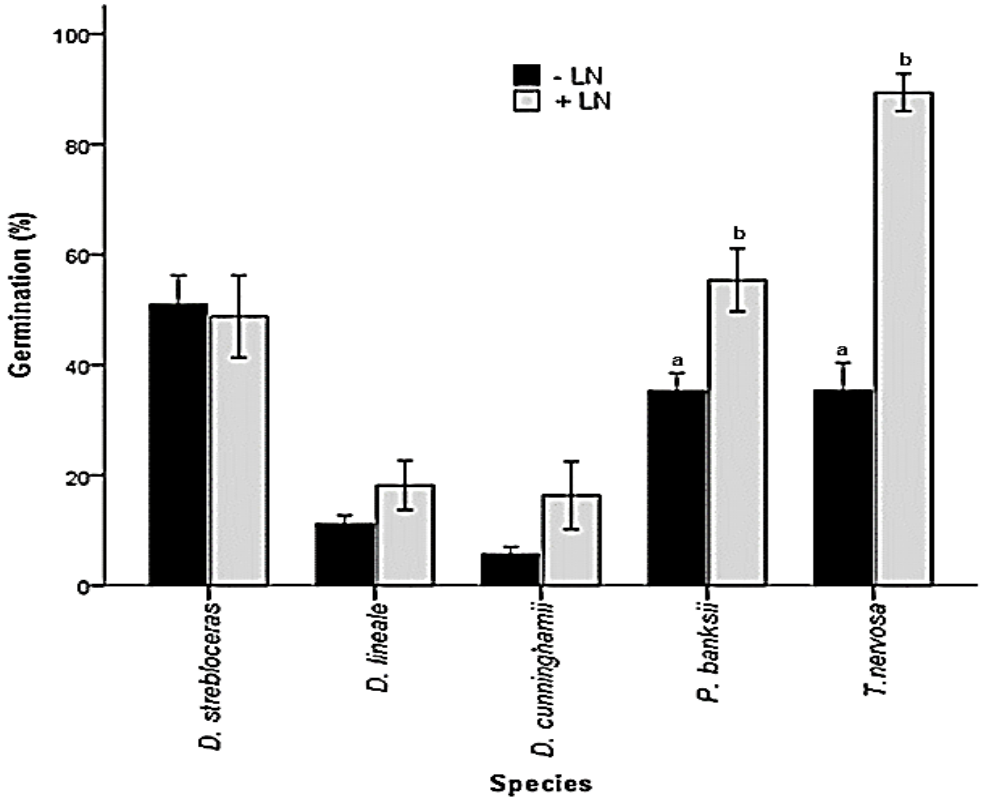


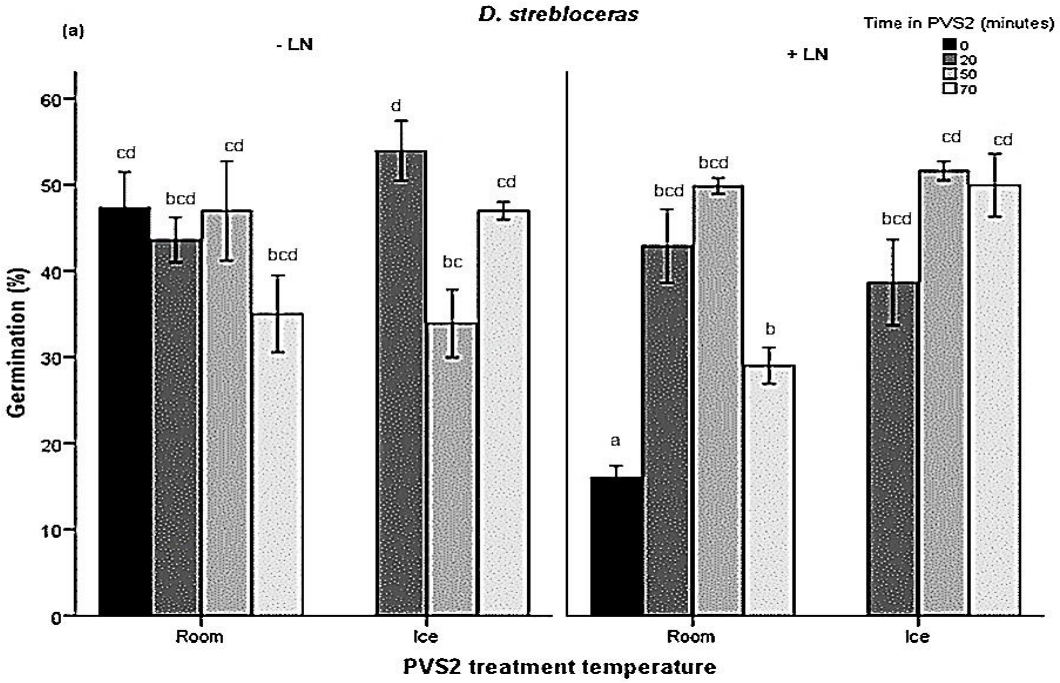
Figure 5.1. Seed germination of five orchid species (*Dendrobium strebloceras, Dendrobium lineale, Dendrobium cunninghamii, Pterostylis banksii, Thelymitra nervosa*) without (-LN) and with (+LN) direct immersion into liquid nitrogen. Bars represent mean ± SE, means labelled with different letters differed significantly at T test (-LN or +LN) compared for each species. (ns = not significant)

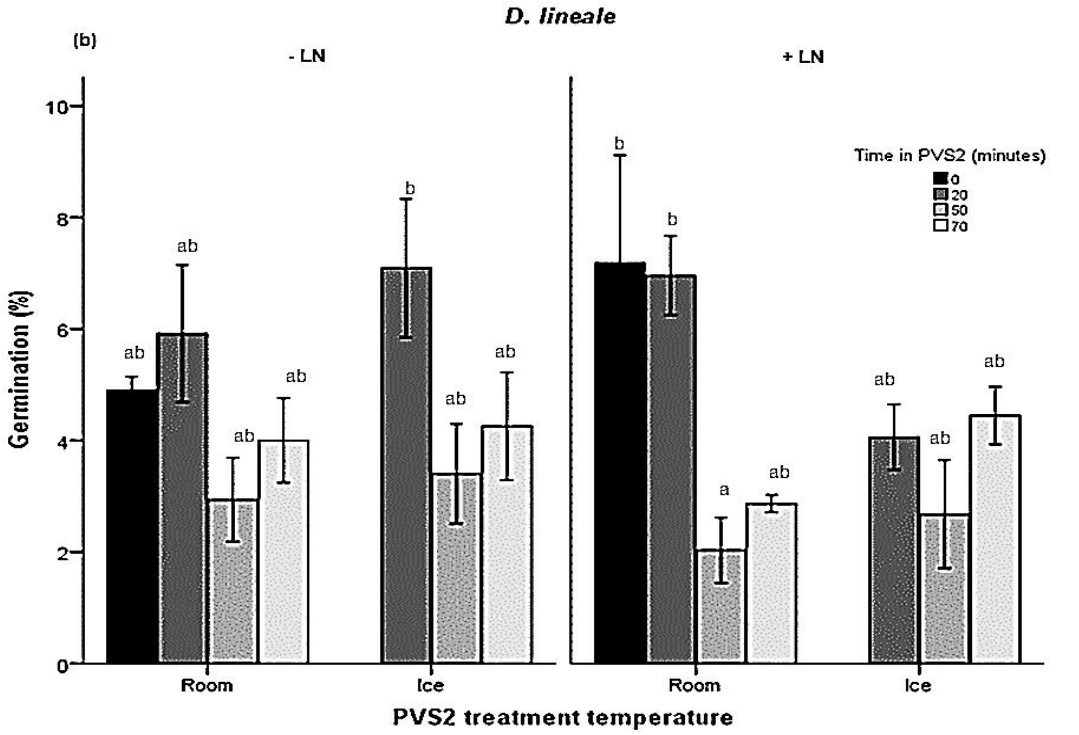
5.4.2. Cryopreservation using PVS2 treatment

