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EX SITU CONSERVATION AND CRYOPRESERVATION OF ORCHID GERMPLASM

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Premise of research. Orchids are among the most enigmatic of plant species. Yet the Orchidaceae comprises more species at risk of extinction than any other plant family. The collection and storage of orchid germplasm—principally seeds and associated mycorrhizal fungi but also protocorm-like bodies using encapsulation and vitrification techniques—allows for secure ex situ conservation. This article reviews the approaches and techniques used for the ex situ conservation of orchid germplasm, with a focus on seed banking and the use of cryopreservation techniques to improve the longevity of germplasm.

Pivotal results. It is increasingly apparent that cryopreservation—the storage of germplasm at ultra-low temperatures (e.g., in liquid nitrogen)—is required for the long-term and low-maintenance conservation of all types of orchid germplasm. For orchid seeds, desiccation tolerance is common, but longevity in storage is poor. Cryopreservation of orchid seeds shows promise, but some complexities in low-temperature storage behavior still require explanation and resolution. The application of more advanced cryopreservation techniques, including encapsulation-dehydration and vitrification, is becoming increasingly common. These techniques provide for the simultaneous storage of orchid propagules with their compatible fungus, while for seeds, vitrification techniques show potential for improving tolerance to the stresses of cryopreservation.

Conclusions. A renewed focus on describing the low-temperature storage physiology of orchid seeds to more precisely define the relationship between seed water content, storage temperature, and seed survival is required, as is perhaps the wider adoption of the use of cryoprotectants for seeds. This research, coupled with the development of improved methods of seed viability testing, will support the growing work of germplasm banks to protect orchid biodiversity in the face of habitat loss and potential species extinction.

Keywords: cryostorage, encapsulation, germination, Orchidaceae, seed banking, vitrification.

Online enhancement: appendix table.

Introduction

As one of the most species-rich families of flowering plants, the Orchidaceae is well known for its immense diversity and broad global distribution. Yet just as well known is the high level of extinction threat that many species within the family face as a consequence of a wide and dynamic range of intrinsic and extrinsic factors (Swarts and Dixon 2009*a*, 2009*b*; Seaton et al. 2010). Many orchids are inherently rare or locally restricted. Because this rarity is coupled with an often intimate and obligate association with soil mycorrhizal fungi and/or animal pollinators for survival, many species are susceptible to habitat and environmental change. Extrinsic factors caused by direct and indirect anthropogenic processes include loss and destruction of natural ecosystems, accelerated environmental and climatic change, weed invasion, changed hydrology and land management regimes, and illegal collection. These factors may push locally restricted or sensitive populations toward complete extinction or an inability to naturally recover. As such, all orchids are now listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. In an earlier review, Swarts and Dixon (2009*b*) identified three considerations for the conservation and recovery of orchids: (1) reserve design and management, (2) effective ex situ germplasm storage, and (3) development of in situ restoration or translocation approaches. Here, we review the development of ex situ conservation of orchid germplasm, with a particular focus on the use of cryopreservation techniques.

Ex Situ Conservation of Orchids

While ex situ conservation should never take the place of in situ conservation, ex situ storage of germplasm provides a necessary backup in the event of species extinction in the wild. For orchids, ex situ conservation refers to the off-site storage of a genetically representative sample of seeds, compatible my-

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corrhizal fungi, germinated protocorms, and/or (where applicable) somatic tissues (Swarts and Dixon 2009*b*). The long-standing challenge is to develop an ex situ conservation strategy that prevents or minimizes genetic and demographic threats associated with the creation of artificial populations of plants and that is efficient in providing material for in situ recovery actions.

Long-term storage of germplasm involves the use of low or ultra-low temperatures (−20° to −196°C) to reduce aging-related damage to macromolecules, spontaneous (synthetic) genetic variation (potentially nonadapted variation), or the gradual breakdown of cellular integrity (Touchell and Dixon 1993). One characteristic of orchids that provides outstanding opportunities for conservation is their dust-like seed. A single seed capsule is capable of producing tens of thousands to millions of seeds (Arditti and Ghani 2000). Seed banking is increasingly being adopted for the ex situ conservation of orchids. For example, Orchid Seed Stores for Sustainable Use, a Darwin Initiative project, aims to collect and store seeds of orchid species from more than 20 countries, using conventional seed banking methodology (Seaton et al. 2010; http://osssu.org). Also included under the umbrella of ex situ conservation (although not discussed here) is the regeneration of plants from stored germplasm and the continued cultivation of species as nursery-based living collections.

The careful selection and collection of germplasm is an important part of an integrated conservation approach that ultimately leads to the creation of self-sustaining populations of plants (Guerrant et al. 2004). Modern molecular methods are increasing technical capacity to identify and select genetic provenances considered most significant from a conservation perspective. Indeed, active restoration of habitats and species now calls for genetic issues to be considered when germplasm is to be translocated or mixed as part of recovery plan actions or to be stored in long-term, off-site genebanks (Rossetto et al. 1995; Fay and Krauss 2003). In these cases, appropriate genetic screening and plant selection are required to avoid inbreeding or outbreeding depression and maximize evolutionary potential of restored populations.

Potential genetic drift during seed storage has been cited as a criticism of seed banking (Basra 2006). Genetic drift is a change in frequency of alleles from the parent population that may arise during storage—or removal from storage—and that may impact the survival of regenerated seedlings from that collection. Hartl and Clark (1989) suggested that the average time a neutral allele will persist in a population is a function of population size and the allele's initial frequency. Given that seed banks are temporary storehouses of genetic variation, the rate of loss or change in allele frequency under the effects of storage and removal is likely to be higher than for natural populations, particularly since the collection is only a sample of the variation within the source population (Hamilton 1994). Though a concern, the phenomenon may not be as significant as the effects of poor sampling in the field and poor regeneration practices. Seed collection from field sites rarely considers measures of phenotypic or genotypic diversity of the sampled population. Although outcrossing between plants may be assumed via natural pollination (although many orchids are self-pollinated) or targeted through hand pollination, poor sampling techniques during seed collection will reduce variation in a seed bank collection, particularly if the variation within the parent population is low. Furthermore, plant regeneration may involve intuitively selecting for the largest or fastest germinating individual under in vitro conditions through assuming that these are most likely to survive outplanting to glasshouse or field conditions. This practice may lead to a loss of allelic diversity previously represented in the source population. As seed collections are becoming an increasingly important tool for orchid conservation and reintroduction, this is an area of research that requires attention.

