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EFFECTS OF DEVELOPMENTAL AGE ON CRYOPRESERVATION AND COLD STORAGE OF SEEDS OF *Arundina graminifolia*, A CLASSIC DAI MEDICINAL ORCHID

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Abstract

BACKGROUND: *Arundina graminifolia* is a traditional medicinal plant of the Dai people in China. To enhance its ex situ conservation greater knowledge is needed on the species' seed storage behavior, particularly the effects of developmental age, moisture content and temperature and storage time on seed survival (germination). **OBJECTIVE:** To investigate whether *A. graminifolia* seeds can be preserved using conventional seed banking and/or cryopreservation in relation to seed developmental age and lipid thermal fingerprinting. **MATERIALS AND METHODS:** Naturally pollinated and artificially pollinated capsules at three maturity stages were collected (i.e., six seed lots), equilibrated to 15%-75% relative humidity (RH), and stored at 4°C, -20°C and in liquid nitrogen (LN). Seed quality, including after 3 and 6 months of storage, were evaluated using asymbiotic germination. Seed water and lipid thermal properties were determined by differential scanning calorimetry. **RESULTS:** Seed maturity state was confirmed by the moisture content decrease of fresh seeds as well as the lipid transition enthalpy increase (from c. 10 to 20 J g^{-1} DW) and melting peak temperatures of dry seeds. Immature seeds (either as green capsules [GC] or 60 days after pollination [DAP60]) had some level of desiccation sensitivity (up to germination halving) and significant losses of germination during storage. Mature seeds (from capsules just before dehiscence [JBD], fully dehisced [FD], DAP90 and DAP120]) retained high germination levels under the vast majority of RH x Temperature x Time combinations. DSC analysis of the enthalpy of transitions during cooling and warming revealed that seeds equilibrated to ≤ 75% RH were below the unfrozen moisture content. **CONCLUSION:** Mature seeds of *A. graminifolia* seem to have orthodox seed storage behaviour, and both cold storage and cryopreservation appear feasible for the long-term ex situ conservation of this species.

Keywords: *Arundina graminifolia*; cryopreservation; differential scanning calorimetry; maturity; Orchidaceae; seed storage behaviour.

INTRODUCTION

With more than 29,000 species, Orchidaceae is among the most species-rich families of flowering plants (1). Most species of this family are economically important in horticulture and in the floral, pharmaceutical, and food industries (2, 3). However, due to land conversion and illegal harvesting, many species are under threat of extinction (4, 5). There are c. 1700 species of orchids in China. They have a broad diversity in morphology, growth form, life history, habitat and physiology, and are of great value in ornamental, medical, conservation, and evolutionary research (6).

Arundina graminifolia (D. Don) Hochr. is a terrestrial orchid belonging to the subfamily Epidendroideae. It is widely recognized as a traditional medicinal plant among the Dai people in China. The species is rich in stilbenoids, phenanthrenes, bibenzyls, and flavonoids, and is used for detoxification, anti-inflammatory purposes, and treating infections (7). Beyond its medicinal value, *A. graminifolia* is also of high ornamental appeal and has promising market potential. However, extensive harvest has led to a decline in wild populations, and this species is listed as a second-class nationally protected plant in China (8).

Various materials of orchid species, including pollen, protocorms, seeds and fungal symbionts, can be cryopreserved using techniques such as programmed freezing, encapsulation-dehydration, and vitrification (9). Several studies have been carried out to preserve *A. graminifolia* germplasm using different approaches. For example, pollen viability was reported to be totally lost after 60 h storage at 4°C, -7°C, or -25°C (10). Several techniques for cryopreservation of protocorms, including droplet vitrification, encapsulation-dehydration, and cryo-plate methods, have been compared, with the highest regrowth (77%) being obtained with the cryo-plate approach (11).

Seeds are considered the most practical form for preserving plant germplasm, as they conserve the full genetic information of a plant (12). Furthermore, the extremely small, micro-seeds of orchids make them ideal for compact storage in seed banks. There are few reports on seed storage of *A. bambusifolia* (synonym of *A. graminifolia*): after cryopreservation at 7.1% moisture content (MC) for 15 days, seeds retained 50% viability and 39% germination; the use of PVS2 and 1% phloroglucinol improved post-cryo viability and germination to 65% and 61% respectively (13). In contrast, > 80% seed germination was obtained after 1 day in liquid nitrogen (LN) using the D cryo-plate method involving bead formation, loading solution and dehydration (14).

Orchid seeds broadly fall into the "orthodox" category for desiccation tolerance (15), and cold storage and/or cryopreservation after drying could be effective conservation methods for *A. graminifolia* seeds. However, such storage research on *A. graminifolia* seeds is still lacking, including considerations of safe MC, or prestorage equilibrium RH, cold storage temperature and time.

Seed maturity also plays a critical role in storage success and longevity. As orthodox seeds mature, they acquire the ability to germinate and have greater tolerance to desiccation and cold storage. Often in orchids, immature seeds from indehiscent fruits germinate better than mature seeds from dehiscent fruits (16). However, seed germination after cryopreservation for *Dendrobium nobile* increased markedly with capsule maturity: 11% at 6 months after pollination (MAP) versus 81-94% at 7-10 MAP (17). In contrast, seed development in *A. graminfolia* is much faster, and the capsules smaller than *D. nobile*, with >80% mature seeds being able to germinate 90 days after pollination (DAP) (18). However, assessing seed maturity for *A. graminifolia* is challenging, as the species flowers and fruits almost all year round. Morphological characters such as capsule coloration and texture as well as seed MC and lipid content need to be considered to enable the evaluation of seed maturity.

Differential scanning calorimetry (DSC) has been widely used to study thermal behavior in plant materials, particularly phase transitions related to water and lipids during cooling and warming (19). DSC transitions observed in dry seeds have been attributed to lipids, mainly in the form of triacylglycerols (TAGs). Due to fatty acid compositional variation between species, substantially different thermal (solidification and melting) properties have been reported, e.g. in orchids (20, 21, 22), *Citrus* species (23, 24), Brassicaceae (25), *Cuphea* species (26, 27) and Australian tropical forest species (28). Coincidence between conventional seed bank storage temperature and the complex melting

transition of seed lipids was hypothesized to compromise long-term seed storage of the orchid *Cattleya aurantiaca* (synonym of *Guarianthe aurantiaca*) based on DSC thermograms and on the fact that storage just above the lipid melting temperature resulted in higher survival over c. 5 years (20). Similar observations of reduced lifespan of dry oilseeds during low temperature long-term (many years) cold storage (25, 28, 29), suggest the importance of generating thermal fingerprints for *A. graminifolia* seeds as part of a storage assessment.