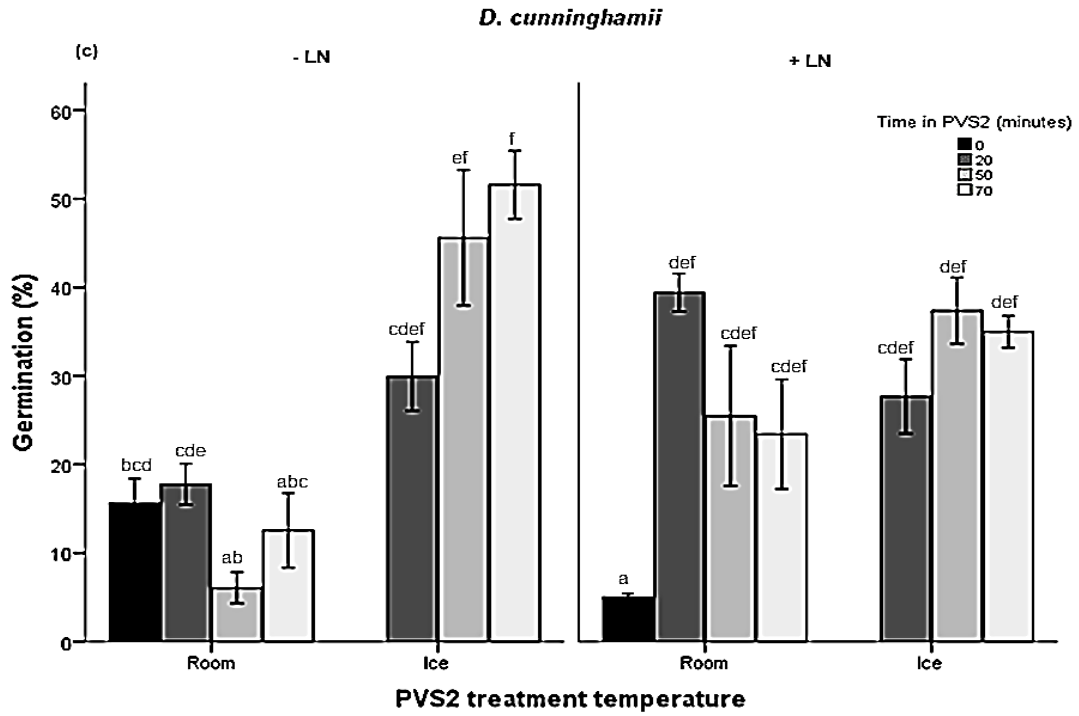
D. strebloceras seeds showed c. 50% post-cryopreservation germination following 50 min PVS2 at room temperature and almost similar germination following 50 and 70 min PVS2 treatment on ice (Figure 5.2a). Loading solution treatment alone is not sufficient for cryopreservation of this species. In contrast, *D. lineale* showed high post-cryopreservation germination with loading solution treatment alone (0 min PVS2) both at room temperature and on ice. However, the highest post-cryopreservation germination achieved was only around 7% compared to c. 20%

that achieved with direct LN freezing for this species (Figure 5.2b). Germination remained at c. 7% for 20 min PVS2 treatment at room temperature, however, PVS2 treatment on ice and increasing the treatment time any further at room temperature showed a detrimental effect.

The temperate epiphytic *D. cunninghamii* was the only species that showed high germination c. 40% following PVS2 vitrification cryopreservation compared to direct LN cryopreservation which was c. 20% (Figure 5.2c). While the temperate terrestrial species *P. banksii*, showed no differences among the liquid nitrogen treatment combinations (Figure 5.2d).




D. lineale
(b)
- LN
+ LN
Time in PVS2 (minutes)
0
20
50
70
Germination (%)
PVS2 treatment temperature
Room
Ice
Room
Ice


D. cunninghamii
(c)
- LN
+ LN
Time in PVS2 (minutes)
0
20
50
70
Germination (%)
PVS2 treatment temperature
Room
Ice
Room
Ice

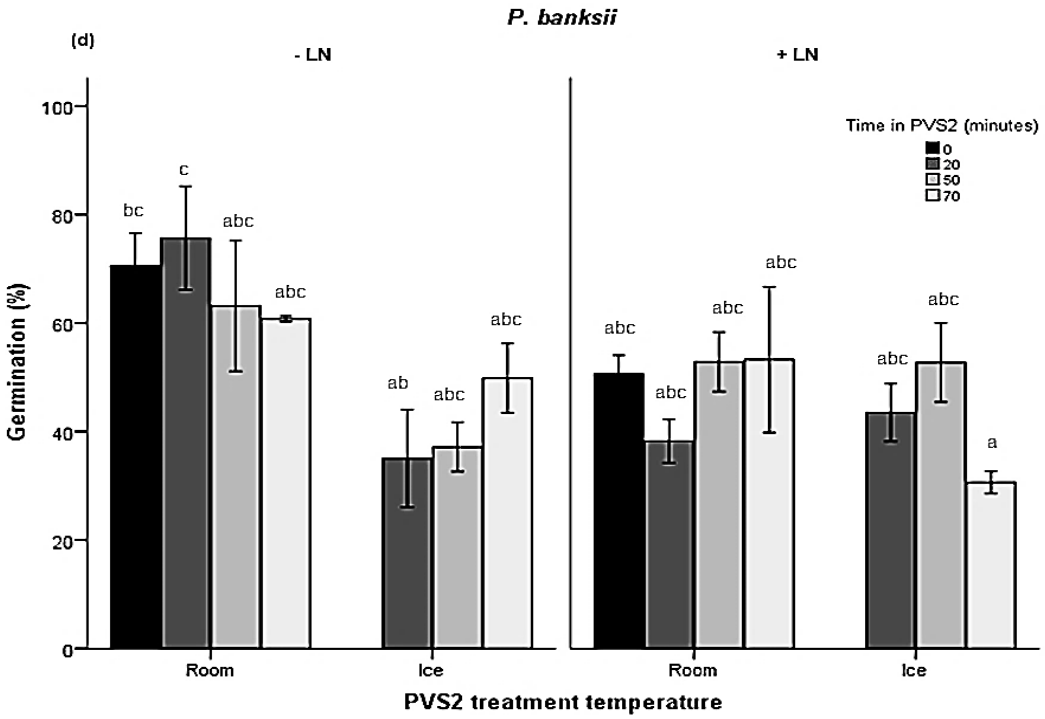


Figure 5.2. Seed germination following PVS2 vitrification treatment at room temperature and on ice without (-LN) and with (+LN) cryopreservation for (a) *Dendrobium strebloceras*, (b) *Dendrobium lineale*, (c) *Dendrobium cunninghamii*, (d) *Pterostylis banksii*. Bars represent mean ± SE, means labelled with different letters, differed significantly at the 5% level at Tukey Test for each species after GLM

5.4.3. Seedling development following cryopreservation

Both the tropical epiphytic *D. lineale* and *D. strebloceras* seeds were able to germinate and develop into complete plantlets after cryopreservation, either with direct LN immersion or following PVS2 treatment. Moreover, for both these species, no difference was found in explant growth and seedling development comparing non-cryopreserved and cryopreserved seeds (Figure 5.3a & b). On the other hand, germination for the temperate epiphytic *D. cunninghamii* seed was only recorded up to stage one of germination (showed by the enlargement or swollen

embryo) for all the treatments. None of the embryos were able to develop into protocorm (Figure 5.3c).

In *P. banksii*, green embryos developed faster in seeds cryopreserved using both direct LN immersion and PVS2 vitrification compared with control (Figure 5.3d). Only cryopreservation with direct immersion in LN was tested on *T. nervosa*. Seed germination increased significantly after cryopreservation, and embryos germinated more rapidly compared with non-cryopreserved seeds (Figure 5.3e).

Cryopreservation improved seedling development in terrestrial orchids compared with non-cryopreserved seeds. Further germination stages up to shoot development were observed on *P. banksii* following both direct LN freezing and PVS2 vitrification cryopreservation (Figure 5.4a & b). Direct immersion in LN enhanced shoot development to about 60% compared to only c. 50% for PVS2 treatment both at room temperature and ice for 20 min for this species (Figure 5.4a & b). Seedling development of the temperate terrestrial species, *T. nervosa*, was superior c. 100% following direct LN cryopreservation (Figure 5.4a).