Orchid Seed Storage Behavior

Seeds of many orchid species are regarded as possessing orthodox storage behavior (Thornhill and Koopowitz 1992; Pritchard and Seaton 1993; Pritchard et al. 1999; Seaton and Pritchard 2003; Seaton et al. 2010). The classification of orchid seeds as orthodox is mainly based on their ability to germinate following desiccation to low moisture contents (5%–7% fresh weight) and storage at subzero temperatures and, in some cases, based on evidence of increased longevity under these storage conditions (Pritchard and Seaton 1993). Orthodox storage behavior is more precisely described as a predictable increase in seed longevity as seed water content and storage temperature is reduced, based on the seed viability equations (Roberts 1973; Ellis and Roberts 1980). Although detailed modeling of the moisture and temperature relations of longevity for orchid seeds is lacking, a progressive increase in longevity with a decrease in seed water content has been demonstrated for three orchid species (Pritchard et al. 1999) and has been shown to be similar in magnitude to that of well-studied crop species (Seaton and Pritchard 2003). Certainly, seeds of the majority of orchid species for which data are available appear to be desiccation tolerant, and this trait seems to be irrespective of geographical origin, life form (e.g., epiphyte, lithophyte, or geophyte), or habitat. For example, seeds of three widely disjunct terrestrial orchids—*Dactylorhiza fuchsii* (a European forest species), *Dendrobium anosmum* (a lowland tropical forest species), and *Eulophia gonychila* (an African woodland species)—all survived drying to water contents in equilibrium with 5% relative humidity (RH; Pritchard et al. 1999). Similarly, seeds of 10 terrestrial orchid species from Mediterranean and temperate climatic regions of Australia survived drying to equilibrium with either 5% or 23% RH without any detrimental effects on germination (Hay et al. 2010), and 60%–100% germination of a further 10 terrestrial Australian orchids was achieved following drying to 15% RH (Dowling and Jusaitis 2012). For seeds of epiphytic orchids, data reviewed by Pritchard and Seaton (1993) provide examples of desiccation tolerance, with >60% germination recorded for species including *Cattleya aurantiaca, Dendrobium phalaenopsis*, and *Phalaenopsis amabilis* after storage at 5°–8°C for varying periods at ~4%–7% water content, or water contents in equilibrium with anhydrous calcium chloride (~30% RH).

There is some evidence, however, that overdrying orchid seeds may be detrimental. For example, seeds of *D. fuchsia*, *D. anosmum*, and *E. gonychila* equilibrated at 5% RH had reduced longevity compared with seeds equilibrated at 11% or 22% RH (Pritchard et al. 1999). In some cases, an im-

provement in longevity brought about by reducing water content to very low levels has been offset by a subsequent decrease in seedling vigor, as seen in *Disa uniflora* (Thornhill and Koopowitz 1992).

While desiccation tolerance in orchid seeds appears common and widespread among orchid genera, indicating the potential for long-term storage, data on the viability of orchid seeds following years or decades of storage are still relatively scarce. Pritchard and Seaton (1993) compiled data from studies dating back to the 1940s and showed that up to 80% germination was possible following storage for up to 10 yr at 8°C in a number of *Brassocattleya* and *Cattleya* hybrids. Low levels of germination (5%–30%) of these hybrids were also reported following 19–22 yr of storage at 8°C (Pritchard and Seaton 1993). However, across other species and genera (including *Dactylorhiza* spp., *Dendrobium* spp., and *Epipactis* spp.), seed viability declined rapidly to negligible levels within a few weeks or months of storage at room temperature, or at 4°–8°C (Pritchard and Seaton 1993).

Further recent evidence that the longevity of orchid seeds is poor relative to those of other species can be drawn from the increasingly large body of data on the longevity of orthodox seeds of agricultural and wild species (Walters et al. 2005; Kochanek et al. 2009; Probert et al. 2009; Tuckett et al. 2010; Mondoni et al. 2011). Much of this data is based on the application of rapid-aging techniques, whereby seeds are stored at elevated temperature and RH, usually at 45° or 60°C and 60% RH (Newton et al. 2009). During these controlled aging experiments, seeds are retrieved from storage periodically and assessed for viability, with the longevity of species ranked relative to each other, most commonly via comparing the time for 50% seed viability loss (p_{50}; Probert et al. 2009; Tuckett et al. 2010; Mondoni et al. 2011). This approach assumes that seed aging kinetics at high temperature and RH are the same as those when seeds are stored under cool (e.g., −20°C), dry (e.g., 15% RH) conditions, and although this correlation is yet to be verified because of a scarcity of data from long-term studies (Walters et al. 2005), a highly significant relationship has been shown to occur between families aged under standard seed banking protocols and those aged under controlled aging conditions (Probert et al. 2009). Utilizing this comparative longevity approach, seeds of 10 terrestrial orchid species were determined to be very short-lived (Hay et al. 2010). Seed longevity for these 10 species (p_{50}) ranged from ~0 to 36 d (mean, 10.9 d), when seeds were aged at 40°C and 60% RH. This compares with p_{50} values exceeding 500–700 d for long-lived seeds aged at 45°C (Probert et al. 2009). Comparative longevity studies of hundreds of phylogenetically diverse species across the globe (Probert et al. 2009; Tuckett et al. 2010; Mondoni et al. 2011) demonstrate that longevity varies by at least four orders of magnitude between species and that orchid seeds appear to be among the shortest-lived species yet found (Hay et al. 2010).

Low-Temperature Storage and Cryopreservation of Orchid Seeds

The apparent short-lived nature of orchid seeds compels not only the use of internationally accepted seed banking procedures through storage at −20°C but also the development of alternative storage techniques, such as cryopreservation, to prolong longevity (Batty et al. 2001; Li and Pritchard 2009; Hay et al. 2010). Reports on the germination of orchid seeds following storage at conventional seed banking temperatures provide some mixed results. For example, seed viability of nine species of epiphytic and geophytic orchids was reportedly >75% after storage for 2–7 mo at −10°C, but viability of a further 20 species was <50% or even <20% under the same conditions (as reviewed by Pritchard and Seaton 1993). Hosomi et al. (2012) reported that seeds of nine species of *Cattleya* maintained viability during storage at −18°C for 3 mo, but an earlier study on seeds of *C. aurantiaca* found seed viability to decline from ~80% to near 0% over 50–100 d in storage at −18°C (Seaton and Hailes 1989).

The potential for ultra-low-temperature storage (i.e., −70° to −196°C) to be employed for the conservation of orchid seeds has been explored in a number of studies. Seed viability was maintained for a number of orchid species—including *Dactylorhiza* spp., *D. anosmum*, *E. gonychila*, and *Paphiopedilum rothschildianum*—during storage for up to 12 mo at −70° and −196°C (Pritchard et al. 1999). In a study of 12 epiphytic and terrestrial orchid species from Thailand and Singapore, after 5 yr of storage at −70°, −20°, and 4°C, germination was found to be greatest following storage at −70°C (Huehne and Bhinija 2011). Storage at −80°C was also suitable for maintaining the viability of a number of Australian terrestrial species over 2 yr of storage (Hay et al. 2010). In some cases, storage at ultra-low temperatures has been found to improve germination. For example, germination was enhanced for seeds of the terrestrial orchid *Orchis morio* following drying to 5%–11% moisture content and immersion for 15 minutes in liquid nitrogen, with the germination of a further eight species being maintained (Pritchard, 1984). Similarly, for the tropical terrestrial and epiphytic orchids *Angraecum magdalenae*, *Calanthe vestita*, and *Trichopilia tortilis*, cryopreservation for 1 mo was found to enhance germination (Nikishina et al. 2001).

Examples such as these provide a basis for the adoption of ultra-low temperatures for conservation of orchid seeds, but this must be tempered by the fact that most studies have assessed seed viability after only relatively short storage periods and that storage behavior can be complex for orchid seeds stored at subzero temperatures (Pritchard et al. 1999; Batty et al. 2001; Sommerville et al. 2008; Hay et al. 2010; Hosomi et al. 2012). Certainly, the broad conclusion that the vast majority of orchid species are likely to have seeds that might remain viable for many decades when stored under gene banking conditions of −20°C (Seaton and Pritchard 2003; Seaton et al. 2010) requires further scrutiny. There is still little empirical evidence to verify the assumption that conventional approaches to the storage of orthodox seeds at −20°C (or even at lower temperatures) will successfully conserve orchid seeds for decades.

