Seed dormancy concepts in orchids: *Dendrobium cruentum* as a model species

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Abstract

Generally, orchids produce dust-like seeds in which endosperm reduction and embryo undifferentiation represent a derived state shared with species in about 11 other plant families. Orchid seeds are proposed to have a special kind of morphological or morphophysiological dormancy. We test this proposition, overcoming several design limitations of earlier studies, specifically that the *in vitro* germination method for orchid seeds uses pro-oxidants for disinfection and incorporates nitrate in the medium; both 'treatments' might contribute to dormancy breaking, potentially confounding judgement on the depth and nature of the dormant state. Seeds of the tropical orchid *Dendrobium cruentum* Rchb. f., were sown both *in vitro*, on a nutrient medium, and *ex vitro*, on plain agar omitting prior disinfection with sodium hypochlorite. Seeds previously stored and fresh seeds were incubated under combinations of vitro conditions, light treatments, constant or alternating temperatures and nitrate concentration. Seeds of *D. cruentum* are very small but have a large embryo that occupies most of the seed. Over a range of constant temperature seeds germinated to the spherical protocorm stage just as well *ex vitro* as *in vitro*. Neither light nor nitrate were prerequisites for *ex vitro* germination. The ability of *D. cruentum* seed to germinate in the absence of environmental or chemical stimuli suggests that mature seed can be non-dormant. Our results support the proposition that neither all DUST seed fit a dormancy class nor all orchids produce morphological or morphophysiological seeds. Finally, embryo/seed volume determinations in orchids may prove as valuable in studies on the evolution and ecology of germination and dormancy as embryo:seed ratios in other angiosperm species.

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Introduction

Dust-like seeds represent a derived state that has evolved separately in at least 12 families, including Burmanniaceae, Gesneriaceae Orobanchaceae and Orchidaceae (Eriksson and Kainulainen, 2011). Purportedly, a reverse evolutionary trajectory resulted in dwarf seeds of these families undergoing endosperm reduction and the production of an undifferentiated embryo. One ecological consequence of these physical changes to orchid seeds is a trade-off between plant competition and colonization. Miniaturization of the seed increased hugely the potential seed yield per plant. For example, about 2000 seeds per capsule in the temperate species *Dactylorrhiza fuchsia* (Druce) Soó (Marks et al., 2014) and about 4 million seeds in capsules of the tropical epiphyte *Cycnoches ventricosum* Bateman (Arditti and Ghani, 2000). Such seed yields are only possible as seed weights are reduced to micrograms; for example, 1.9 and 3.6 µg for *D. fuchsii* and *C. ventricosum*, respectively (Arditti and Ghani, 2000; Marks et al., 2014). In addition to being flimsy, a lack of endosperm has resulted in the seeds of many species having an air space between the embryo and the testa. There is considerable inter-species variation in this balloon-like space as a proportion of seed volume, for example, 92% air space in *Epipactis palustris* (L.) Crantz (Arditti and Ghani, 2000), and 13–36% (Prasongsom et al., 2017) and 32–92% (Diantina et al., 2020a) among a range of *Dendrobium* species. Geographical differences (tropical *vs* temperate) and life traits (epiphyte *vs* terrestrial) are critical factors affecting seed traits (Arditti and Ghani, 2000; Diantina et al., 2020b). The lighter (less mass) and more buoyant (more air space) the seed is the greater the potential dispersal distance from metres to kilometres. Thus, miniaturization of the seed affects the likelihood of the progeny reaching favourable environments for germination and thus colonization (Mitra, 1971; Arditti and Ghani, 2000; Hashimoto et al., 2012).