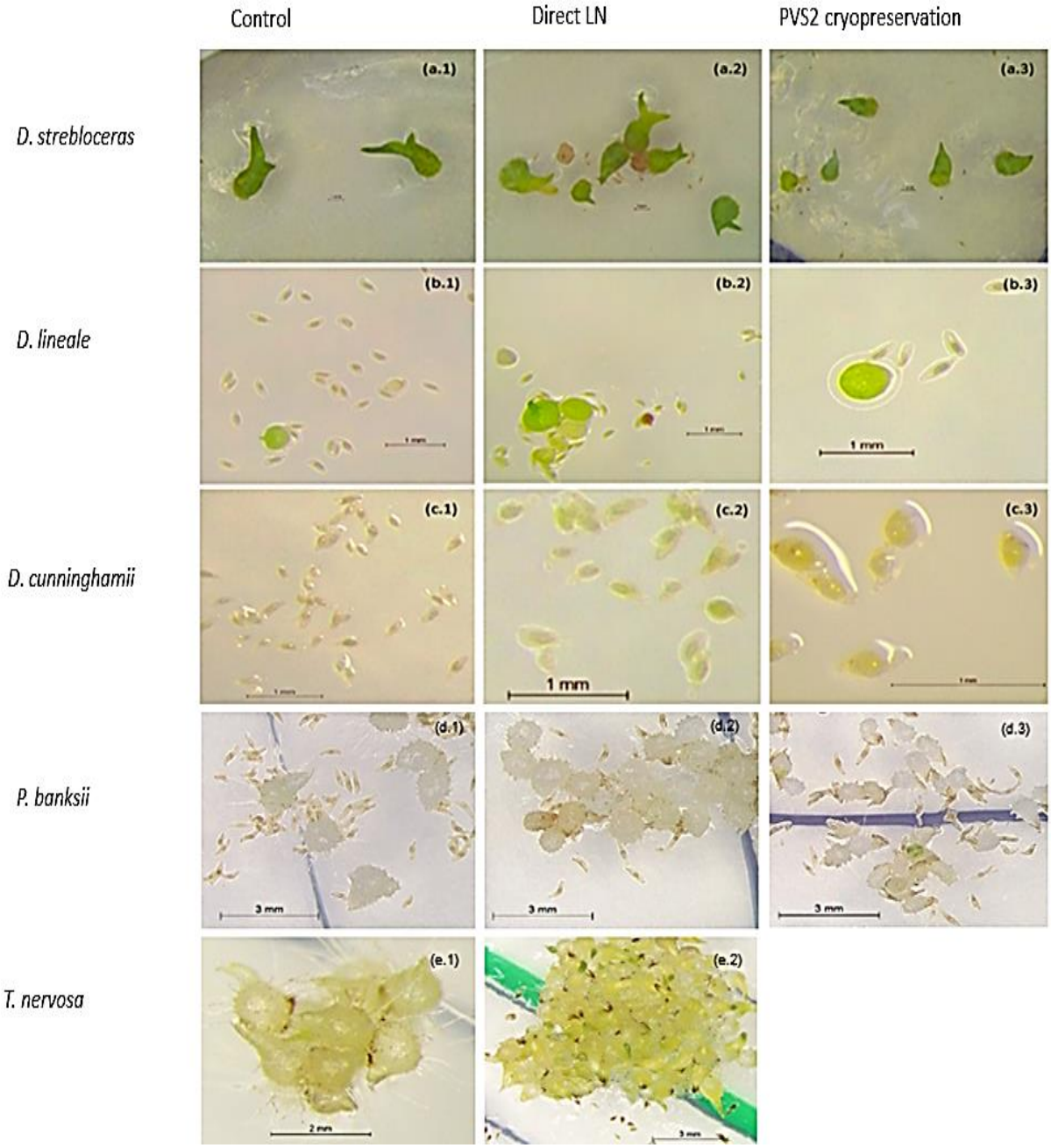


Figure 5.3. Seed germination of (a*) Dendrobium strebloceras*, (b) *Dendrobium lineale*, (c) *Dendrobium cunninghamii* at two months after treatment; and (d) *Pterostylis banksii*, (e) *Thelymitra nervosa* at four months following (1) control (without PVS2 or direct cryopreservation), (2) direct cryopreservation, (3) PVS2 vitrification cryopreservation

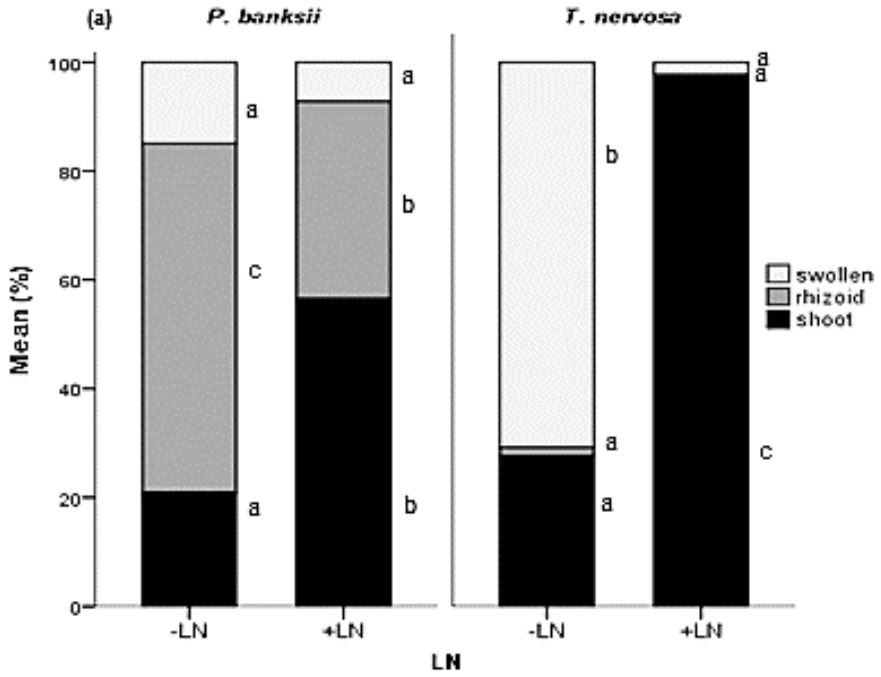


Figure 5.4.a. Seedling development following cryopreservation for direct immersion in liquid nitrogen for terrestrial orchid, *Pterosylis banksii* and *Thelymitra nervosa*. Bars represent mean (%), means labelled with different letters, differed significantly at the 5% level at Tukey Test for each development stage after GLM

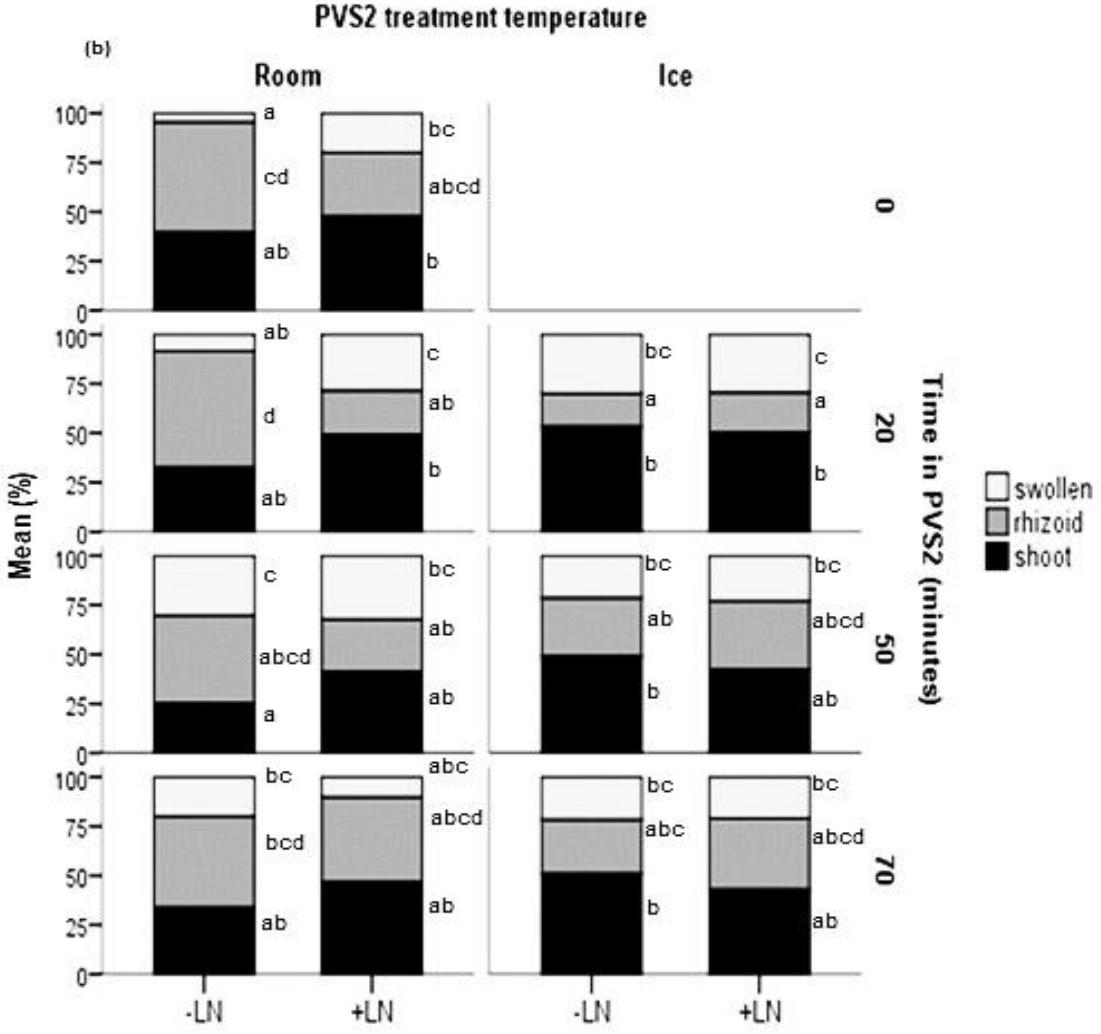


Figure 5.4.b Seedling development following PVS2 vitrification treatment for *Pterostylis banksii*. Bars represent mean (%), means labelled with different letters, differed significantly at the 5% level at Tukey Test for each development stage after GLM

5.5. Discussion

The threat to orchid germplasm *in situ* and the difficulty of storing most orchid species in a conventional seed bank environment, demands development of alternative methods for *ex situ* storage to enable conservation of Orchidaceae. Cryopreservation has been recommended as an alternative to standard seed banking for many orchid species (Engelmann 2011). Cryopreservation at ultra-low

temperatures below -150 °C is reported to suspend all metabolic activity prolonging seed longevity. Development of cryopreservation protocols can be species-specific as post-cryopreservation germination involves *in vitro* technology. It is therefore important to compare different cryopreservation strategies on temperate and tropical species with different growth habits i.e., epiphytic versus terrestrial.