Intermediate-type seed storage behavior, whereby short periods of storage of dry seeds at subzero temperatures result in dramatically reduced germination, is apparent in some orchid data (Pritchard and Seaton 1993; Hay et al. 2010). Such complexities in low-temperature storage behavior were highlighted by Pritchard et al. (1999), who found that seed viability of *P. rothschildianum* and two species of *Dactylorhiza* was stable

during storage at −196°C for 12 mo but that germination significantly declined when seeds were stored at −30° or −50°C. A more recent study examining low-temperature storage of seeds of 10 Australian orchids also found evidence of complex storage behavior (Hay et al. 2010). For example, the germination of some species (e.g., *Caladenia flava*, *Diuris laxiflora*, and *Pterostylis recurva*) was greatly reduced within 3–6 mo when stored at −196°C, regardless of water content (seeds were dried before storage at between 5% and 92% RH at 23°C). Seeds of *P. recurva* had greater germination when stored at −80° or −18°C, but for seeds of *D. laxiflora*, storage at −18°C appeared to be optimal. For seeds of *Microtis media* subsp. *media*, germination was greatly reduced at −18°, −80°, and −196°C and all water contents. Many species showed a significant reduction in germination after 3 mo of storage but then little further reduction in germination for up to 2 yr of storage (Hay et al. 2010; fig. 1).

Do Plant or Seed Traits Relate to Seed Storage Behavior?

An analysis of the published data from which conclusions can be made regarding seed storage behavior reveals that data are available for fewer than 100 species, a mere 0.4% of the estimated total number of orchid species (table A1, available online). Almost half of this data is derived from the review of Pritchard and Seaton (1993), who in turn derived much of their data from a handful of studies published as early as 1943. Given this lack of empirical data and the diversity of the Orchidaceae, an ability to predict seed storage behavior on the basis of phylogenetic, phylogeographic, or seed morphological characters would be powerful. However, any such analyses are hindered by the fact that the current knowledge of storage behavior is not uniformly spread across the diversity of Orchidaceae but rather is biased strongly toward the subfamilies Epidendroideae and Orchidoideae (table A1). Data on only one species of Cypripedioideae are available, the Vanilloideae is poorly represented by only one genus, and data on the species-poor but widely distributed Apostasioideae are entirely absent. Within the Epidendroideae (commonly epiphytes), 64% of species for which data are available possess orthodox or probably orthodox seeds. Within the Orchidoideae (commonly terrestrial), the proportion of species with orthodox seeds is higher at 72%. But storage behavior can be divergent at the generic level, with some examples of genera that likely comprise species with both orthodox and intermediate seeds (e.g., *Cattleya*, *Dendrobium*, *Polystachya*). In terms of plant life form, intermediate seed storage behavior appears to be a little more common in epiphytic orchids, with ~39% possessing or likely to possess intermediate storage behavior compared with ~28% of the perennial geophytes (table A1). Most epiphytic orchids are found in tropical environments, and it has similarly been found that many other (nonorchid) species that have cold-sensitive seeds also originate in tropical regions (Hong and Ellis 1996). Tropical regions are centers of orchid diversity (Cribb et al. 2003), placing a strong impetus on assessing and managing intermediate storage behavior to ensure effective orchid conservation.

Predicting seed behavior on the basis of phylogeny or seed morphological traits is likely to be flawed, since the Orchidaceae is highly evolved and capable of rapid adaptation, meaning that the range of seed characters will also be great. Species of *Vanilla*, for example, produce hard seeds adapted to bird dispersal, but within other clades of Vanilloideae, there exist widely divergent seed morphologies, from filliform, to balloon, to highly elongated seeds (Pridgeon et al. 1999). Similarly, within the Cypripedioideae, species of *Selenipidium* have seeds with thick testae, whereas other Cypripedioideae have rather more conventional orchid seeds (Pridgeon et al. 1999). Seeds with a relatively thick, hard testa (deemed to be a primitive character) are also found within the other basal subfamily Apostasioideae (Pridgeon et al. 1999). Such seeds—as opposed to seeds with a thin, papery testae—are outgroups for which there is no information, and further investigations of the storage behavior of these outgroups is needed. But while it is tempting to suppose that the diverse morphological traits of the

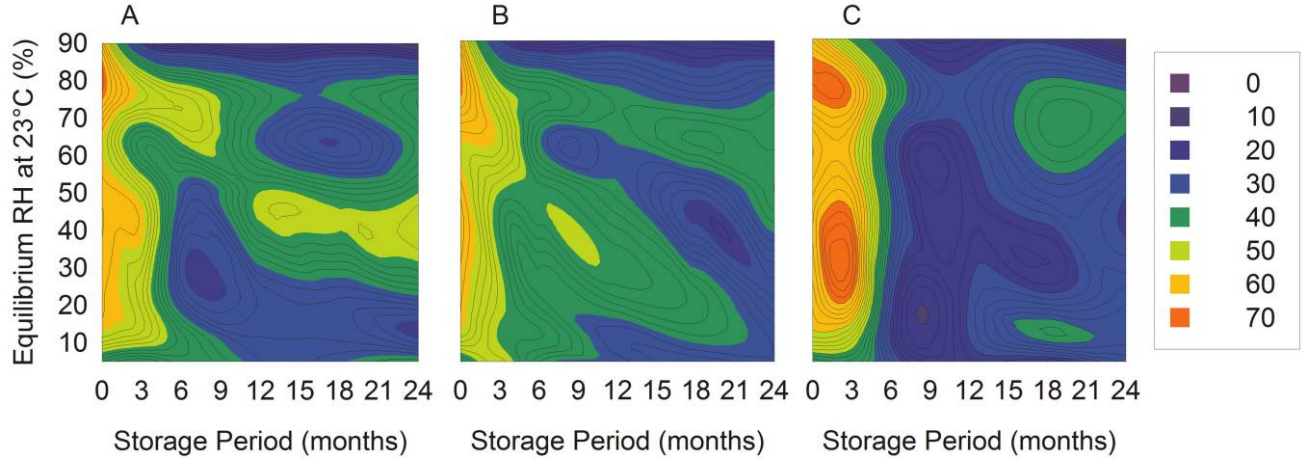

Fig. 1 Contour plot of the combined germination response (%; graded by color) of seeds of *Caladenia flava*, *Diuris laxiflora*, *Microtis media*, *Pterostylis recurva*, and *Thelymitra crinita* following storage for up to 2 yr at a range of moisture contents (equilibrium relative humidity [RH] at 23°C) and at −18°C (*A*), at −80°C (*B*), or in liquid nitrogen (−196°C; *C*). Data from Hay et al. (2010).