This study aimed to determine if seeds of *A. graminifolia* were tolerant of drying to different relative humidities (RH) and subsequent storage under cool, cold and ultra-cold storage. We hypothesized that any cold storage stress would be associated with particular features of the lipid thermal fingerprint. We also evaluated storage performance of seeds at different maturity levels determined by morphological traits and days after pollination (DAP) to infer optimal timing for seed collection.

MATERIALS AND METHODS

Capsules of *A. graminifolia* were collected from Xishuangbanna Tropical Botanical Garden on 6th November, 2024. Naturally fruiting capsules were classified into three maturity grades based on morphological differences: 'green capsules' (GC), 'just before dehiscence' (JBD), and 'fully dehiscent' (FD). GC capsules had a relatively firm pericarp; JBD capsules had a green pericarp tinged with brown and a softer texture, indicating imminent splitting; FD capsules had split open with a distinctly brown pericarp (Fig. 1). As plants in the same collection were flowering at this time, artificial pollination was carried out on 5th November 2024 and capsules collected at specific DAP (i.e., 60, 90 and 120). All collected capsules were placed in envelopes and transported to the laboratory within 2 days.

Seed desiccation

Seeds were removed from capsules, and placed for 48 h to dry at 15 °C: to 15% RH (dry room conditions), 30% and 50% RH over LiCl solutions, and 75% RH over a saturated solution of NaCl. As the seeds are very small, the equilibrium conditions in the containers were confirmed using a Rotronic HC2-AW probe connected to a Hygrolab C1 unit (Rotronic Ltd., Crawley, UK).

Seed and pericarp moisture content

The moisture content (MC) of fresh pericarps, and of seeds before and after desiccation, was determined gravimetrically after drying at 103°C for 17 h (30). Four to 10 randomly selected replicates were used and MC was calculated on a fresh weight basis:

MC (%) = [(Fresh weight - Dry weight) / Fresh weight] × 100.

Differential scanning calorimetry (DSC)

Water and lipid transition temperatures in fresh seeds, and seeds that had been equilibrated to different RHs, were characterized using a differential scanning calorimeter (DSC2500 TA instruments, Delaware, US), calibrated using indium. Approximately 4 mg of seeds were placed in pre-weighed aluminum pans, non-

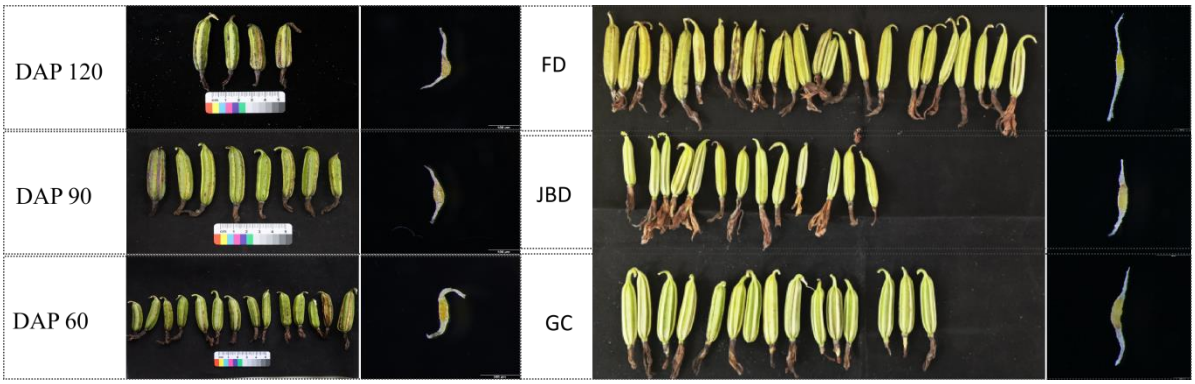

Figure 1. *Arundina graminifolia* capsules at different maturity stages: 60 days after pollination (DAP60); 90 days after pollination (DAP90); 120 days after pollination (DAP120); green capsules (GC); just before dehiscence (JBD); fully dehiscent (FD).

hermetically sealed with pre-weighed aluminum caps. Samples were equilibrated at 20°C for 5 min, cooled at 10°C min^{-1} to -80°C, held for 5 min, and then rewarmed at 10°C min^{-1} to 20°C. Single cooling and warming runs were made for each of the six developmental stages and four RHs, plus six fresh seed lots (i.e. 30 runs). The melting peak temperatures and enthalpies for lipid transitions were analyzed using the TA instruments TRIOS software, in relation to the scanning baseline.

Seed germination

Seeds were surface-disinfected by immersion in 0.5% sodium dichloroisocyanurate (NaDCC) with 1% Tween 20 for 3 min and repeated for three times, followed by rinsing five times in sterile distilled water. Seeds were aseptically sown on hormone-supplemented medium in 90 mm Petri dishes. All operations took place in a laminar flow cabinet. The germination medium consisted of half-strength Murashige and Skoog (MS) basal salts supplemented with 1.0 mg/L NAA, 2.0 mg/L 6-BA, 0.5 g/L Hyponex No.1, 30 g/L sucrose, 7 g/L agar, and 1 g/L activated charcoal, at pH 5.7-5.8 (18). Cultures were incubated at 25±2°C under a 12 h light/12 h dark photoperiod (light intensity 1800 lux).

Germination percentage was scored after 60 days and protocorm formation was regarded as germination (n=3 replicates with >50 full seeds assessed in each).

Seed storage and thawing

Desiccated seeds were transferred to cryovials and stored at 4°C, -20°C and in liquid nitrogen (LN). After 3 or 6 months of storage, seeds were retrieved for germination tests. Cryovials stored at 4°C and -20°C were equilibrated to room temperature. Cryovials preserved in LN were thawed in a 42°C water bath for 2 min, and the vial surfaces were dried before the seeds were retrieved for use.

Satistical analysis

Germination percentage (%) was determined as the number of protocorms formed from the total full seeds sown (x100).

Data were compiled using Microsoft Excel, ver 2010. Statistical significance was evaluated by a one-way analysis of variance (ANOVA) using R (31), followed by a post hoc pairwise comparison using the Student-Newman-Keuls (SNK) method (significance level $p < 0.05$). All percentage data were arcsine transformed to meet the requirement of normality distribution.

Table 1. Moisture content of fresh pericarp, fresh seeds and seeds after equilibration at different relative humidities (RH) for 48 h.