The feasibility of cryopreserving dry orchid seeds in liquid nitrogen without the need for highly toxic cryoprotectants has been previously reported (Schofield *et al*. 2018). Drying seeds to their lowest safe moisture content (LSMC) is crucial for successful cryopreservation (Kim *et al*. 2008; Wen *et al*. 2010). Hughes and Kane (2018) reported no significant difference in seed germination of non-cryopreserved terrestrial *Bletia purpurea* and epiphytic *Epidendrum nocturnum* when cryopreserved seeds were equilibrated on saturated salt solution to 33% and 55% RH respectively, prior to cryopreservation. Lowering seed moisture content (up to 5 – 8% H_2O) under silica gel for 24 h before direct cryopreservation in some Western Australian terrestrial orchid species was also promoted seed germination compared to germination of freshly harvested seeds (Batty *et al*. 2001). In this study, seeds were equilibrated in the drying room to around 15 – 17% equilibrium relative humidity (eRH) before cryopreservation. All the studied species germinated well following direct freezing into liquid nitrogen and no difference was noted in the seedling development between cryopreserved and non-cryopreserved seeds in both tropical epiphytic species, demonstrating the ability of seeds of these species to tolerate sub -150°C temperatures.

The results showed that direct immersion in liquid nitrogen has no significant effect on post-cryopreservation germination tropical and temperate epiphytic orchids. However, this method of cryopreservation enhanced seed germination and shoot formation in both the temperate terrestrial orchids *T. nervosa* and *P. banksii*. Physical dormancy is common in some terrestrial orchid seeds (Rasmussen *et al*. 1995) and exposure of liquid nitrogen following direct cryopreservation increased seed germination of some species and mostly related to seed physical dormancy breaking. For example, Acosta *et al*. (2019) showed in their morphological investigation of the forage species *Teramnus labialis* with scanning electron

microscopy, cracking of the seed coat and breaking physical dormancy due to exposure to liquid nitrogen. Exposure to liquid nitrogen was also reported to break physical dormancy caused by the impermeable or hard alfalfa seed coat (Acharya *et al*. 1999). Meanwhile, other reports suggested that lipid characteristics, specifically lipid biothermal status, determined successful seed germination following cryopreservation (Pritchard 1984, Nadarajan and Pritchard 2014). There is evidence suggesting that some species in this study contain lignin (Diantina *et al*. 2020b), which the exsistance of lignin has been linked to physical dormancy in other species (Pierce *et al* 2019). However, its direct relation to dormancy for the studied orchids remains unknown. Thus, further research in seed lipid biophysics and morphological investigation with scanning electron microscopy is suggested to identify the factors leading to improved germination after cryopreservation in these species

Due to the simplicity of direct seed cryopreservation techniques, successful seed cryopreservation with direct freezing has been reported in various orchid species (Popova *et al*. 2003; da Silva *et al*. 2014; Merritt *et al*. 2014). Wu *et al*. (2016) reported that dehydrated seeds of more than 30 out of the 54 orchid species tested were successfully cryopreserved with direct immersion in liquid nitrogen, including *Pterostylis sp.* and *Thelymitra sp.*, which supports the findings in this study. Vendrame *et al*. (2007) and Galdiano *et al*. (2014) found detrimental effects with direct immersion into LN on freshly harvested mature seeds of *Dendrobium*, compared with vitrification cryopreservation. Also, *D. strebloceras* and *D. cunnighamii* immersed in LN without PVS2 solution (0 min), had lower germination rates than those exposed to PVS2 for 20, 50, or 70 min.

Seed moisture content is important in successful cryopreservation (Batty *et al* 2001). The tendency of fresh seeds to have high moisture content means they are prone to crystallization and cellular damage during cryopreservation (Wu *et al*. 2016). Altering residual cellular water to the vitrification state is crucial to avoid detrimental effects during freezing (Fujikawa and Jitsuyama 2000). The PVS2 cryoprotectant solution is known to remove cellular water content by permeating

into the cells and promoting glass transition, therefore avoiding ice crystallization during the freeze-thaw processes (Nadarajan and Pritchard 2014).

Cryopreservation with vitrification-based procedures has been recommended for recalcitrant seeds or plant tissues that are extremely sensitive to direct desiccation for example cell suspension, somatic embryo, zygotic embryos or embryonic axes of recalcitrant seeds, and apical meristem or shoot tips (Engelmann 2000). Successful orchid seed germination following cryopreservation using PVS2 vitrification has also been widely reported (Vendrame *et al*. 2007; da Silva 2013; Merritt *et al*. 2014; Vendrame *et al*. 2014; Popova *et al*. 2016). Vitrification techniques can also be used for cryopreservation of protocorms and protocorm like bodies (PLB) in orchid species (da Silva *et al*. 2014; Popova *et al*. 2016).

The application of PVS2 cryoprotectant solution needs to be carried out with caution due to its high toxicity to plant tissues (Fahy *et al*. 2004). Treatment time and treatment temperature of PVS2 solutions are crucial factors during acquisition of dehydration through vitrification while avoiding toxic effects (Nadarajan and Pritchard 2014). According to the results here, dehydration in PVS2 cryoprotectant was required for seed cryopreservation of *D. strebloceras* and *D. cunninghamii*. While *D. lineale* showed low seed germination percentages on all treatments (<10%), obscuring the interpretation of treatments effect on this species. Vendrame *et al*. (2007) found no difference in seed germination of the tropical hybrid orchid, *Dendrobium swartz*., following one to three hours in PVS2 on ice after cryopreservation for the 'Sena Red', 'Mini WRL' and 'BFC pink' hybrids; while the 'Jaquelyn Thomas' hybrid required shorter exposure (1 h) in PVS2. Moreover, Vendrame *et al*. (2007) also found vitrification solution treatment on ice was better than treatment at room temperature for those hybrids. Longer PVS2 dehydration on ice for 60 to 90 min was required for better post-cryopreservation germination in the tropical terrestrial *Phaius tankervilleae* orchid (Hirano *et al*. 2009) and longer exposure time in PVS2 for up to 3 hours on ice was also needed for seed cryopreservation of the immature epiphytic orchid *Vanda tricolor* (Jitsopakul *et al*. 2012).

Overall, high interspecific variation in seed germination and seedling development responses to vitrification treatment (exposure time in PVS2 and treatment temperature) following cryopreservation were observed. *D. cunninghamii* was the only species that showed improved germination following PVS2 cryopreservation. Therefore, different protocols may be necessary to optimise cryopreservation of different species. A different treatment time requirement even within the two tropical epiphytic was noted with 20 and 50 min as optimum for *D. strebloceras* and 20 min for *D. lineale* both at room temperature. *P. banksii* required slightly longer PVS2 exposure i.e. 70 min at room temperature for optimum post-cryopreservation germination, however, this treatment time on ice was found to be detrimental for this species. Thammasiri (2000) found that PVS2 exposure for 50 min at room temperature was optimum for the terrestrial *Doritis pulcherrima* seeds. While the best seed germination of the temperate terrestrial orchid *Bletilla formosana* was found for 30 min PVS2 treatment on ice (Hu *et al.* 2013). This again, supports the need to develop species-specific cryopreservation protocols.

Cryopreservation is regarded as the alternative long-term conservation strategy with the advantages of minimal space requirement, maintenance of genetic stability of the collection over a long period and as a satisfactory solution to overcome problems associated with conventional seed banking of orchid species. Optimization of cryopreservation procedures is still needed for the studied epiphytic *Dendrobium* species. Nevertheless, there was no significant difference between control and direct cryopreservation of those *Dendrobium* species, indicating no detrimental effect of direct freezing on seed germination and seedling performance. Direct freezing, a much simpler procedure, also proved to be effective for improving seed germination particularly for dry seed of temperate terrestrial *P. banksii* and *T. nervosa*, therefore it is recommended as feasible strategy to support conservation of these species.

5.6. Conclusions

In summary, this finding results suggest that cryopreservation with direct seed immersion in LN is applicable for desiccated orchid seeds. Direct freezing offers a simple and high throughput protocol with a minimum skill or training required making it appealing for large-scale genebank operations. Considering the effectiveness and ease of a direct seed cryopreservation protocol, this method of preservation is recommended for seed conservation of both tropical epiphytic and temperate terrestrial species of Indonesia and New Zealand orchid.

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CHAPTER 6

General Discussion, Findings and Conclusions

6.1. General discussion and findings

6.1.1. Seed morphological characters: qualitative and quantitative performances

In this study, seed morphology in terms of seed qualitative (seed and pod colour, shape) and seed quantitative (seed and embryo length, width, volume, as well as airspace volume) attributes for orchid seeds of six species of tropical epiphytic, temperate epiphytic and temperate terrestrial origin was first characterized (Chapter 2).

Epiphytic *D. strebloceras* in this study had very large seed and air space compared to other species. This exception is contrary to the results of Tsutsumi *et al.* (2007) that shorter seed length was associated with epiphytic habitats. Exception to the general observation of reduced seed size in epiphytic species have also been observed in other species within the *Spatulata* section (Lavarack *et al.* 2000). Consequently, a further study on a wider range of species, especially within *Dendrobium* genus vs *Spatulata* section, to validate any association between seed morphometry and taxonomy in this genus is needed.