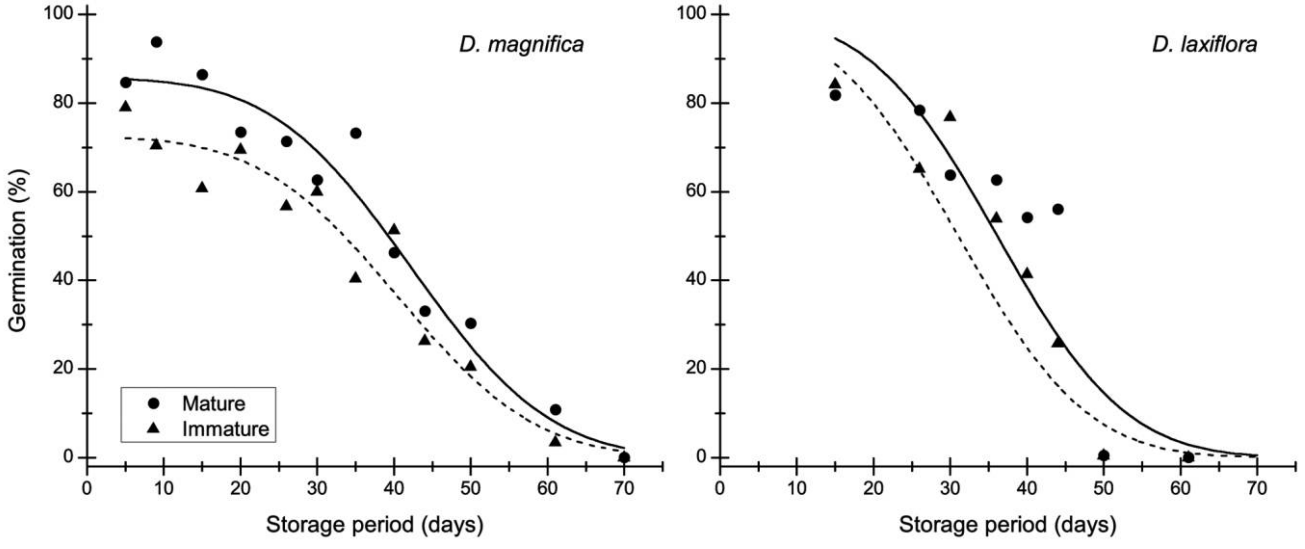

Fig. 2 Survival curves of *Diuris magnifica* and *Diuris laxiflora* seeds aged at 40°C and 60% relative humidity. Seeds from wild plants were harvested from fully matured, dehiscing pods (mature; circles; solid line) or from immature green pods that were collected and stored in the laboratory, with pod stems immersed in water until dehiscence (immature; triangles; dashed line). Survival curves were fitted by probit analysis, and the time for 50% viability loss (p_{50}) was determined to be 39.3 d (mature) and 33.3 d (immature) for *D. magnifica* seeds and 36.1 d (mature) and 31.1 d (immature) for *D. laxiflora* seeds.

wafer-thin testae of orchid seeds might play a role in storage behavior through, for example, a varying susceptibility to mechanical damage during handling, freezing, or thawing, there is no evidence of any such damage following low-temperature storage across diverse terrestrial genera (Batty et al. 2001). Ultimately, the morphological variability in orchid seeds is likely to be more of functional significance as related to environmental or adaptive needs, with the tolerance to the stresses of ex situ storage more closely tied to seed physiology.

Why Are Orchid Seeds Short-Lived?

Seed Maturity

Seed maturity at the point of collection can have a marked effect on the storage behavior and longevity of seeds. During seed development, the dry weight increases to a point that is termed "mass maturity" (Ellis and Pieta Filho 1992). In general, orthodox seeds acquire germinability (the ability of freshly harvested seeds to germinate without drying) and desiccation tolerance before or around this time (immature orthodox seeds in effect show recalcitrant behavior, since they are not desiccation tolerant). However, seed quality (including longevity in subsequent storage) continues to increase as the seeds dry to equilibrium with ambient conditions, reaching a maximum at the point of natural dispersal (Hay et al. 1997; Probert et al. 2007). It is therefore important that seeds are not harvested too much in advance of natural dispersal to ensure that their maximum potential longevity is realized. This is of paramount importance for very short-lived seeds such as those of orchids, as illustrated for *Diuris magnifica* and *Diuris laxiflora*, whereby seeds from mature pods were longer-lived in experimental storage than seeds from immature pods, with the time for viability to fall to 50% (p_{50}) increasing by 16%–18% (fig. 2). Unfortunately, it is common for orchid seeds to be collected while the capsules—and the seeds within—are still partly immature. This practice arises from difficulties in locating plant populations (particularly of those species that are rare, uncommon, or epiphytic) and is compounded by the risk of losing many tens of thousands of seeds upon splitting of the mature capsules. Furthermore, the timing of maturation is not always obvious. For example, in a glasshouse study of hand-pollinated *Phalaenopsis* hybrids, chlorosis of the seed capsules was observed at 120 d after pollination, but desiccation tolerance occurred at 180 d after pollination, and dehiscence did not commence until 220 d after pollination (Schwallier et al. 2011). For species such as these *Phalaenopsis* hybrids, seed maturity could be easily misjudged if based on capsule colour alone, leading to early collection and poor seed longevity. Though data are lacking, it is also conceivable that peculiarities in pollination mechanisms of orchids might complicate seed collection. For some orchids, temporal variation in the developmental status of seeds in different fruit capsules may result from the timing of pollination events of individual flowers across a population or even on a single plant, such as seen along the inflorescence of *Myrosmodes cochleare* (Berry and Calvo 1991). A better appreciation of the effects of seed maturity on desiccation tolerance and longevity, and of how to manage seed collection programs accordingly, would benefit the orchid seed banking community.

Biophysical Aspects of Orchid Seed Storage

While a level of variability in germination following low-temperature storage of orchid seeds has been apparent for some time, there remains uncertainty as to the underlying phys-