Orchid seeds with a very small embryo and no endosperm have a limited internal energy supply. Consequently, in the natural environment, the seed embryo may imbibe but fail to develop further unless an exogenous supply of carbohydrate is provided or mycorrhizal association occurs (Smith and Read, 1997; Chugh et al., 2009). In the laboratory, orchid seed germination is possible *in vitro* on a medium that varies in complexity depending on the species but generally includes macro- and micro-elements and a source of carbon (Arditti et al., 1982;

Deb and Pongener, 2011). Norstog medium, with amino acids, may be effective with epiphytic species, while Knudson C, with activated charcoal, can support the germination of tropical terrestrial species (Nadarajan et al., 2011). Alternatively, the seed can be sown on a simple oat-yeast medium in the presence of a symbiotic fungus (Muir, 1989), such symbiosis reflecting so-called mycovitalism in nature (Vujanovic and Vujanovic, 2007). These means of germinating orchid seeds are relatively complicated compared to the sowing of seeds of other species on an inert, moistened substrate (e.g. filter paper, sand, agar) to which stimulants can be added, if appropriate (Baskin and Baskin, 2014).

An understanding of the type of stimulant required for seed germination (e.g. nitrate, gibberellic acid), the combinations of temperatures needed (warm, cold, warm followed by cold, etc.) and insights on seed morphology (especially embryo size and form) have all contributed to the development of a classification system for seed dormancy that has been applied to thousands of species (Baskin and Baskin, 2004, 2021). This system identifies key classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD). Because of their dust-like form, undifferentiated embryo and other features, orchids are proposed to have a special kind of MD or MPD (Baskin and Baskin, 2004, 2014), and most recently, the 'dust seeds' of mycoheterotrophs, including orchids, have been placed within the seven subclasses of MD (Baskin and Baskin, 2021). Of the relatively few orchid species studied for seed dormancy and germination out of the >25,000 species in the family, the most detailed studies have been undertaken with temperate species. One of the main pieces of evidence for seed dormancy in orchids is less germination in mature compared with immature seeds (Rasmussen, 1995). For example, *Phaius tankervilleae* (Banks) Blume seed maturation was accompanied by a decrease in water content and a concomitant increase in ABA content and PtNCED1 mRNA level along with a marked decrease in germination percentage (Lee et al., 2018). Other evidences for orchid seed dormancy include a delay of several months before embryo growth occurs; restriction of growth by the testa; a requirement for cold stratification or after-ripening and responsiveness to gibberellic acid (Baskin and Baskin, 2014). While physical dormancy is not a general feature of orchid seeds (Baskin and Baskin, 2014), ultra-low temperature treatment of dry seeds can improve germination in *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase (Pritchard, 1984) and laccase enzyme scarification facilitates lignin degradation, water uptake and germination in the same species (Pierce et al., 2018). Surface disinfection with bleach can also partially scarify orchid seeds (Magrini and De Vitis, 2017; Pierce et al., 2018) and facilitate the permeation of the vital stain triphenyl tetrazolium chloride (Custódio et al., 2016).

Disinfection with bleach is applied as a standard, and necessary, treatment for orchid seeds from mature capsules prior to sowing on *in vitro* medium, primarily as a means of ensuring clean cultures. However, the application of pro-oxidants to seeds may also affect a physiological block to germination. For example, germination of tea seeds increased with H_2O_2 treatment (Chen et al., 2012) and exposure of mature seeds of the orchid *P. tankervilleae* to NaOCl solution lowers ABA content and improves seed germination (Lee et al., 2018). In addition, nutrient media for orchid seed germination incorporate relatively high levels of nitrate, for example, ammonium nitrate and potassium nitrate in Murashige and Skoog medium, although rarely have the potential dose effects of nitrate on orchid seed germination

been explored (Ponert et al., 2013). This is important, as nitrate is known to facilitate both endosperm and testa rupture in *Sisymbrium officinale* (L.) Scop. seeds (Toorop, 2015), and to act as a signal, in a wide range of species, to stimulate seed germination, particularly in association with light and alternating temperatures (Probert et al., 1987; Baskin and Baskin, 2014; Toorop, 2015; Duermeyer et al., 2018). On occasions, *Dendrobium* species seed has been successfully germinated on nutrient-poor medium containing oat meal agar (OMA), usually in the presence of potato dextrose agar (PDA), following disinfection of the capsule or seed directly and conditions of light (16 h d^{-1}) following dark (Swangmaneecharern et al., 2012; Mala et al., 2017). As seeds of terrestrial and epiphytic species tend to prefer dark and light conditions, respectively, for germination (Rasmussen, 1995), determining the nature of seed dormancy in orchids requires an assessment of the effects of a range of factors (including disinfection, nitrate, and light and temperature alternation) on the germination response.