All *Dendrobium* species in this study displayed yellowish seed and green-yellowish pods, supporting that it might be a useful taxonomic marker in this genus (Wang and Xiao 2010). While Barthlott *et al.* (2014) did not suggest for other genera. This study on terrestrial *G. cunninghamii*, *P. banksii* and *T. nervosa* showed brown seeds with variable intensity. Some reports suggest that brown seed or testa colour is associated with the presence of phenolic compounds and seed germinability (Thomson *et al.* 2001), and lignification that increase embryo survival during dispersal (Yang and Lee 2014). Therefore, further research in orchid seed morphological properties in a wider range of species and genera associated with seed germination, lignification and survival adaptability in nature are needed to confirm these observations. Another important trait observed was seed air space volume. *D. strebloceras* had a very large air volume whereas the tropical epiphytic *D. lineale* and temperate terrestrial *T. nervosa* had the lowest seed air volume. These results contradict those of Tsutsumi *et al.* (2007) that epiphytic species have a smaller air space than terrestrial. Moreover, Chaudhary *et al.* (2014) related seed

air volume to biogeographical region and suggested that tropical species had the smallest air space, followed by sub-tropical and temperate species. The results from *D. strebloceras* (tropical epiphytic, *Spatulata* section) and *T. nervosa* (temperate terrestrial) in this study need to be validated with wider observations within the same genera (*Dendrobium, Thelymitra*) or section (*Spatulata*).

Air surrounding the embryo within the seed provides an advantage for seed dispersion (Arditti and Ghani 2000; Tsutsumi *et al.* 2007; Leck *et al.* 2008). Meanwhile, the proximity of air to the embryo in orchid seed also raises a number of issues including: (1) is there a harmful association between oxygen surrounding the embryo, seed fatty acids and ageing? Previous studies show that oxygen accelerated seed ageing in conventional dry seed banks (Groot *et al.* 2012) while this study found no correlation between air space and seed viability after ageing (Chapter 3); on the other hand, (2) there may be benefits from the airspace under anoxic environments (Groot *et al.* 2014). The airspace may also improve seed tolerance to freezing in liquid nitrogen (Mweetwa *et al.* 2007; Seaton *et al.* 2013), supporting the development of cryopreservation methods to prolong storage longevity for orchid species.

To summarise, results from seed qualitative and quantitative measurement showed that orchid seed morphology varied between species. However, no association between bio-geographical origin and ecological niche with seed micro-morphology traits was identified. Seed micro-morphometric characters possibly reflect the ecological adaptation and modes of dispersal among the species included in the current study, and examination of a much greater number of species within genera is required to test this possibility.

6.1.2. Biochemical characters: seed fatty acids in fresh seed and changes following subsequent natural ageing

Environmental factors, e.g. high temperature, humidity and oxygen pressure, are recognized as detrimental factors in seed storage, thus common seed storage protocols use low humidity and temperature to extend seed longevity (Rao *et al.*

2006). Apart from air-trapped around the embryo (Chapter 2), orchid seeds are also known to contain lipids (Pritchard and Seaton 1993; Colville *et al.* 2016). In this study, differences in lipid composition and content (in term of fatty acids) between the six studied species, and their changes after natural ageing storage was investigated (Chapter 3).

As an oily seed, some orchid seeds are known to have low storability in conventional seed banking (-20 ºC, 15% RH) compared with other crop species (Seaton *et al.* 2013). The time for stored seed to lose viability (zero germination) varies between species. This can be from 3 to 48 months within *Coelogyne* genus (Puspitaningtyas and Handini 2014). While other species from different genera, such as *Dendrobium stratiotes* (Puspitaningtyas and Handini 2011) and *Papiophedilum supardii* (Handini and Puspitaningtyas 2016) may survive for up to 12 months in seed banks. Embryo survival in nature is associated with seed lignification (Yang and Lee 2014). In this study, lignoceric acid, a by product of lignin, was found in most species but not detected in the brown seeded species, *G. cunninghamii* and *T. nervosa*. This limitation may be due to its presence at levels below the detection limits of the method used. Sample mass of 10 mg seeds were applied in this study. Previous report from Colville *et al.* (2016) suggested this minimum sample size is adequate to provide reliable fatty acid composition data. However, there was limitation on small sample size, that minor fatty acid with oil content below the limit of detection size of the analytical method may not be detected. Therefore, increasing sample quantities is strongly advised for further research investigating fatty acid compounds and compositions in orchid seeds.

This study revealed that the polyunsaturated fatty acid, linoleic acid, (18:2) was the most abundant fatty acid in all species investigated. The second most abundant fatty acid varied between species and was either monounsaturated oleic acid, (18:1) or saturated palmitic acid, (16:0). Sanyal and Decocq (2016) indicated that biogeographical region determines seed oils since tropical families had more seed fatty acids than temperate. However, results from this study showed little difference among tropical and temperate epiphytic *Dendrobium* species while three terrestrial species had lower fatty acid content than epiphytic. Despite their biogeographical differences, habitat was suggested as an influence on orchid seed fatty acids status.

However, limited testing was a constrait in this study, consequently further research is required to validate this result.

Lipid degradation can result from oxygen exposure (Ponquett *et al.* 1992) in combination with other environmental stressors such as heat stress (Slimen *et al.* 2014). Pritchard (1984) and Hulbert (2005) suggested that variation in lipid composition within species is a significant factor determining seed ageing and lifespan due to fatty acid degradation. Variation within species has been suggested as a cause of differences in equilibrium moisture contents and isotherms at specific humidity levels (Pritchard *et al.* 1999) and may consequently, result in different seed lifespans within species (Vertucci 1993; Walters *et al.* 2005).

Seed ageing or deterioration associated with the oxidation of storage reserves or membrane lipids has been extensively investigated (Hartman and Mattick 1976; Ohlrogge and Kernan 1982; Leprince *et al.* 1990; Sun and Leopold 1995; Sung and Chiu 1995; Khan *et al.* 1996; Al-Maskri *et al.* 2003; Burthon and Jauniaux 2011; Kumar *et al.* 2015; Kurek *et al.* 2019), exploring both enzymatic and non-enzymatic mechanisms (Murthy *et al.* 2003; Burthon and Jauniaux 2011; Vigor *et al.* 2014; Oenel *et al.* 2017). Auto-oxidation of membrane lipids (a non-enzymatic mechanism) has been identified as the primary cause of damage due to the action of reactive oxygen species (ROS) (Shaban 2013; Oenel *et al.* 2017). The occurrence of lipid peroxidation (Groot *et al.* 2012; Morscher *et al.* 2015) was confirmed during natural ageing.

Other studies investigating biochemical changes in ageing seeds also report an increase in free fatty acids (Balešević-Tubić *et al.* 2005), accumulation of malondialdehyde (Kibinza *et al.* 2006; Vigor *et al.* 2014) and thermal degradation of lipid hydroperoxides resulting in the production of volatile aldehydes (Colville *et al.* 2012). Also, an overall increase in volatile compounds were derived from alcoholic fermentation, lipid peroxidation, and Maillard reactions (Akimoto *et al.* 2004; Colville *et al.* 2012; Mira *et al.* 2016). All these factors were suggested as predictors of seed viability loss.

This study utilised a natural ageing treatment at close to ambient 60% RH at 20 °C to investigate any changes or differences between fatty acids in fresh and aging

seeds. Reduction in polyunsaturated linoleic acid and monounsaturated oleic acid during aging was found to be correlated with seed viability loss during ageing. However, correlation between seed viability loss and seed air volume was not found. Calculation of the S:U ratio revealed a significant increase for *D. strebloceras,* indicating a significant decrease in unsaturated fatty acid content after storage. This may relate to lipid degradation, which is associated with seed deterioration and ultimately, seed viability loss, during storage. A weak correlation between seed air volume and viability loss in our results suggests that other factors influenced ageing. However, neither enzymatic nor non-enzymatic changes were assessed in this study.

Considering variation in fatty acid compounds and seed viability loss in the species studied (except for high viability loss on terrestrial *P. banksii*), suggests that lipid biothermal properties (biophysical status of lipid contents) and storage conditions (humidity and temperature), rather than air volume that determines seed lifespan in storage. Therefore, further study, particularly in seed biophysical properties, seed storability in various environmental conditions (humidity, temperature, and oxygen pressure), and lipid peroxidation metabolisms are needed to reveal the key contributors to orchid seed viability loss during storage.

This study suggests that a reduction in the proportion of polyunsaturated fatty acids over time can also be used as an ageing predictor, as a decrease in unsaturated fatty acid content had been proven as evidence of free-radical driven lipid peroxidation (Pichardo-Gonzales *et al.* 2014; Vigor *et al.* 2014). Further research on the correlation between losses of seed unsaturated fatty acid and production of specific bioactive markers (for example malondialdehyde (MDA) and 4-hydroxynonenal (HNE)) is also suggested to further investigate seed ageing under storage.

6.1.3. Asymbiotic seed germination and seedling development

One of the challenging aspects of orchid seed conservation is to achieve good seed germination. Asymbiotic orchid seed germination requires appropriate nutrients that may be species-specific (Nadarajan *et al.* 2011), resembling those produced by

symbiotic relationships and macro-culture conditions found in the natural environment (Pritchard 2017).