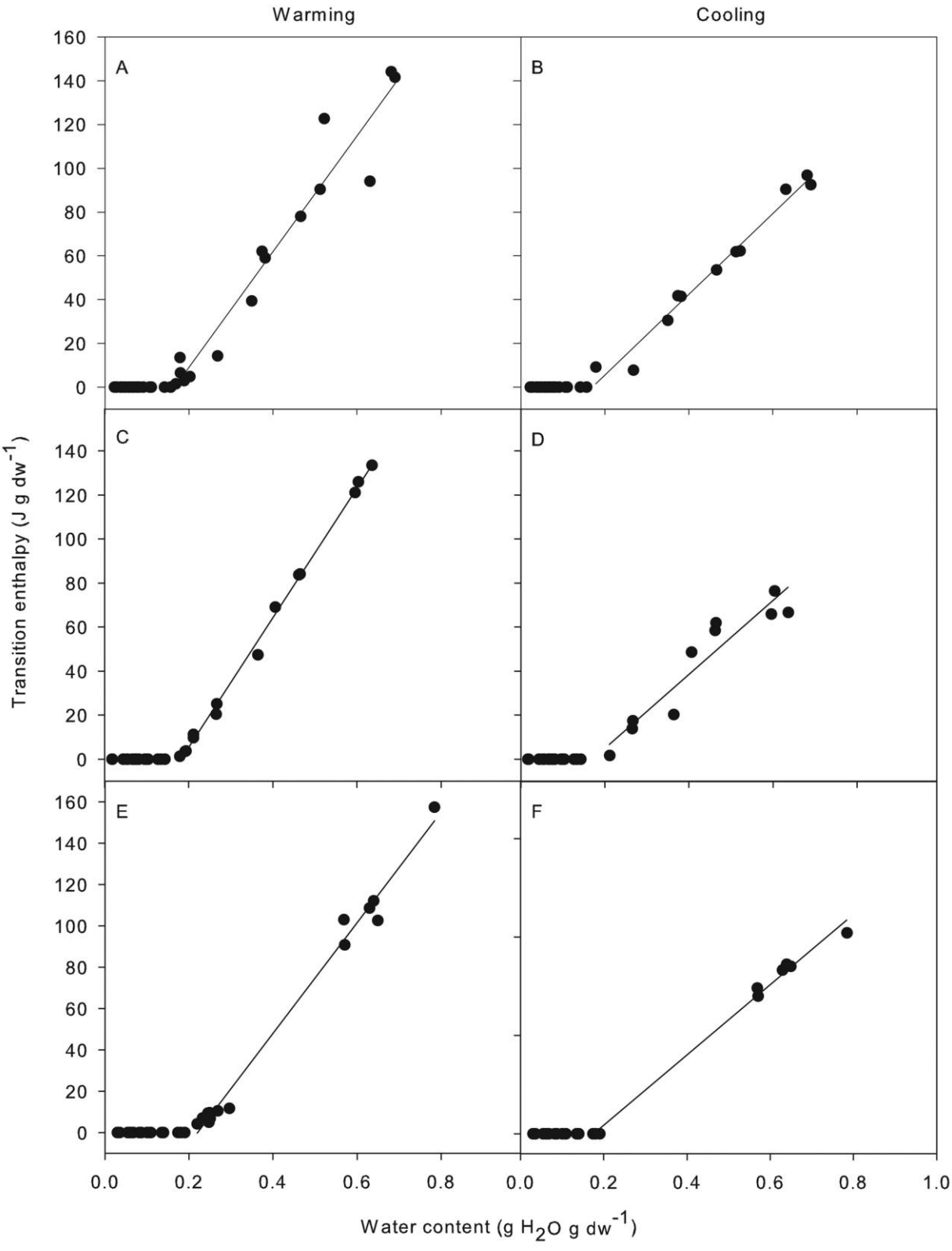

Fig. 3 Relationship between seed water content and enthalpy of melting (*left*) or freezing (*right*) transitions for *Caladenia flava* (*A*, *B*), *Microtis media* (*C*, *D*), and *Pterostylis recurva* (*E*, *F*) seeds. Unfrozen water contents: 0.17 g H_2O g dry weight^{-1} (dw^{-1}) for *C. flava*, 0.18 g H_2O g dry weight^{-1} for *M. media*, and 0.20 g H_2O g dry weight^{-1} for *P. recurva* seeds. Transition enthalpies were calculated from warming thermograms similar to those presented in fig. 4.

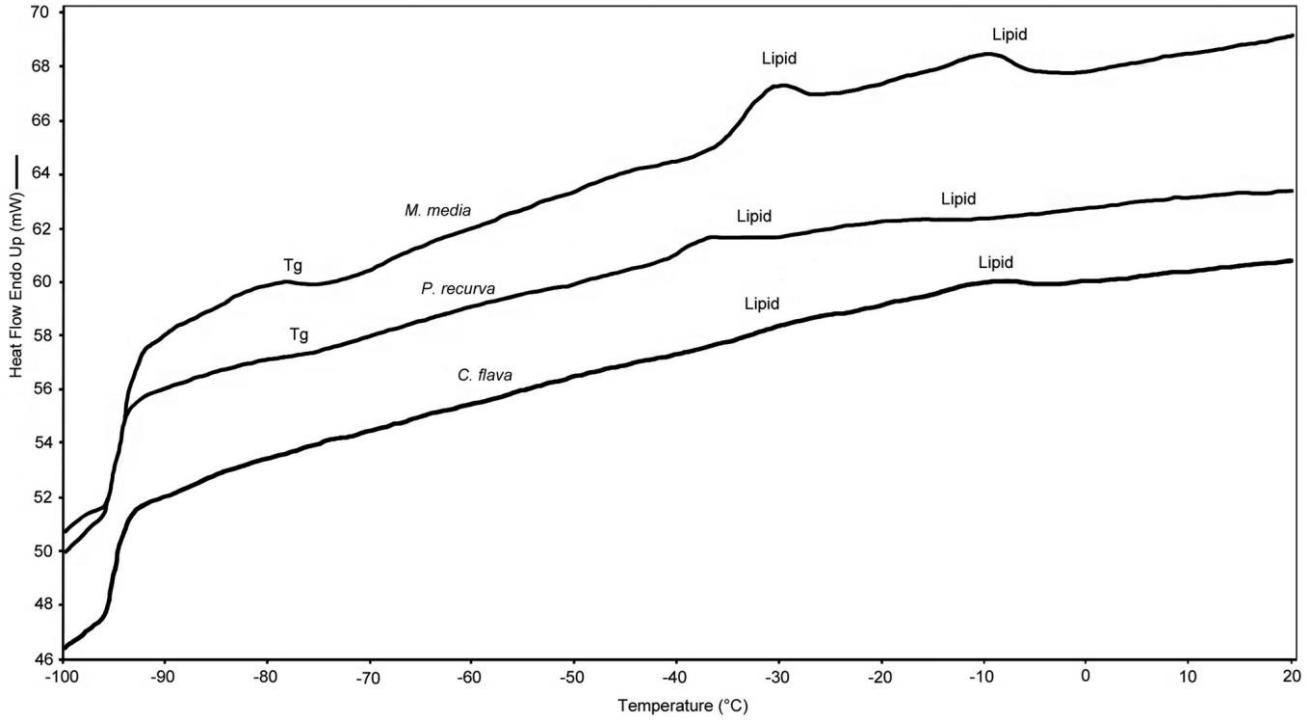

Fig. 4 Warming thermograms of *Caladenia flava*, *Microtis media*, and *Pterostylis recurva* seeds after being dried over silica gel (5% relative humidity) for 5 d (seed water content of 0.02–0.03 g H_2O g dry weight^{-1}). Samples of seeds (5 mg) were scanned at a warming rate of 20°C min^{-1} from −100° to 20°C, using a Perkin-Elmer DSC-7 (Perkin-Elmer LAS, Beaconsfield).

iology. There has been little in the way of biophysical or biochemical investigations that might explain the relatively short longevity of orchid seeds in storage at low temperatures. Research on numerous crop and wild species with orthodox seeds demonstrates that successful storage at cryogenic temperatures requires careful drying of seeds to avoid the lethal injury associated with the formation of ice crystals while also avoiding potentially damaging effects resulting from overdrying (Vertucci 1989, 1990; Vertucci and Roos 1993). For orchids, almost no data on thermodynamic properties are available that may assist to explain complex storage behavior. However, as one example, differential scanning calorimetry thermograms for seeds of three Australian orchid species indicate that the unfrozen water content is between 0.17 and 0.20 g H_2O g dry weight^{-1} (fig. 3) and, therefore, that seeds should be dried below this level before exposure to subzero temperatures. These water contents are a little lower than those observed for orthodox seeds, such as pea and soybean (Vertucci 1990), but higher than those observed for seeds of some nonorthodox species (Sacandé et al. 2000) and for fern spores, which, similar to orchid seeds, are short-lived in storage (Ballesteros and Walters 2007; Ballesteros et al. 2012).