Developmental stage*	Moisture content (%)					
	Fresh pericarp	Fresh seeds	75% RH seed	50% RH seed	30% RH seed	15% RH seed
DAP60	72.39 ±1.33[a]	51.11 ±0.67[a]	11.10 ±0.21[a]	6.90 ±0.28[a]	5.71 ±0.09[a]	3.69 ±0.19[a]
DAP90	71.76 ±0.78[a]	42.43 ±0.23[b]	7.94 ±0.12[b]	5.42 ±0.08[b]	3.99 ±0.11[b]	2.95 ±0.08[b]
DAP120	67.63 ±2.71[a]	22.83 ±0.20[c]	9.31 ±0.09[c]	6.70 ±0.10[a]	4.45 ±0.03[c]	3.50 ±0.08[a]
GC	77.40 ±0.23[b]	56.23 ±1.25[d]	9.90 ±0.15[d]	5.99 ±0.07[bc]	5.20 ±0.12[d]	3.18 ±0.14[b]
JBD	71.16 ±1.48[a]	32.36 ±0.14[e]	8.50 ±0.15[e]	5.87 ±0.13[bc]	4.79 ±0.10[e]	2.52 ±0.10[c]
FD	68.70 ±0.98[a]	28.29 ±0.19[e]	8.55 ±0.14[e]	6.45 ±0.21[ac]	5.32 ±0.07[d]	4.21 ±0.23[d]

*DAP60, 90, 120 indicates days after pollination; GC, JBD and FD signifies green capsule, just before dehiscence and fully dehisced, respectively. Data in each column with differing lower case letters are significantly different ($p < 0.05$).

RESULTS

Seed development and thermal behaviour

The initial MC of fresh seeds of both collections decreased progressively with increasing maturity from > 50 % for DAP60 and GC to < 30% for DAP120 and FD (Table 1). In contrast, fresh pericarp MCs of each collection remained relatively stable (68-72 %) and were not significantly different as development progressed, except for the slightly wetter GC pericarps (Table 1). Therefore, fresh seed MC serves as a better maturity index than pericarp MC.

Initial germination levels of c. 80% were relatively consistent for DAP60, DAP90 and DAP120 seeds (Fig. 7). And initial germination of GC, JBD and FD seeds were all >85% (Fig. 8).

Drying reduced seed MC significantly, reaching 7.9-11.1% at 75% RH, 5.4-6.9% at 50% RH, 4.0-5.7 % at 30% RH and 2.5-4.2% at 15% RH, across developmental stages (Table 1). Generally, the MC of DAP60 seeds was higher than the other developmental stages irrespective of the RH, suggesting a lower level of reserve lipid accumulation.

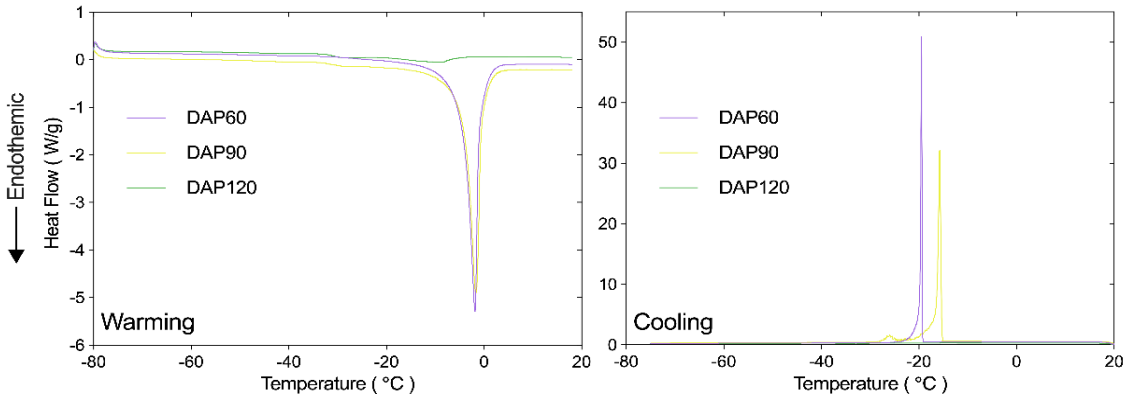

Figure 2. DSC warming (left) and cooling (right) thermograms for fresh seeds of *Arundina graminifolia* collected at 60, 90 and 120 d after pollination (DAP). The main transitions observed as sharp peaks relate to water.

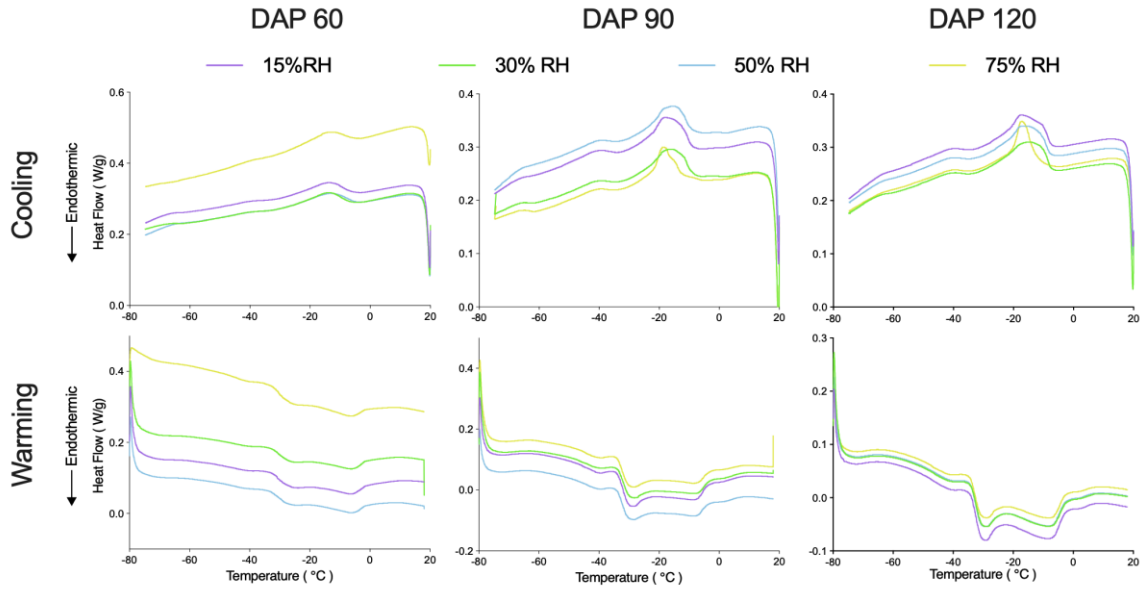

Figure 3. DSC warming (below) and cooling (above) thermograms for dried (15, 30, 50 and 75% RH) seeds of *Arundina graminifolia* at different maturity stages: DAP60, DAP90 and DAP120. The main transitions observed relate to a change in state of the embryo lipids.