Both the evolution of seed dormancy (Nikolaeva, 2004; Willis et al., 2014) and seed embryos (Finch-Savage and Leubner-Metzger, 2006) have received considerable attention, indicating key evolutional transitions between dormancy classes, including the class 'DUST' seeds. DUST seeds may have arisen from MPD seeds but not from MD seeds, and the non-dormant (ND) state primarily evolved from PD but rarely from MPD (Willis et al., 2014). The evolution of PD was also associated with increased speciation rates. Thus, physiologically regulated environmental cueing of dormancy appears to have influenced major evolutionary patterns in the seed plants (Willis et al., 2014).

The genus *Dendrobium* in the Orchidaceae has undergone one of the greatest levels of speciation in the plant kingdom. Around 800–1500 species, which are mainly epiphytic (occasionally lithophytic), have considerable morphological diversity and occur across a wide range of microenvironments (Xiang et al., 2013). Thus, this genus could be an excellent model in which to consider the evolution of dormancy in orchids (Prasongsom et al., 2017). Seeds of some *Dendrobium* species can germinate *ex vitro* on plain agar-water in the absence of soluble sugars, with the enlarging embryo turning green, as in several other tropical orchids, forming a spherical protocorm and breaking through the testa. Thus, spherical protocorm development *ex vitro*, the first stage of germination in orchids, has been reported for nine species of the genus: *D. chrysotoxum*, *D. draconis*, *D. fimbriatum*, *D. findlayanum*, *D. hercoglossum*, *D. lituiflorum*, *D. parishii*, *D. pulchellum* and *D. schildhaueri* (synonym of *D. kontumense*) (Prasongsom et al., 2017). In these species, the air space is relatively small from 13 to 35% of seed volume, suggesting that variation in embryo size might contribute to germination performance in DUST seeds of orchids. However, micromorphometric studies on orchid seeds are often limited to the external dimensions and appearance of the seeds in relation to taxonomy. In contrast, far less attention has been given to seed micromorphometry and germination in orchids (Arditti and Ghani, 2000).

The objective of this study is to explore the possible presence and type of dormancy in *Dendrobium cruentum* Rchb. f. seeds. This epiphytic orchid originates from southwest Thailand, growing on small trees in open forests at low elevations, and flowers all year (Vaddhanaphuti, 2005). It is facing extinction due to climate change and loss of natural habitats and is listed in Appendix I of CITES. We assessed the effects of various factors (disinfection, nitrate, light/dark, constant and alternating temperatures) on

the dormancy release or germination response and characterized embryo growth both *in vitro* and *ex vitro*. Finally, we consider for fresh and stored seed whether the DUST seeds of some orchids can be non-dormant.

Materials and methods

Plant and seed materials

Wild plants of *D. cruentum* were collected from their natural environments and then cultivated in a greenhouse (Fig. 1A) at the Institute of Science and Technology for Research and Development, Salaya Campus, Mahidol University, Thailand. Flowers (Fig. 1B) were hand pollinated and mature, just dehiscing capsules harvested (Fig. 1C). Seeds were extracted from single capsules (containing many thousands of seeds) maturing in two different years. Seed lot 1 was held in the laboratory briefly (approximately 80% relative humidity, RH) until dispatch to the UK; thereafter, the seeds were dried for about 3 weeks in a room operating at 15% RH and 15°C and then stored in vials at −20°C for 7 months until experimentation. Seed lot 2 was not subjected to artificial drying, with the freshly isolated seeds committed to experimentation soon after extraction. The collected capsule was cleaned under running tap water followed by dipping briefly in 95% alcohol. The seeds were removed from the capsule and used immediately in the experimentation to reduce any likelihood of after-ripening. During this handling procedure, the seeds may have equilibrated to ambient conditions (approximately 80% RH).

An initial seed viability of the seed lots was assessed using a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate buffer (Custódio et al., 2016). Seeds were added to 20 ml of TTC solution in a screw cap tube and kept in the dark at 30°C for 24 h. While viable embryos appeared to turn yellow-red from yellowish, as judged under a light microscope (Fig. 1D), the colour change was not always strong.