It has been suggested that crystallization of triacylglycerols during storage of desiccation-tolerant organisms at subzero temperatures may cause damage and hasten the rate of viability loss (Crane et al. 2003; Ballesteros and Walters 2007). There are little data for orchids, but seeds of *Cattleya aurantiaca* have been found to have a relatively high oil content (29%; Pritchard and Seaton 1993; Pritchard et al. 1999), and the isotherms presented by Hay et al. (2010) for seeds from nine orchid species similarly suggest a high oil content. Storing seeds at temperatures at or near those at which lipid phase transitions occur may be detrimental (Vertucci 1989; Pritchard and Seaton 1993; Crane et al. 2003). In orchids, conformational changes of lipids between −5° and −35°C were proposed to contribute to the loss of viability of dry seeds of *C. aurantiaca* stored at −18°C (Pritchard and Seaton 1993). For seeds of *Caladenia flava*, *Microtis media*, and *Pterostylis recurva*, such lipid phase transitions are evident at ~−10° and −30°C in warming thermograms (fig. 4), which may contribute to their poor longevity at −18°C (fig. 1; Hay et al. 2010). A glass transition (the transformation of the intracellular solution into a highly viscous fluid, or amorphous solid) for the latter two species was also evident at −80°C (fig. 4), although there was apparently better survival of *P. recurva* seeds during storage at a range of moisture contents at −80°C than at either −18° or −196°C (Hay et al. 2010). While the formation of intracellular glasses during cooling and/or drying is known to influence storage stability and aging as the extreme viscosity of the glass restricts molecular mobility (Walters et al. 2010), phase transition temperatures can vary somewhat, depending on cooling and thawing rates, and those detected via commonly used methods (such as differential scanning calorimetry; fig. 4) do not necessarily reflect exactly what might occur during storage. The relationship between storage behavior and glass transitions as they are influenced by temperature and seed moisture content is still an evolving field (Walters et al. 2010). With such data for orchids restricted to a handful of species,

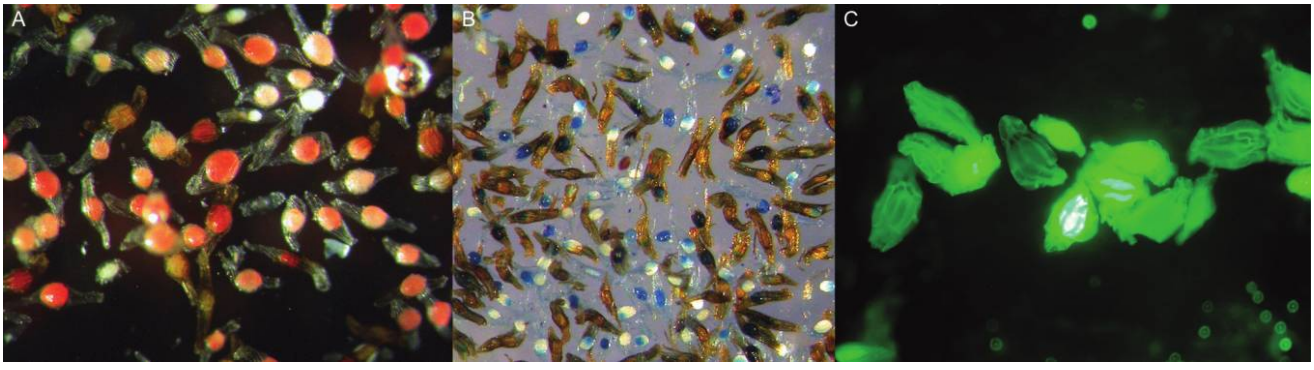

Fig. 5 Examples of staining patterns observed for *Caladenia arenicola* seeds stained with tetrazolium chloride (*A*), Evans blue (*B*), and fluorescein diacetate (*C*). Variability in staining patterns and intensity can make interpretation of viability challenging.

there is a clear opportunity to apply thermodynamic studies to orchid seeds with complex low-temperature storage behavior.

Challenges in Handling Orchid Seeds

Orchid seeds are difficult to handle in the laboratory. Germination and viability testing is technically demanding, and a lack of precision in seed testing may be a contributing factor to inconsistencies in results sometimes observed when seed samples are periodically retrieved and tested during storage. For example, Pritchard et al. (1999) reported that seed germination of five orchid species stored at seven different temperatures between 6° and −196°C varied considerably within a single storage environment over a 1-yr period, regardless of the environment. Hay et al. (2010) reported a similar result for 10 orchid species monitored over 2 yr of storage at five different temperatures between 23° and −196°C. Seed sampling and viability testing of orchid seeds in the laboratory is challenging because of their small size. Also, there are inherent difficulties in initiating germination and subsequent protocorm development that arise from the seeds' reliance on a suitable mycorrhizal fungus inoculum. In the absence of the fungus, the commonly used technique is to incubate seeds on an asymbiotic growth medium. But indiscriminate use of asymbiotic media can be problematic and inferior to symbiotic media (Johnson et al. 2007), and inappropriate culture media can confuse the interpretation of seed viability. The variation in germination that can occur among different asymbiotic media has been recently illustrated in a study of four Australian orchids (Dowling and Jusaitis 2012). Germination, when defined as the early stages of testa rupturing and enlargement of the protocorm, varied significantly between six germination media, differing by as much as 50% for seeds of *Thelymitra pauciflora*. Further, when considering those seeds that progressed through to the final stage of germination (i.e., shoot differentiation), for two species (*T. pauciflora* and *Prasophyllum pruinosum*), none of the media were suitable (Dowling and Jusaitis 2012).

Alternative methods of seed viability testing include the use of vital stains. For orchids, these offer some advantages in the potential to assess viability more rapidly through bypassing the need for development of suitable germination media and the long incubation periods necessary for germination. However, the use of vital stains such as tetrazolium chloride, fluorescein diacetate (FDA), and Evans blue has been met with varying degrees of success for orchid seeds (Waes and Debergh 1986; Batty et al. 2001; Nikishina et al. 2001; Wood et al. 2003; Wood and Pritchard 2004; Johnson et al. 2007; fig. 5). For example, disparities between seed germinability and viability were noted during storage of *Phaius tankervilleae* seeds (Hirano et al. 2009). Germination of this species decreased by 60% over a 6-mo storage period at 4°C, yet viability was apparently maintained on the basis of assessment via tetrazolium chloride (the most commonly used vital stain in seed testing). Wood and Pritchard (2004) had better success correlating germination with viability using FDA for 10 orchid species, and Batty et al. (2001) also concluded that FDA was a superior stain, while noting that it tended to overestimate viability in comparison to the germination tests.

The complexities involved in assessing the viability and/or germinability of orchid seeds in the laboratory are well recognized, but the consequences for evaluating storage behavior are not always appreciated. As orchid seed conservationists endeavour to manage an ever increasing diversity of species, the considered development and use of appropriate growth media and viability testing regimes will be fundamental to the accurate monitoring of seed quality during storage.

Other Germplasm Storage Techniques

The storage of seeds carefully dried under controlled conditions (e.g., 15%–20% RH at 15°–20°C) is not the only method available for the conservation of orchid germplasm. Other techniques for cryopreserving orchid germplasm include three that are routinely used for preserving desiccation-sensitive material produced in vitro: vitrification, encapsulation-dehydration, and encapsulation-vitrification.