Using DSC, water crystallization and melting peaks were observed in fresh seeds (at 23-56% MC) at all maturity stages; after seed equilibration to or below 75% RH, these peaks disappeared (Figs 2, 3, 4, 5). The free water crystallization/melting peaks for DAP120 (Fig. 2) and FD (Fig. 4) seeds were much less prominent compared to those of DAP60, DAP90 (Fig. 2), GC and JBD (Fig. 4) consistent with their lower MC.

In lower moisture contents seeds, i.e., after equilibration to 15 – 75% RH, DSC traces revealed additional peaks, during cooling and warming (Figs. 3, 5), attributed here to lipid phase transitions as small seeds of orchids tend to accumulate storage lipids. Broadly, the lipid

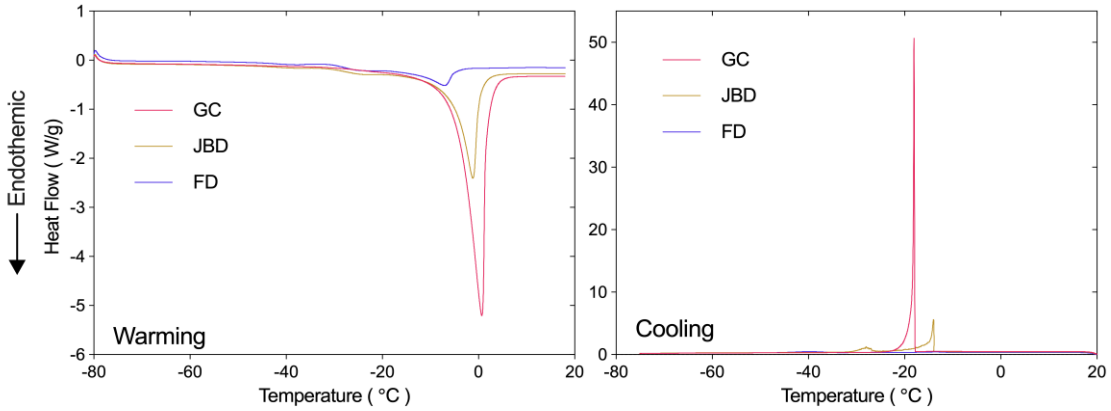

Figure 4. DSC warming (left) and cooling (right) thermograms for fresh seeds of *Arundina graminifolia* collected at green capsule (GC), just before dehiscence (JBD) and fully dehisced (FD) stages of development. The main transitions observed as sharp peaks relate to water.

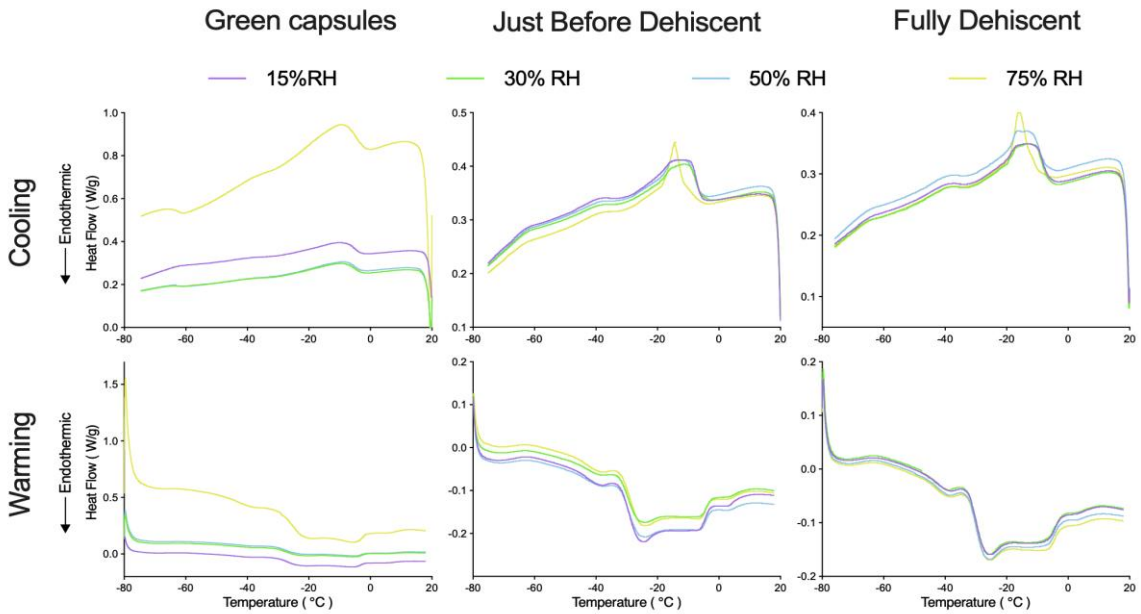

Figure 5. DSC warming (below) and cooling (above) thermograms for dried (15, 30, 50 and 75% RH) seeds of *Arundina graminifolia* at different maturity stages: green capsule (GC), just before dehiscence (JBD) and fully dehisced (FD). The main transitions observed relate to a change in state of the embryo lipids.

crystallization during cooling had a main peak centered around -18°C, with a smaller, flatter peak at c. -40°C; during warming, melting peaks coincided with temperatures of -30°C and -10°C, with the higher temperature peak being somewhat broader; a smaller shoulder peak was observed at 5-10°C (Figs. 3, 5). During cooling and warming the transition peaks were connected and not distinctly separated.

More detailed analysis of the DSC thermograms for dry seeds (15% RH) revealed generally higher crystallization and melting temperature peaks and onsets for the immature seeds (DAP60, GC) compared to more mature seeds (Table 2). The lipid transition enthalpy was also lower in immature seed: 4-5 and 10-11 J g^{-1} DW during cooling and warming, respectively, for the DAP60 and GC seeds compared to 7-9 and 16-21 J g^{-1} DW during cooling and warming, respectively, for the more mature seeds. Across all RH treatments, the enthalpy for lipid melting during warming was 9.2 ± 1.5 J g^{-1} DW for immature seed (DAP60, GC) versus 18.3 ± 1.7 J g^{-1} DW for mature seeds (DAP90, DAP120, JBD, FD), which were significantly different (p < 0.05).