In Chapter 4, an asymbiotic *in vitro* germination test to compare seed germination and seedling development on various media was developed. Embryo volume (larger embryo) is correlated with ease of germination (Tsutsumi *et al.* 2007; Prasongsom *et al.* 2017; Colville and Pritchard 2019). *D. strebloceras*, *P. banksii* and *T. nervosa* have a larger embryo ($P > 0.001$) and consequently are expected to germinate faster than *D. lineale*, *D. cunninghamii*, and *G. cunninghamii*. However, this study found that both tropical epiphytic, *D. strebloceras* and *D. lineale* seeds, germinated faster and had wider media tolerance than temperate terrestrial species, while temperate terrestrial species required more specific media germination and a longer incubation period was required for germination and seedling development.

Temperate epiphytic *D. cunninghamii* and temperate terrestrial *G. cunninghamii* both experienced seed dormancy. Being saprophytic, the lack ofchlorophyll in *Gastrodia* means that seeds germinate to produce tuberous protocorms and spend most of their life cycle in the tuberous form (Yuan *et al.* 2018). This contributes to the difficulty in shoot production through *in vitro* seed germination. In addition, seeds of these two species might have morphophysiological dormancy thus were unable to develop into protocorms and seedlings. Morphophysiological dormancy is a common phenomenon in temperate species (Rasmussen 1995; Baskin and Baskin 1998; Poff *et al.* 2016; Voronkova *et al.* 2018).

Some temperate plants, especially from alpine or subalpine species, require temperature stratification (Fernandez-Pascual *et al.* 2017) to interrupt physiological dormancy (Baskin and Baskin 1998). Further correlation between biogeographical background and lipid composition was suggested by Linder (2000), who found that *Helianthus* species from temperate regions with higher latitude (or altitude) have a higher proportion of unsaturated fatty acids (smaller S:U ratio) than lower latitude (or altitude) plants with higher S:U ratio, and thus germinated rapidly at low temperatures.

The epiphytic *D. cunninghamii* seed had the lowest S:U ratio (0.13) among the species assessed, suggesting it should be capable of germinating at lower

temperatures. *Platanthera chapmanii*, a temperate terrestrial orchid, required cold stratification (8 months at 5 °C) to achieve good germination and seedling development (Poff *et al.* 2016). Other research has also indicated the benefits of cold stratification for temperate species (Rasmussen 1992; Miyoshi and Mii 1998; Nikishina *et al.* 2001; Nikishina *et al.* 2007). This suggests that future research in the effects of cold stratification on seed germination and seedling establishment in *D. cunnninghamii* is warranted.

Differences in seedling development between epiphytic and terrestrial species were observed in this study. Djordjevic & Tsiftsis (2020) suggest that the reaction to environmental factors depends largely on the below ground strategies of orchids. Differences in initial seed development were observed between epiphytic and terrestrial species. Epiphytic seed produced protocorms with initial root and shoots produced at the same time, while terrestrial species produced more adventitious roots. These differences may be a consequence of morphological anatomy; *Dendrobiums* species are rhizomatous while the other three terrestrial species are tuberous.

6.1.4. Seed cryopreservation protocols and orchid seed conservation

Air surrounding orchid seed embryos may benefit cryopreservation of orchid seed, assisting *ex situ* orchid conservation. Vitrification with PVS2 solution or direct immersion in liquid nitrogen was tested in this study (Chapter 5). Variation in optimum exposure time to PVS2 was observed and terrestrial species need longer exposure in PVS2 than epiphytic confirming the results of Hirano *et al.* (2011). This suggests possible species differences in seed permeability properties (Popova *et al.* 2016).

Morphological and biochemical factors may explain the improvement in seed germination after cryogenic treatment in liquid nitrogen. Orchid seeds have a hard or thick seed coat, associated with the accumulation of lignin (Yang and Lee 2014). Some authors (Pence 1991; Tikhonova *et al.* 1997) report damage to the seed coat during the freezing-thawing process, increasing seed permeability (Kauth *et al.*

2008), allowing more rapid seed germination due to increased accessibility to nutrient by the embryo (Nikishina *et al.* 2001). However, other observations reported no damaging effects on orchid seed coats after cryopreservation (Thompson *et al.* 2001; Hughes and Kane 2018). Direct immersion into liquid nitrogen is strongly recommended as the most effective and efficient method for cryopreservation storage of dry orchid seed for the species used in this study. Further research involving more orchid species from various genera is needed to test the broad effectiveness of direct cryopreservation of orchid seed.

Optimal storage temperatures for oily seeds (including orchid seeds) may be species specific, in relation to fatty acid composition and thermal behaviour (Pritchard 2004). Other factors such as seed water content, maturity status, initial viability or germination status may also determine the success of seed cryopreservation. More detailed studies in seed storability and seed-biophysics (lipids, biothermal) are needed for successful conservation of specific orchid species.

6.2. Conclusions

This study compared the seed characteristics of six orchid species originating from different habitats and biogeographical regions. It also compared the asymbiotic *in vitro* germination and seedling development, and the optimal cryopreservation protocols in these species.

Seed morphological traits were related to their ecological adaptation reflecting their modes of dispersal (Chapter 2). There were similarities in fatty acid composition in the seeds of epiphytic species within the same genera (*Dendrobium*); terrestrial orchid seeds displayed less variation in fatty acid composition compared with epiphytic (Chapter 3). Fatty acid profile changed over time, in particular linolenic and oleic acid content declined with seed viability, suggesting an association with loss of seed viability. However, a relationship between seed morphological and biochemical characters was not established. Further research in seed storability and seed-biophysics (lipid biothermal) is strongly recommended to achieve successful conservation through orchid seed banking.

Epiphytic orchid species required less complex nutrient media for germination compared to terrestrial species; tropical species were more easily germinated than temperate species (Chapter 4). In relation to our results on lipid status (Chapter 3), future research into the correlation between morpho-physiological dormancy (MPD), ratio of saturated and unsaturated fatty acid, and germination temperature to break MPD, especially in temperate species, is needed to support successful asymbiotic orchid seed germination.

Application of a cryopreservation protocol utilising direct immersion of dry orchid seeds into liquid nitrogen (Chapter 5) is suggested. Further studies involving other orchid species are strongly recommended to support *ex situ* conservation of orchid in seed banking.

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APPENDICES

Appendix 1. Published papers

plants

MDPI

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Comparative Seed Morphology of Tropical and Temperate Orchid Species with Different Growth Habits

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Associated Data

▸ Supplementary Materials

Abstract

Go to:

Seed morphology underpins many critical biological and ecological processes, such as seed dormancy and germination, dispersal, and persistence. It is also a valuable taxonomic trait that can provide information about plant evolution and adaptations to different ecological niches. This study characterised and compared various seed morphological traits, i.e., seed and pod shape, seed colour and size, embryo size, and air volume for six orchid species; and explored whether taxonomy, biogeographical origin, or growth habit are important determinants of seed morphology. We investigated this on two tropical epiphytic orchid species from Indonesia (*Dendrobium strebloceras* and *D. lineale*), and four temperate species from New Zealand, terrestrial *Gastrodia cunnninghamii*, *Pterostylis banksii* and *Thelymitra nervosa*, and epiphytic *D. cunninghamii*. Our results show some similarities among related species in their pod shape and colour, and seed colouration. All the species studied have scobiform or fusiform seeds and prolate-spheroid embryos. Specifically, *D. strebloceras*, *G. cunninghamii*, and *P. banksii* have an elongated seed shape, while *T. nervosa* has truncated seeds. Interestingly, we observed high variability in the micro-morphological seed characteristics of these orchid species, unrelated to their taxonomy, biogeographical origin, or growth habit, suggesting different ecological adaptations possibly reflecting their modes of dispersal.