Vitrification

Vitrification is a process in which intracellular fluids form an amorphous glass without the formation of ice crystals (Benson 2008). This is achieved by the addition of cryoprotective solutions, such as glycerol, dimethylsulfoxide, glycols, and plant vitrification solution 2 (PVS2), a mixture of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimeth-

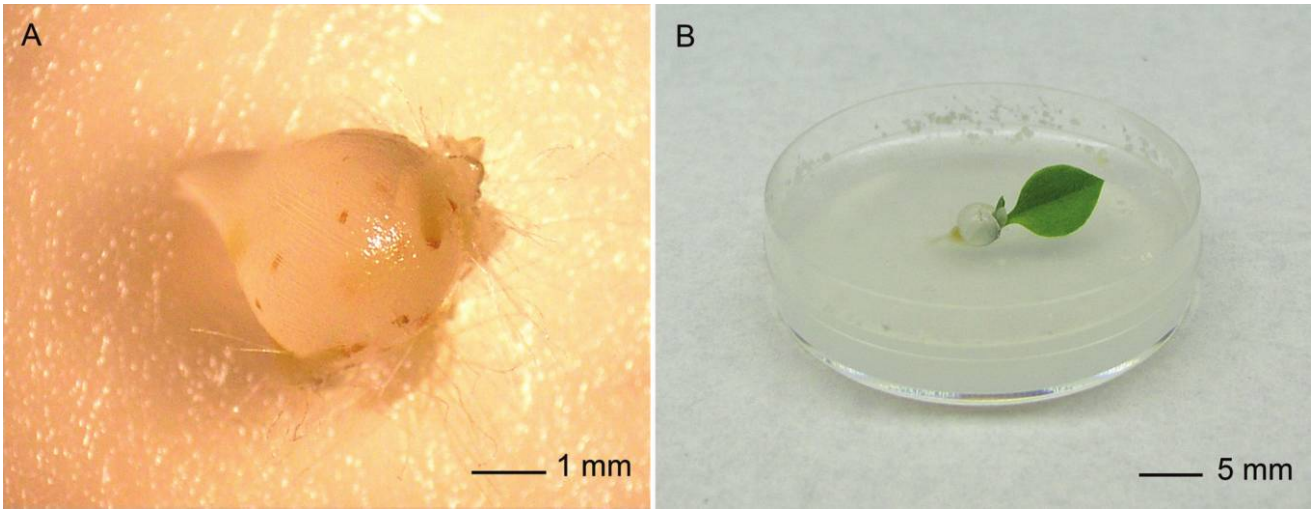

Fig. 6 Germination of *Pterostylis saxicola* seed encapsulated in 2% sodium alginate with a compatible mycorrhizal fungus. *A*, Initial protocorm development. *B*, Subsequent shoot formation.

ylsulfoxide (Sakai and Engelmann 2007). Vitrification techniques—developed principally to allow for the cryopreservation of desiccation-sensitive plant material, such as in vitro tissue culture–derived shoot tips, somatic embryos, and cell cultures—are becoming increasingly common for the cryostorage of orchid seeds and protocorms (Ishikawa et al. 1997; Hirano et al. 2005, 2011; Nikishina et al. 2007; Vendrame et al. 2007; Huehne and Bhinija 2011; Galdiano et al. 2012; Watanawikkit et al. 2012). Vitrification appears to enhance survivability of orchid seeds. Seeds of four *Dendrobium* hybrids, for example, did not survive cryostorage at water contents of 11%–19% (fresh weight), but germination of >50% was achieved following preconditioning of seeds with PVS2 and sucrose over ice (Vendrame et al. 2007). Similarly, the germination of seeds of *Phaius tankervilleae* was maintained at ~70% during storage in liquid nitrogen for at least 12 mo when seeds were first precultured on a sucrose solution and then treated with PVS2; in the absence of PVS2, no survival was recorded (Hirano et al. 2009). For seeds of two species of *Dendrobium* and the hybrid *Vanda* Miss Joaquim, which do not survive storage at −70°C, the cryoprotectants mannitol and *meso*-inositol enhanced survival; after 5 yr of storage, up to ~30% germination was achieved for *Dendrobium crystallinum* seeds (Huehne and Bhinija 2011). These compounds, along with glucose, were also found to be effective at enhancing seed survival for a number of other *Dendrobium* spp. as well as *Grammatophyllum speciosum* and *Pholidota articulata* over a 6-mo storage period at −70°C (Huehne and Bhinija 2011). Vitrification techniques have also been used to good effect by allowing for the cryostorage of immature seeds of *Bletilla striata*, providing readily germinable seeds on retrieval from storage by avoiding the dormancy present once seeds mature (Hirano et al. 2005).

Encapsulation-Dehydration

Encapsulation-dehydration involves the encapsulation of germplasm in a sodium alginate bead, pretreatment of the bead in a concentrated sucrose solution, then desiccation of the bead over silica gel or in a flow of sterile air (fig. 6). Once dried sufficiently, the alginate beads can be treated as orthodox seeds. An advantage of this technique over vitrification lies in its use of nontoxic materials. The encapsulation-dehydration technique was initially developed for the production of artificial seeds (Janick et al. 1989) and was adapted for the cryopreservation of *Solanum* shoot tips by Fabre and Dereuddre (1990). Over the past decade, it has also been found to be effective in the cryopreservation of orchid seeds (Wood et al. 2000; Flachsland et al. 2006; Surenciski et al. 2007; Sommerville et al. 2008), orchid protocorms (Maneerattanarungroj et al. 2007; Jitsopakul et al. 2008; Gogoi et al. 2013), protocorm-like bodies (Poobathy et al. 2009; Antony et al. 2011; Yin et al. 2011), and shoot tips (Lurswijidjarusa and Thammasiri 2004).

Flachsland et al. (2006) found that survival of mature *Oncidium bifolium* seeds stored in liquid nitrogen for 1 h was significantly greater for seeds treated by encapsulation-dehydration (67% survival) than for seeds dried over silica gel and stored without additional cryoprotection (0% survival). Few examples of long-term storage using this method are available; however, Wood et al. (2000) demonstrated that encapsulated seed of *Dactylorhiza fuchsii* could be stored at −196°C for 1 mo with no change in viability, and Sommerville et al. (2008) showed that encapsulated *Pterostylis saxicola* and *Diuris arenaria* seed could be stored at −18° or −196°C for 6 mo with no change in germination percentage compared with freshly encapsulated seeds.

To date, encapsulation-dehydration has been used more commonly to preserve orchid protocorms (partially germinated seeds that will eventually develop a shoot and root) and protocorm-like bodies generated by in vitro culture of orchid tissues. Successful storage of this germplasm at −196°C in the short term (1–24 hours) has been achieved for *Cleisostoma arietinum* (Maneerattanarungroj et al. 2007), *Vanda coerulea* (Jitsopakul et al. 2008), *Phalaenopsis bellina* (Khoddamzadeh

et al. 2011), and cultivars of *Dendrobium* (Antony et al. 2011; Zainuddin et al. 2011), *Brassidium* (Yin et al. 2011), and *Ascocenda* (Poobathy et al. 2009). When each step in the procedure is optimized, the percentage recovery of stored material can be high. Gogoi et al. (2013), for example, achieved recovery rates (generation of complete plantlets) of 70%–72% for *Cymbidium eburneum* and *Cymbidium hookerianum*.