The dependency of enthalpy on MC was plotted to enable a separation of water and lipid transition enthalpies during warming (Fig. 6). For seeds at all developmental stages, two lines could be fitted for MCs between 2.5 % and 11.1% (R^2 = 0.072, p = 0.204) and between 22.8 % and

Table 2. Thermal parameters measured by DSC for seeds of *Arundina graminifolia* at different stages of maturity and equilibrated to 15% RH at 15°C. ΔHmelt and ΔHcrys represent the lipid enthalpy of melting and crystallization respectively. T peak and T onset values are shown for the main lipid peaks during cooling and warming. Data is shown as a representative of the dry seed responses, i.e., including 30, 50 and 75% RH..

Seed or capsule development stage	Thermal parameters					
	During warming			During cooling		
	ΔHmelt （J g-1 dw）	T peak (°C)	T onset (°C)	ΔHcrys （J g-1 dw）	T peak (°C)	T onset (°C)
DAP60	9.53	-6.8	-33.8	4.30	-14.3	-6.6
DAP90	19.34	-28.9	-34.4	9.07	-18.3	-8.6
DAP120	15.67	-29.5	-34.7	8.65	-17.7	-7.4
GC	11.43	-20.8	-30.8	5.19	-10.2	-3.5
JBD	20.92	-24.9	-32.8	7.66	-13.6	-6.0
FD	16.08	-25.5	-32.8	6.67	-13.6	-6.5

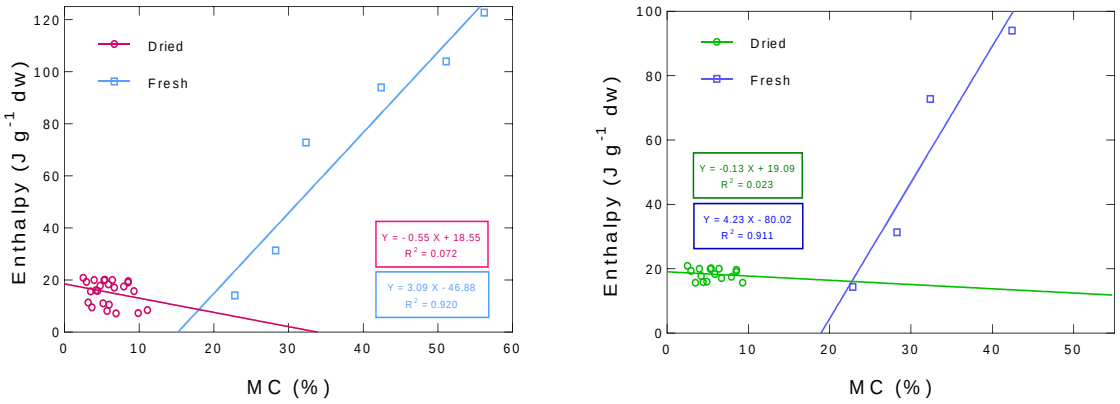

Figure 6. Effect of moisture content on the enthalpy of the transitions in seeds of *Arundina graminifolia* during warming. (Left) Data for all six developmental stages, including immature (DAP60, GC); (Right) data included only for seeds from mature capsules (DAP90, DAP120, JBD, FD). The lines drawn are the least squares best fit for the data. The intersection of the two lines is x = 17.98 and y = 8.66 (Left panel) and x = 22.73 and y = 16.14 (Right panel), indicating the unavailability of water for a melting transition below 18.0 % (all seeds) and 22.7% MC (mature seeds).

56.2 % MC (R^2 = 0.92, p = 0.0025). The intersection of the two lines was calculated as x =17.98 and y = 8.66. The same analysis for seeds of mature capsules only (DAP90, DAP120, JBD, FD) yielded lines of R^2 = 0.023, p = 0.5731 for MCs of 2.5% to 9.3% and R^2 = 0.911, p = 0.0453 for MCs of 22.8% to 42.4%; with an intersect of x = 22.73 and y = 16.14 (Fig. 6). Comparable plots and analyses for the seed during cooling yielded intersects of x = 20.53 and y = 4.41 and x = 22.61 and y = 5.69 for all seeds and mature seeds only, respectively (plots not shown).

Seed cryopreservation and cold storage

The first step in the cold storage of seeds is predrying to moisture levels low enough to avoid intracellular ice formation on cooling and warming.

Seeds of each developmental stage were equilibrated to 15-75% RH. Relatively immature seeds from DAP60 capsules had lower germination on drying, and significantly so after the 15% RH treatment (Fig. 7). Drying of DAP90 and DAP120 seeds did not result in lower germination (Fig. 7). On subsequent storage, DAP60 seed germination fell significantly in 22 of 24 combinations of RH X Temperature X Time. The lowest survival was c. 10% (30% RH and