Keywords: air-space, epiphytic, terrestrial, tropical, temperate, micro-morphometric

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Comparative in vitro seed germination and seedling development in tropical and temperate epiphytic and temperate terrestrial orchids

Surya Diantina, Suskandari Kartikaningrum, Andrea Clavijo McCormick, James Millner, Craig McGill, Hugh W. Pritchard & Jayanthi Nadarajan

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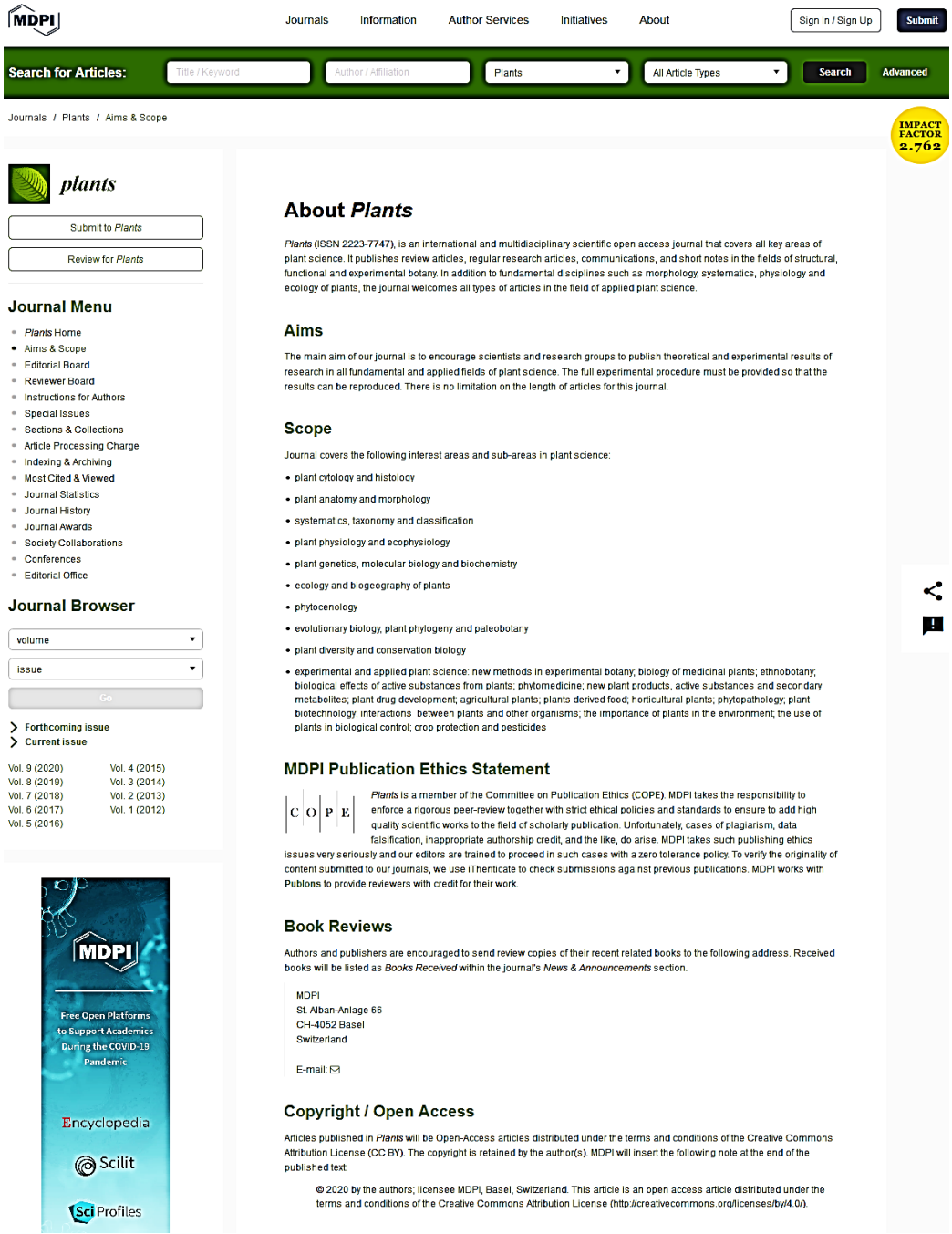
Abstract

Ex situ conservation of orchid seeds requires the application of modern biotechnology to determine seed quality and to optimise in vitro regeneration. The nutrient requirements for germination and seedling growth in vitro can be species-specific and influenced by life history traits and habitats. Therefore, this study aimed to explore in vitro seed germination and subsequent seedling development of selected tropical and temperate epiphytic and terrestrial orchids on different growth media. Seeds of three epiphytic orchids [*Dendrobium strebloceras* (tropical), *D. lineale* (tropical) and *D. cunninghamii* (temperate)] were sown on four different media supplemented with 2% sucrose: (1) Murashige and Skoog (MS); (2) Vacin and Went (VW); (3) Norstog; and (4) water agar. In addition, *D. strebloceras* seed were sown on the media above supplemented with 3% sucrose and half-strength MS supplemented with either 2% or 3% sucrose. Seed germination of three temperate terrestrial orchids (*Gastrodia cunninghamii, Pterostylis banksii* and *Thelymitra nervosa*) was assessed on media (1–4) but with VW replaced by terrestrial orchid medium-BM1. Whilst all epiphytic orchids germinated on all media tested, the best seedling development for the two tropical species was on full or half-strength MS media. The temperate epiphyte *D. cunninghamii* germinated best on Norstog medium but did not develop further. Norstog and BM1 media supported germination and seedling development better in *P. banksii* and *T. nervosa*. This study concludes that temperate terrestrial *P. banksii* and *T. nervosa* need more excating media (Norstog and BM1 media) for seed germination and subsequent seedling development compared to tropical epiphytic species *D. strebloceras* and *D. lineale*. Optimising these nutrient requirements *in vitro* will underpin successful *ex situ* conservation of orchid species.

Appendix 2. Authorship and copyright information for publication

Chapter 2.



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In which chapter is the manuscript /published work:	Two

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Diantina, S., McGill, C., Millner, J., Nadarajan, J., Pritchard, H.W., McCormick, A.C. (2020). Comparative Seed Morphology of Tropical and Temperate Orchid Species with Different Growth Habits. Plants, 9, 161. https://doi.org/10.3390/plants9020161 (Published online).

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Appendix 4. Statistical analysis

Chapter 2. Seed Morphology

GLM for species, taxonomy (Epidendroideae or Orchidoideae), climate (tropical or temperate) or habit (epiphytic or terrestrial) on seed traits.

Multivariate test[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1	165725.065b	5	282	0
	Wilks' Lambda	0	165725.065b	5	282	0
	Hotelling's Trace	2938.388	165725.065b	5	282	0
	Roy's Largest Root	2938.388	165725.065b	5	282	0
Species	Pillai's Trace	1.508	173.439	10	566	0**
	Wilks' Lambda	0.027	290.001b	10	564	0
	Hotelling's Trace	16.563	465.416	10	562	0
	Roy's Largest Root	15.24	862.587c	5	283	0
Climate	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Climate * Habit	Pillai's Trace	0	.b	0	0	.

	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Climate * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Habit * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate * Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Habit * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Climate * Habit * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate * Habit * Taxonomy	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1

a Design: Intercept + Species + Climate + Habit + Taxonomy + Species * Climate + Species * Habit + Species * Taxonomy + Climate * Habit + Climate * Taxonomy + Habit * Taxonomy + Species * Climate * Habit + Species * Climate * Taxonomy + Species * Habit * Taxonomy + Climate * Habit * Taxonomy + Species * Climate * Habit * Taxonomy

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	EL	1.483a	5	0.297	15.923	0
	EW	12.401b	5	2.48	140.781	0
	SL	73.418c	5	14.684	723.743	0
	SW	30.219d	5	6.044	286.239	0
	AS	62.754e	5	12.551	223.956	0
Intercept	EL	8499.304	1	8499.304	456197.666	0
	EW	6668.182	1	6668.182	378484.885	0
	SL	12739.114	1	12739.114	627896.892	0
	SW	7622.971	1	7622.971	361035.532	0
	AS	4619.781	1	4619.781	82435.399	0
Species	EL	1.28	2	0.64	34.353	0
	EW	6.104	2	3.052	173.237	0
	SL	60.101	2	30.051	1481.158	0
	SW	24.267	2	12.134	574.671	0
	AS	56.177	2	28.089	501.214	0
Error	EL	5.328	286	0.019		
	EW	5.039	286	0.018		
	SL	5.803	286	0.02		
	SW	6.039	286	0.021		
	AS	16.028	286	0.056		
Total	EL	8515.485	292			
	EW	6694.05	292			
	SL	12851.331	292			
	SW	7672.952	292			
	AS	4718.278	292			
Corrected Total	EL	6.812	291			
	EW	17.44	291			
	SL	79.221	291			
	SW	36.257	291			
	AS	78.782	291			

a R Squared = .218 (Adjusted R Squared = .204)

b R Squared = .711 (Adjusted R Squared = .706)

c R Squared = .927 (Adjusted R Squared = .925)

d R Squared = .833 (Adjusted R Squared = .831)

e R Squared = .797 (Adjusted R Squared = .793)

GLM for species, climate (tropical or temperate) or habit (epiphytic or terrestrial) on seed traits.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1	162540.246b	5	282	0
	Wilks' Lambda	0	162540.246b	5	282	0
	Hotelling's Trace	2881.919	162540.246b	5	282	0
	Roy's Largest Root	2881.919	162540.246b	5	282	0
Species	Pillai's Trace	1.89	96.686	15	852	0**
	Wilks' Lambda	0.013	198.214	15	778.879	0
	Hotelling's Trace	18.208	340.688	15	842	0
	Roy's Largest Root	15.259	866.734c	5	284	0
Climate	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Climate * Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1
Species * Climate * Habit	Pillai's Trace	0	.b	0	0	.
	Wilks' Lambda	1	.b	0	284	.
	Hotelling's Trace	0	.b	0	2	.
	Roy's Largest Root	0	.000b	5	281	1

a Design: Intercept + Species + Climate + Habit + Species * Climate + Species * Habit + Climate * Habit + Species * Climate * Habit