Encapsulation-Vitrification

Encapsulation-vitrification is a combination of the above two techniques. The germplasm to be conserved is encapsulated in sodium alginate, immersed in a loading solution of glycerol and sucrose, and finally exposed to a cryoprotective solution such as PVS2 before storage in liquid nitrogen. The method has been successfully used to preserve the protocorm-like bodies of *Dendrobium candidum* (Yin and Hong 2009) and *Dendrobium nobile* (Mohanty et al. 2012). Optimizing each step of the process resulted in a survival rate of 89% for *D. candidum* (Yin and Hong 2009) and 78% for *D. nobile* (Mohanty et al. 2012). In the latter case, optimized encapsulation-vitrification resulted in significantly higher survival of the protocorm-like bodies compared with encapsulation-dehydration (78% vs. 53%). Encapsulation-vitrification has not yet been applied extensively to orchid seeds, although one report comparing this method to vitrification and encapsulation-dehydration on six species found it to be optimal for *Dendrobium hercoglossum* seeds (Thammasiri 2008).

While orthodox seeds generally do not benefit from cryoprotection (since their desiccation tolerance provides for the removal of freezable water before storage), vitrification, encapsulation-dehydration, and encapsulation-vitrification appear to offer some benefits for the cryostorage of orchid seeds, potentially through providing better resilience to the rapid cooling (and subsequent thawing) associated with plunging germplasm directly into liquid nitrogen. These alternatives may ultimately prove a superior option for orchid seeds by allowing for cryostorage while avoiding the difficulties surrounding the complex and species-specific interactions between seed water content, storage temperature, and survival.

Cryopreservation of Mycorrhizal Fungi

Terrestrial orchid seeds require a mycorrhizal association to germinate in natural environments. Therefore, effective conservation of orchids also requires the simultaneous conservation of compatible fungi. While orchid seed can be germinated via asymbiotic in vitro methods, transfer of terrestrial orchid seedlings to field sites is considerably more successful when a compatible mycorrhizal fungus is transferred with each plant. In addition, when orchids are translocated to new sites from which compatible fungi may be absent, symbiotically grown germplasm ensures that the new populations have the potential to become self-sustaining.

A number of methods are available for storing cultures of fungi, including, for example, on agar plates stored at room temperature or lower, on agar submersed in sterile water or mineral oil, and inoculation onto sterile soil or millet (Smith and Onions 1994). These methods pose a particular set of problems for long-term storage, the most important of which are potential changes in morphology, physiology, and/or genetic structure; the loss of key functional characteristics, such as the ability to increase plant growth and to initiate germination (Sneh and Adams 1996; Ryan and Smith 2004); the need for repeated subculturing; and the risk of contamination during subculture.

Of the preservation methods currently available, cryostorage is thought to be best for the retention of viability and genetic integrity of the fungi associated with orchids (Sneh and Adams 1996; Wood et al. 2000). A variety of genera in the Basidiomycota, a taxon that includes many of these fungi, have been found to tolerate storage at −165°C for 12 mo when 10% (w/w) glycerol is used as a cryoprotectant. Of 1615 strains tested by Nagai et al. (2005), 96% survived cryopreservation in 10% glycerol for 1 yr. The isolates successfully preserved included strains from three genera commonly associated with orchids: *Ceratobasidium*, *Sebacina*, and *Thanatephorus*. Similarly, Batty et al. (2001) found that fungal isolates associated with three Australian orchids (*Caladenia arenicola*, *Diuris magnifica*, and *Pterostylis sanguinea*) survived storage in liquid nitrogen for 12 mo (when treated with various cryoprotectants), with no apparent impact on viability other than a delay in initiating hyphal growth.

The use of cryoprotectants appears to provide the key to successful storage of Basidiomycetes at ultra-low temperatures. For example, 39 of 106 isolates of *Rhizoctonia solani* showed a significant decrease in viability after 10 yr of storage in the vapor phase of liquid nitrogen without cryoprotectants (Webb et al. 2011). By contrast, 30 strains of a variety of Basidiomycetes stored in liquid medium supplemented with 5% glycerol showed no change in viability or morphology, no mutations in ITS or ribosomal DNA sequences, and very little change in the activity of a range of enzymes after storage in liquid nitrogen for 10 yr (Homolka et al. 2010). However, while many fungi in the Basidiomycota can be preserved cryogenically, some cannot (Ryan and Smith 2007). A potential alternative method for storing these fungi is encapsulation-dehydration. For the species associated with orchids, this technique can be modified to encapsulate orchid seed and fungi simultaneously (Wood et al. 2000; Sommerville et al. 2008). Simultaneous encapsulation has several advantages: it simplifies the procedures associated with the maintenance of individual collections of orchid seed and fungi, it reduces the need for frequent subculturing of the fungi, and it provides a simple method for checking the physiological integrity of the fungal isolate through the ability of the fungi to initiate germination of the seed. This method was used successfully by Wood et al. (2000) to store the seed and compatible fungi of *Anacamptis morio* and *Dactylorhiza fuchsii* for up to 1 mo with no loss in viability of either the seed or the fungi. Similarly, Sommerville et al. (2008) reported successful storage of seed and compatible fungi of *P. saxicola* and *D. arenaria* for up to 6 mo, with no reduction in fungal recovery or germination percentage.

Conclusion

Programs for the ex situ conservation of wild plant germplasm are fundamental to the increasingly coordinated and global-scale actions to conserve the world's declining biodiversity. Orchids are widely acknowledged as being among the most threatened of plants, and ex situ conservation requires

efforts to secure genetically representative germplasm of both the plants and their fungal symbionts. Much has been learned and achieved in the past 20 yr to provide the platform for the prevention of orchid species extinction through ex situ conservation. Biotechnological approaches, including vitrification and encapsulation-dehydration, although somewhat labor-intensive, have proved effective for the simultaneous cryopreservation of fungi and orchid germplasm. Seed banking is the most widely adopted, practical, and effective means for conserving orchid biodiversity, and cryopreservation is arguably the superior option for seed banking, given the short-lived nature of orchid seeds in storage.

Yet data are still limited regarding the behavior of orchid seeds in storage at ultra-low temperatures, and there is clearly a need for a renewed focus on scientifically defining storage procedures. Such studies should incorporate a greater phylogenetic coverage of the Orchidaceae to include poorly represented subfamilies, including the Apostasioideae, Cypripedioideae, and Vanilloideae. The vast majority of orchid species appear to possess desiccation tolerant seeds, meaning that they are potentially suitable for low-temperature storage. However, desiccation tolerance per se does not guarantee seed survival during storage at subzero temperatures, and identifying species that possess intermediate-type storage behavior is the most urgent task. In the absence of any prior knowledge, seed banks would be well advised to closely monitor the seed viability of a phylogenetically diverse representation of species during the first months of storage to screen for intermediate storage behavior. Where nonorthodox storage behavior is identified, the development of alternative approaches for low-temperature storage of seeds, such as the treatment of seeds with cryoprotectants, may benefit. For high-value species, the cryopreservation of protocorms using encapsulation and/or vitrification techniques is another viable option. The use of multiple storage environments—for example, the storage of seeds at both −18° and −196°C—is also a useful strategy to mitigate the risk of viability loss.

Limits to the available data should not limit nor delay the adoption of ex situ conservation measures. It is unfortunate that the ever diminishing habitat for wild plant populations requires such drastic action as the freezing of germplasm in a global network of repositories to arrest extinction events. Nevertheless, the successful adoption of cryopreservation will at least create within the modern seed bank an enduring legacy of conserved biodiversity and, ultimately, provide for the repatriation of species into restored landscapes.

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