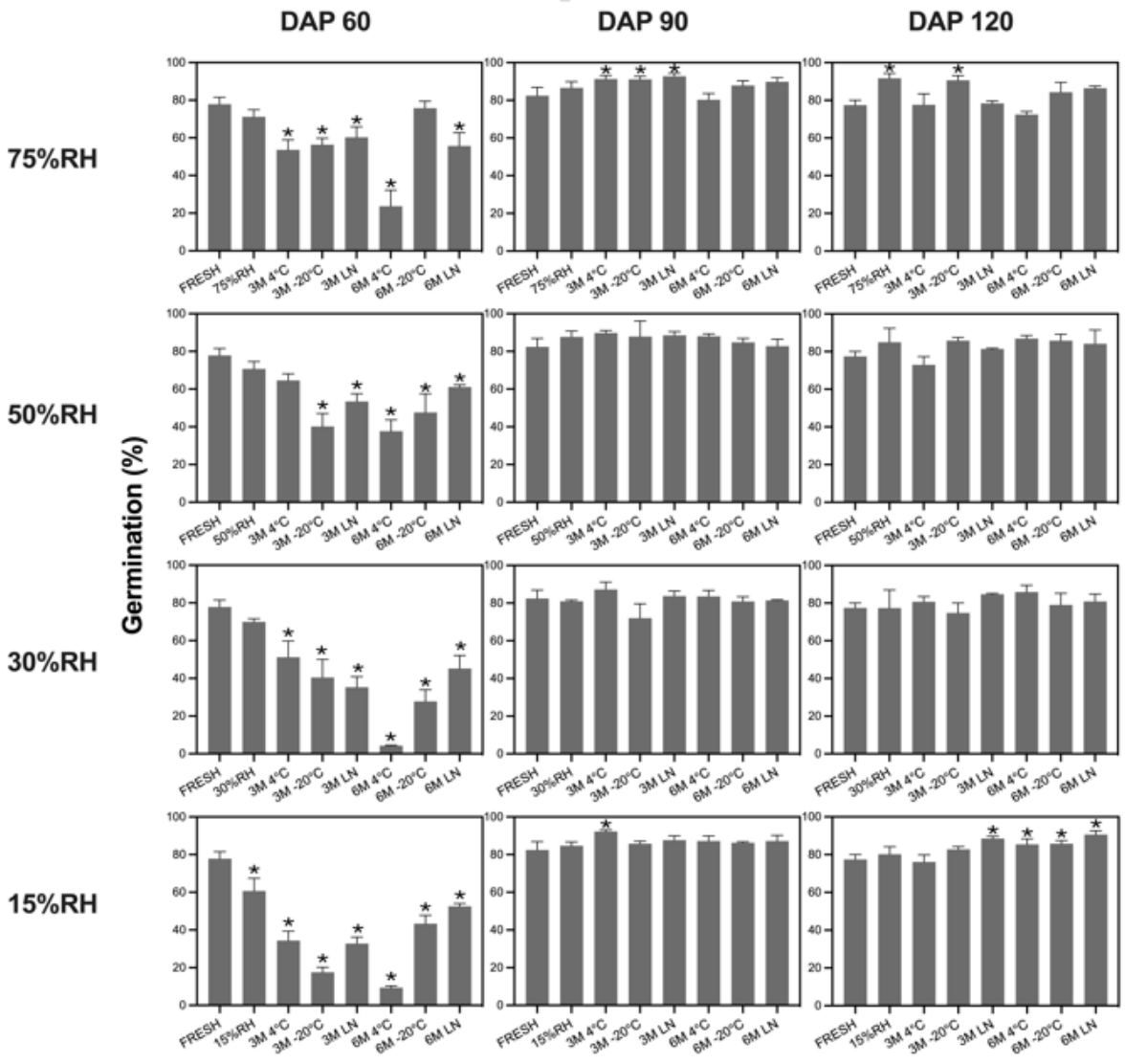

Figure 7. Germination percentage of fresh *Arundina graminifolia* seeds collected at 60, 90 and 120 days after pollination (DAP) and seeds after storage at 4°C, -20°C and in liquid nitrogen (LN) following equilibration to different relative humidities (RHs). Bars show means and error bars show standard error; asterisks indicate significant difference (p < 0.05) compared with initial germination. N=3.

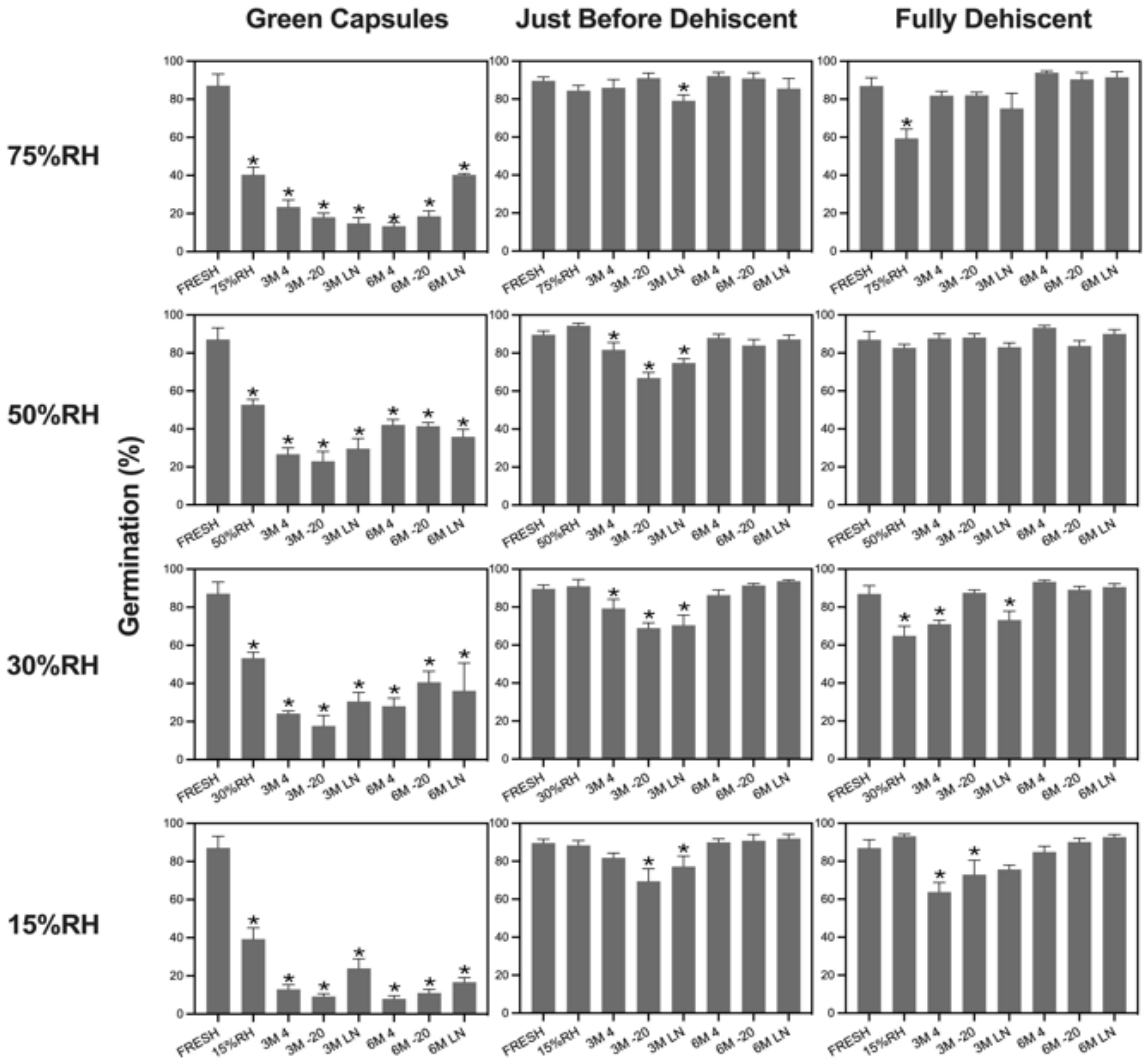

Figure 8. Germination percentage of fresh *Arundina graminifolia* seeds at different morphological maturity stages (green capsules, just before dehiscence and fully dehiscent) and seeds after storage at 4°C, -20°C and in liquid nitrogen (LN) following equilibration to different relative humidities (RH). Bars show means and error bars show standard error; asterisks indicate significant difference ($p < 0.05$) compared with initial germination. N=3.