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	EL	1.483a	5	0.297	15.923	0
	EW	12.401b	5	2.48	140.781	0
	SL	73.418c	5	14.684	723.743	0
	SW	30.219d	5	6.044	286.239	0
	AS	62.754e	5	12.551	223.956	0
Intercept	EL	8333.326	1	8333.326	447288.819	0
	EW	6536.238	1	6536.238	370995.784	0
	SL	12488.221	1	12488.221	615530.673	0
	SW	7513.22	1	7513.22	355837.571	0
	AS	4539.67	1	4539.67	81005.89	0
Species	EL	1.284	3	0.428	22.981	0.000
	EW	10.256	3	3.419	194.038	0.000
	SL	60.405	3	20.135	992.428	0.000
	SW	26.883	3	8.961	424.408	0.000
	AS	60.854	3	20.285	361.962	0.000
Error	EL	5.328	286	0.019		
	EW	5.039	286	0.018		
	SL	5.803	286	0.02		
	SW	6.039	286	0.021		
	AS	16.028	286	0.056		
Total	EL	8515.485	292			
	EW	6694.05	292			
	SL	12851.331	292			
	SW	7672.952	292			
	AS	4718.278	292			
Corrected Total	EL	6.812	291			
	EW	17.44	291			
	SL	79.221	291			
	SW	36.257	291			
	AS	78.782	291			

a R Squared = .218 (Adjusted R Squared = .204)

b R Squared = .711 (Adjusted R Squared = .706)

c R Squared = .927 (Adjusted R Squared = .925)

d R Squared = .833 (Adjusted R Squared = .831)

e R Squared = .797 (Adjusted R Squared = .793)

Chapter 3. Seed Lipid

Seed viability

Tests of Between-Subjects Effects

Dependent Variable: viability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	30321.313[a]	11	2756.483	115.925	.000
Intercept	181227.863	1	181227.863	7621.579	.000
Species	15037.057	5	3007.411	126.477	.000
Treatment	12523.518	1	12523.518	526.679	.000
Species * Treatment	2108.756	5	421.751	17.737	.000**
Error	665.791	28	23.778		
Total	208050.324	40			
Corrected Total	30987.104	39			

a. R Squared = .979 (Adjusted R Squared = .970)

Seed Saturated Fatty Acid (SAFA)

Tests of Between-Subjects Effects

Dependent Variable: fame_safa

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	748.036[a]	11	68.003	24.986	.000
Intercept	13979.121	1	13979.121	5136.215	.000
Species	472.739	5	94.548	34.739	.000
Treatment	179.024	1	179.024	65.777	.000
Species * Treatment	96.273	5	19.255	7.075	.000**
Error	65.320	24	2.722		
Total	14792.478	36			
Corrected Total	813.356	35			

a. R Squared = .920 (Adjusted R Squared = .883)

Seed Mono-Unsaturated Fatty Acid (MUFA)

Tests of Between-Subjects Effects

Dependent Variable: fame_mufa

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2181.188[a]	11	198.290	57.695	.000
Intercept	15092.532	1	15092.532	4391.343	.000
Species	2105.794	5	421.159	122.541	.000
Treatment	50.718	1	50.718	14.757	.001
Species * Treatment	24.676	5	4.935	1.436	.247
Error	82.485	24	3.437		
Total	17356.205	36			
Corrected Total	2263.673	35			

a. R Squared = .964 (Adjusted R Squared = .947)

Seed Poly-Unsaturated Fatty Acid (PUFA)

Tests of Between-Subjects Effects

Dependent Variable: fame_pufa

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3632.653[a]	11	330.241	111.219	.000
Intercept	128821.174	1	128821.174	43384.358	.000
Species	3067.091	5	613.418	206.587	.000
Treatment	420.250	1	420.250	141.532	.000
Species * Treatment	145.313	5	29.063	9.788	.000**
Error	71.263	24	2.969		
Total	132525.090	36			
Corrected Total	3703.917	35			

a. R Squared = .981 (Adjusted R Squared = .972)

Chapter 4. *In vitro* seed germination

Univariate Analysis of Variance for *in vitro* seed germination of each species

D. lineale

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	413.119[a]	3	137.706	20.499	.000
Intercept	14015.381	1	14015.381	2086.382	.000
media	413.119	3	137.706	20.499	.000
Error	80.611	12	6.718		
Total	14509.111	16			
Corrected Total	493.730	15			

a. R Squared = ,837 (Adjusted R Squared = ,796)

D. cunninghamii

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	3248.814[a]	3	1082.938	41.086	.000
Intercept	21889.103	1	21889.103	830.454	.000
media	3248.814	3	1082.938	41.086	.000
Error	316.296	12	26.358		
Total	25454.214	16			
Corrected Total	3565.110	15			

a. R Squared = ,911 (Adjusted R Squared = ,889)

D. strebloceras

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4298.938[a]	10	429.894	4.855	.000
Intercept	92190.847	1	92190.847	1041.086	.000
media	4298.938	10	429.894	4.855	.000
Error	3896.313	44	88.553		
Total	100386.098	55			
Corrected Total	8195.250	54			

a. R Squared = .525 (Adjusted R Squared = .417)

P. banksii

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1095.636[a]	3	365.212	9.614	.005
Intercept	6253.155	1	6253.155	164.612	.000
media	1095.636	3	365.212	9.614	.005
Error	303.898	8	37.987		
Total	7652.689	12			
Corrected Total	1399.534	11			

a. R Squared = ,783 (Adjusted R Squared = ,701)

G. cunninghamii

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	766.899[a]	3	255.633	15.946	.001
Intercept	2855.428	1	2855.428	178.121	.000
media	766.899	3	255.633	15.946	.001
Error	128.246	8	16.031		
Total	3750.573	12			
Corrected Total	895.146	11			

a. R Squared = ,857 (Adjusted R Squared = ,803)

T. nervosa

Tests of Between-Subjects Effects

Dependent Variable: %germ_3mo

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	968.129[a]	3	322.710	13.377	.002
Intercept	8365.047	1	8365.047	346.751	.000
media	968.129	3	322.710	13.377	.002
Error	192.993	8	24.124		
Total	9526.168	12			
Corrected Total	1161.122	11			

a. R Squared = ,834 (Adjusted R Squared = ,771)

Chapter 5. Cryopreservation with Direct Immersion and Vitrification Treatment

T test comparison for in vitro seed germination after direct immersion or control (without liquid nitrogen) treatment for each species

D. lineale

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
germination	Equal variances assumed	2.861	0.166	-1.480	4	0.213	-7.02625	4.74805	-20.20895	6.15645
	Equal variances not assumed			-1.480	2.485	0.253	-7.02625	4.74805	-24.07018	10.01768

D. cunninghamii

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
germination	Equal variances assumed	3.664	0.128	-1.701	4	0.164	-10.65359	6.26469	-28.04717	6.73998
	Equal variances not assumed			-1.701	2.192	0.220	-10.65359	6.26469	-35.46717	14.15998

D. strebloceras

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
germination	Equal variances assumed	0.391	0.566	0.239	4	0.823	2.17727	9.10962	-23.11510	27.46964
	Equal variances not assumed			0.239	3.604	0.824	2.17727	9.10962	-24.24846	28.60300

P. banksii

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
germination	Equal variances assumed	2.222	0.210	-3.079	4	0.037	-20.03911	6.50847	-38.10951	-1.96871
	Equal variances not assumed			-3.079	3.134	0.051	-20.03911	6.50847	-40.25897	0.18075

T. nervosa

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
germination	Equal variances assumed	0.805	0.399	-10.400	7	0.000	-50.10563	4.81797	-61.49832	-38.71295
	Equal variances not assumed			-9.009	2.968	0.003	-50.10563	5.56156	-67.91285	-32.29842

Univariate Analysis of Variance for *in vitro* seed germination after vitrification cryopreservation of each species

D. cunninghamii

Tests of Between-Subjects Effects

Dependent Variable: germ

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.883	13	0.299	12.653	0.000
Intercept	73.848	1	73.848	3127.989	0.000
PVS treatment	3.883	13	0.299	12.653	0.000
Error	0.661	28	0.024		
Total	78.393	42			
Corrected Total	4.544	41			

R Squared = .855 (Adjusted R Squared = .787)

D. lineale

Tests of Between-Subjects Effects

Dependent Variable: germ

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	115.116[a]	13	8.855	3.413	0.003
Intercept	843.611	1	843.611	325.141	0.000
PVS treatment	115.116	13	8.855	3.413	0.003
Error	72.649	28	2.595		
Total	1031.376	42			
Corrected Total	187.764	41			

a. R Squared = .613 (Adjusted R Squared = .433)

D. strebloceras

Tests of Between-Subjects Effects

Dependent Variable: germ

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.759[a]	13	0.058	13.245	0.000
Intercept	107.658	1	107.658	24407.518	0.000
PVS treatment	0.759	13	0.058	13.245	0.000
Error	0.124	28	0.004		
Total	108.541	42			
Corrected Total	0.883	41			

a. R Squared = .860 (Adjusted R Squared = .795)

P. banksii

Tests of Between-Subjects Effects

Dependent Variable: germ

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6814.665[a]	13	524.205	3.238	0.005
Intercept	105463.217	1	105463.217	651.410	0.000
PVS treatment	6814.665	13	524.205	3.238	0.005
Error	4371.297	27	161.900		
Total	119120.139	41			
Corrected Total	11185.962	40			

a. R Squared = .609 (Adjusted R Squared = .421)