15% RH, 6 months at 4°C), whilst 6 months LN stored seeds generally retained around 50% germination, compared to c. 70% germination post-drying (Fig. 7). In contrast, DAP90 and DAP120 seed germination was around 80% after all 24 combinations of storage treatments (RH x Temperature x Time) for each DAP, and around the same as that of pre-dried (fresh) seeds (Fig. 7). The exception to this broad pattern was that about one fifth of the storage treatments (4 or 5) for DAP90 and DAP120 had significantly increased germination, which was also the case for DAP120 seed after drying to 75% RH (Figure 7).

GC seeds were highly sensitive to drying, with germination reduced by about half (from c. 85% to c. 40 – 50%) after equilibration to 15-75% RH (Fig. 8). For JBD seeds, the effect of drying was insignificant, whilst two treatments (75% and 30% RH) had slightly, but significantly, reduced germination in FD seeds (to c. 60%). On subsequent storage, GC seeds often had further falls in germination level (c.f. the drying treatment) to around 10-40%, with the poorest performance evident in 15% RH seed (Fig. 8). For JBD and FD seeds, c. 60% and c. 80%, respectively, of the 24 storage combinations (RH x Temperature x Time) applied to each retained

germination levels c. 85%, i.e., round that of the fresh (control) seeds (Fig. 8). For the nine treatments in which significant falls in germination were observed, all were associated with the 3-month storage assessment, for reasons that remain unclear (Fig. 8). But the seeds held for 6 months at 4°C, -20°C and LN germinated to >80%, and were not significantly different to that recorded for fresh (control) seed.

DISCUSSION

Seed development and thermal behaviour

The assignment of seed maturity to orchid seeds is complicated by the very small seeds precluding the standard determination of average seed mass, particularly as thousands of seeds would be used for a single determination. Therefore, seed developmental age was inferred from the quality (colour and firmness) of the capsules, the DAP and the response of the seeds to drying. On this basis, GC was the most immature as the capsules were green and hard, and the seeds sensitive to all drying environments (15-75% RH). Nonetheless, some seeds from GC must have acquired a level of desiccation tolerance as not all viability was lost on drying (Fig. 8). DAP60 seeds were evidently more mature than GC as the seeds had a lower MC (51 vs 56%; Table 1), and higher level of desiccation tolerance (Fig. 7) and the capsules were slightly brown and remained in good shape when gently squeezed.

Based on fresh seed MC, seeds of JBD (32% MC) and FD capsules (28% MC) were assumed to have reached a developmental stage between that of DAP90 (42% MC) and DAP120 (23% MC). This interpretation is not supported fully by the response to drying, as FD seeds had reduced germination by about 20% on two occasions whilst DAP90 and DAP120 seeds did not (Figs 7, 8).

Further evidence that GC and DAP60 seeds were the more immature of the six developmental stages relates to their thermal fingerprints (Figs 4, 5, 6, Table 2). As seeds mature, reserve materials accumulate, which in orchids includes lipids (20). Thus, we could expect that dry seeds of the GC and DAP60 capsules would have a smaller transition enthalpy in the DSC. This proved to be the case for 15% RH seeds (Table 2) and across all RH treatments. For example, the mature seed (DAP90, DAP120, JBD, FD) enthalphy during warming was 18.3 J g^{-1} DW, and twice that of the immature seed (DAP60, GC). This difference is evident from the enthalpy plots in Figure 6, such that the DAP60 and GC data in the 'all seeds' plot sit below the fitted line at low moisture.

In this study, the lipid enthalpy values in dry seeds at the DAP60 and GC stages appear to indicate a relatively low level of lipid accumulation. Compared with mature seeds of other orchids such as those of the genus *Lycaste* (enthalpy range: 14–32 J g^{-1} DW) (32), the low-lipid species *Cattleya granulosa* (enthalpy: ca. 2 - 10 J g^{-1} DW) (33) and Mediterranean terrestrial orchids (lipid content: 3 - 27%; enthalpy: 2 – 24 J g^{-1} DW) (34), the enthalpy values of the DAP60 and GC seeds fall at the lower end of these ranges, possibly reflecting incomplete lipid accumulation during early seed development. Similar development program-driven changes have been reported in other plant species. For example, enthalpy increases from c. 2 to 28 J g^{-1} DW as citrus seeds mature (24) and from 42 to 53 J g^{-1} DW in avocado as seeds mature and coincidental with an increase in oil content (35). Whilst an increase in lipid transition enthalpy may correlate with lipid content in dry seeds, such a correlation between melting enthalpy and lipid content for different species is relatively poor (28).

The DSC data for dry seed in Table 2 and Figs. 3 and 5 also showed that the less mature seed (DAP60, GC) tended to have higher transition temperatures by about 3°C and more equally sized melting peaks (centered around c. -6 and -26°C). In contrast, the lower temperature peak was dominant in more mature seed (DAP90, DAP120, JBD, FD). These findings imply that there is an increase in fatty acid unsaturation late in development, consistent with the general findings in plant cells that saturated FAs are first synthesized and then enter the unsaturation program administered by a series of FA desaturases in most plants (36).

Seed cryopreservation and cold storage

Although immature orchid seeds are usually preferred for propagation (37), they may not be fully tolerant of drying. In contrast, mature seeds of *A. graminifolia* have a higher level of tolerance of drying. The acquisition of this seed trait is important for their subsequent storage at cold temperature. In the case of *A. graminifolia*, fresh seeds had initial MCs (23-56%, Table 1) at or

above the estimated unfrozen water content of c. 18-23 % (Fig. 6), with the likely consequence of damaging ice formation if the seeds had been exposed to subzero temperature. The lack of additional drying *ex planta* may provide an explanation for the fall in viability (79 to 50%) of *A. graminifolia* seed from 'matured capsules' when cryopreserved for 15 d, whilst 65% viability and 61% germination was achieved when cryoprotectant treatment was used (13). Other examples of cryoprotection facilitating orchid seed cryopreservation include the D cryo-plate technique, involving a loading solution, for DAP90 seeds of *A. graminifolia* (82% regrowth after 1 d cryopreservation) (14), and 40 min PVS2 treatment to ensure the successful cryopreservation (82% germination vs. 75% for the control) of fresh seeds (46% MC) of *Coelogyne nitida* from green capsules (38).

Seed unfrozen moisture / water content has negative linear relations with seed oil content (23, 39) and a value of c. 20% unfrozen MC (or high moisture freezing limit) estimated for *A.graminifolia* (Fig. 6) would be expected of seeds with a c. 15 % oil content. Slightly lower estimates for unfrozen water contents of 14.5-16.7 % MC (0.17 and 0.20 g H_2O.g DW) have been made for seeds of three Australian species, *Caladenia flava, Microtis media* and *Pterostylis recurva* (21, 22). However, the relations between MC and RH over 15-75% when averaged across the four more mature developmental stages (DAP90, DAP120, JBD, FD) indicates that *A.graminifolia* seed isotherm is similar to that of *Dendrobium anosmum* (15) and *Cattleya aurantiaca* (synonym of *Guarianthe aurantiaca*), the latter having an oil content of 29% (20). Even higher oil contents of 28-51% have been estimated across eight *Cattleya* orchid species (33). Future studies should determine the precise oil content and fatty acid composition in *Arundina graminifolia* seeds using methods specifically designed for orchid seed (40).

Across species, with varying seed oil content, the safe MC threshold for cryopreservation is equivalent to c. 75-85% RH (23, 38, 41), hence our choice of four precooling RHs (15, 30, 50, 75%) for the cryopreservation and conventional seed bank storage studies undertraken here. We found that *A. graminifolia* seeds are orthodox in their response to drying, unless the seed is immature (DAP60, GC). Some desiccation sensitivity was noted in more than half of the RH treatments for these seeds, followed by a further fall in viability during 3 months' storage at 4°C, -20°C and LN (Figs. 7, 8). However, low temperature survival at 6 months was not necessarily any worse than after 3 months. Similarly, dry seeds (5-92% RH) of some Australian orchids had significant reductions in germination after 3 months of storage at -18°C, -80°C and -196°C but little further reduction when storage was extended to 2 years (21).

Numerous, sometimes contradictory, studies have made it difficult to agree optimal storage conditions for orchid seeds (15, 20, 21, 22). Thus, some orchid seeds are considered short lived under dry and cold storage, e.g., seeds of four *Coelogyne* species exhibited very poor storability under standard seed bank conditions (-20°C, desiccated), with viability loss exceeding 90% within 9-12 months (42). However, we found that dry, mature seeds of *A. graminifolia* retained >80% germination after 6 months of cold and ultracold storage (Figs. 7, 8). Thus, it is possible that seeds of this species could be conserved long-term in conventional seed banks and / or in cryo-banks. Conventional seed bank storage may also be suitable for dry (c. 23% RH) seeds of some Australian orchids (21, 22). Moreover, seeds of eight *Cattleya* species have been successfully stored for over 10 years under conventional seed bank conditions (-18°C with 3-4% MC), with a predicted lifespan (P_{50}) of c. 30 years or more (33).

A contributory factor in reduced longevity under dry, cold conditions, can be the relatively low initial viability. For example, across three Brazilian *Cattleya* species, *C. amethystoglossa* (88% initial germination) was less tolerant to storage at 1.5% MC and 5°C over 270 days and to more conventional storage at 6% MC and -18°C (43). In comparison *C. kautskyana* and *C. tigrina* seeds (98-99% initial germination) had better longevity over a range of MC and temperature conditions. Although storage was for a shorter time period of 6 months, mature dry seed of *A. graminifolia* with 80-90% germination after drying also survived storage under a wide range of storage conditions. This suggests that the seed of this species is not particularly short-lived.

Another potential contributory factor to reduced longevity in oily seeds is the state of the lipids during longer-term (years) storage (20, 22, 25, 28, 29). This depends on lipid composition and thermal behavior (particularly at melting

temperatures), which exhibit significant species-specific variation, e.g. in orchids (22, 32, 33), brassicas (25), and Australian rainforest species (24, 28). We showed that *A. graminifolia* seed has lipid melting transitions during warming centered on c. -25°C and c. -8°C (with a smaller shoulder event around 5-10°C) (Figs. 3, 5). This pattern of transitions is similar to that observed in some other orchids: *Caladenia flava, Microtis media,* and *Pterostylis recurva* (21) and seven *Cattleya* species (33. The shapes of the endothermic melting in dry seeds may indicate multiple phases of melting. Thus, a sharp melting peak followed by crystallisation that causes the signal to fall below the scanning baseline, followed by another melting event may indicate conversion of lower-density, higher-energy α crystals to higher-density, lower-energy β′ (and β form) via crystallisation during warming (19, 28). However, the DSC melting traces of *A. graminifolia* presented in Figures 3 and 5 indicate two main peaks, nearly equal in extent and without an obvious crystallisation event between peaks. This may simply reflect the melting of different lipid moieties with varying thermal properties, and a full fatty acid analysis of *A. graminifolia* seeds could be illuminating. It is also the case that characteristing crystallisation events during warming can be a function of prior cooling rates and time. Thus, it is possible if *A. graminifolia* seeds had been stored for many years, rather than 6 months, evidence of increased lipid crystallisation may have been observable. It could be that the large (two-fold) difference between the enthalphy for crystallisation during cooling and melting during warming (average 8.1 ± 0.8 J g^{-1} DW, and 18.3 ± 1.7 J g^{-1} DW) in mature seed is a harbinger of future events in the same way that fern spores after many years cold, dry storage have larger lipid enthalpy, indicative of increased crystallisation over time (44) melting. A similar differential between cooling and melting enthalpies in Australian rainforest species has been suggested to be an indication of slow, resistance to crystallisation of seed lipids that could contribute to differential seed longevity in a conventional seed bank (28), which also appears to be a function of higher lipid melting temperatures too (20, 25, 26, 27).

In summary, the developmental age of seeds of *A. graminifolia* can be inferred from the fresh seed MC, the capsule colour and firmness, and the enthalpy of the dry seed lipid signal. Seeds from mature capsules tolerate desiccation to 75-15% RH and subsequent 6 months storage at cool, cold and ultracold temperatures, indicating their potential for *ex situ* conservation. Further studies on orchid seed longevity should focus on the interaction between seed MC, lipid content, lipid composition and lipid physical state over time with the aim of devising low temperature species-specific storage conditions for oil seeds (45).

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