Micromorphology analyses

The seeds were placed on stubs using double adhesive tape and coated with gold palladium alloy for 5 min. Thereafter, the material was examined in a Scanning Electron Microscope (EDS model 6610LV), and the macromorphology of the seed coat (testa cell) surface was photographed.

The micromorphology of the seed was examined at the start of, and during, germination (i.e. emergence of the spherical protocorm through the testa). For each determination, 30 seeds and embryos were measured under a light microscope using AxioVision software (Carl Zeiss: AxioCam). The descriptors included seed and embryo length and width, seed shape and colour, and embryo/protocorm colour. The percentage air space in ungerminated seed was calculated from determinations of the seed and embryo volumes using the following two equations (adapted from Arditti et al. (1980)):

$$\text{Embryo volume,} \quad EV = 4/3 * 22/7 * (EL/2) * (EW/2)^2, \quad (1)$$

where EL is embryo length and EW is embryo width.

$$\text{Seed volume,} \quad SV = 2 * ((SW/2)^2 * (SL/2) * (1.047)), \quad (2)$$

where SL is seed length and SW is seed width and $1.047 = \pi/3$.

Seed sowing

For *ex vitro* germination, fresh (seed lot 2) and dry-stored seeds (lot 1) were sown without disinfection on non-sterile agar-water. For *in vitro* (sterile culture) germination, fresh and dry-stored seeds were placed in filter paper packets and disinfected for 20 min with 1% sodium hypochlorite solution, followed by washing four times in sterile distilled water for 5 min each. Disinfected seeds were transferred to sterile, half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in Petri dishes. Both *ex vitro* and *in vitro* media contained 1% agar (w/v).

Light and temperature treatments

The effect of constant temperature in the light (12 h d^{-1} light; white fluorescent tubes at approximately 15–20 µmol m^{-2} s^{-1}) on seed germination was assessed in eight environmental chambers from 5 to 40°C at 5°C intervals. Fresh and dry-stored seeds were sown *ex vitro* and *in vitro*, as described above.

Fresh and dry-stored seeds were also sown *ex vitro* and germinated at constant 25°C and alternating 30/20°C under different light conditions. Seeds were incubated in the light (12 h photoperiod; applied during the warm temperature phase for the alternating treatment), in dark (aluminium foil wrapped, but opened weekly, and briefly, for germination scoring) and double dark (only unwrapped at the end of the test).

In all instances, three replicates of 100–150 full seeds were used per treatment and germination was assessed, as spherical protocorm production, after 28 d.

Nitrate and light treatments

Fresh and dry-stored seeds were sown *ex vitro* on plain agar with different concentrations (0, 1, 3, 10 and 30 mmol) of the dormancy-breaking chemical KNO$_3$. Seeds were incubated in the light and dark, as described above, at 25°C, the optimum temperature for germination. Three replicates of 100–150 full seeds per nitrate concentration were assessed for germination (spherical protocorm formation) after 28 d.

Statistical analysis

Changes in mean embryo volume ($n = 30$ seeds) during the germination process were subjected to a *t*-test ($P \leq 0.05$). For the germination experiments, data were arc-sine transformed to normalize the distribution and subjected to ANOVA and all pairs of means compared using Duncan's Multiple Range Test (SPSS Statistic 21 software).

Results

D. cruentum plants produced capsules around 2 cm long (Fig. 1). The seeds were fusiform in shape with a relatively large central embryo (Fig. 1). The micropylar and the chalazal cells of the testa appeared shorter than the medial cells, and the raised testa cell walls had frilled edges (Fig. 2), described by Chaudhary et al. (2014), as 'cottony-white substance'. The seeds were extremely small, measuring around 0.285 mm long and 0.060 mm wide ($n = 30$), with some variations in dimensions between seed lots. In particular, fresh seeds (lot 2) were slightly, but significantly ($P < 0.05$) narrower, and had estimated seed and embryo volumes about 15% less than dry-stored seeds (lot 1) (Table 1). The embryo
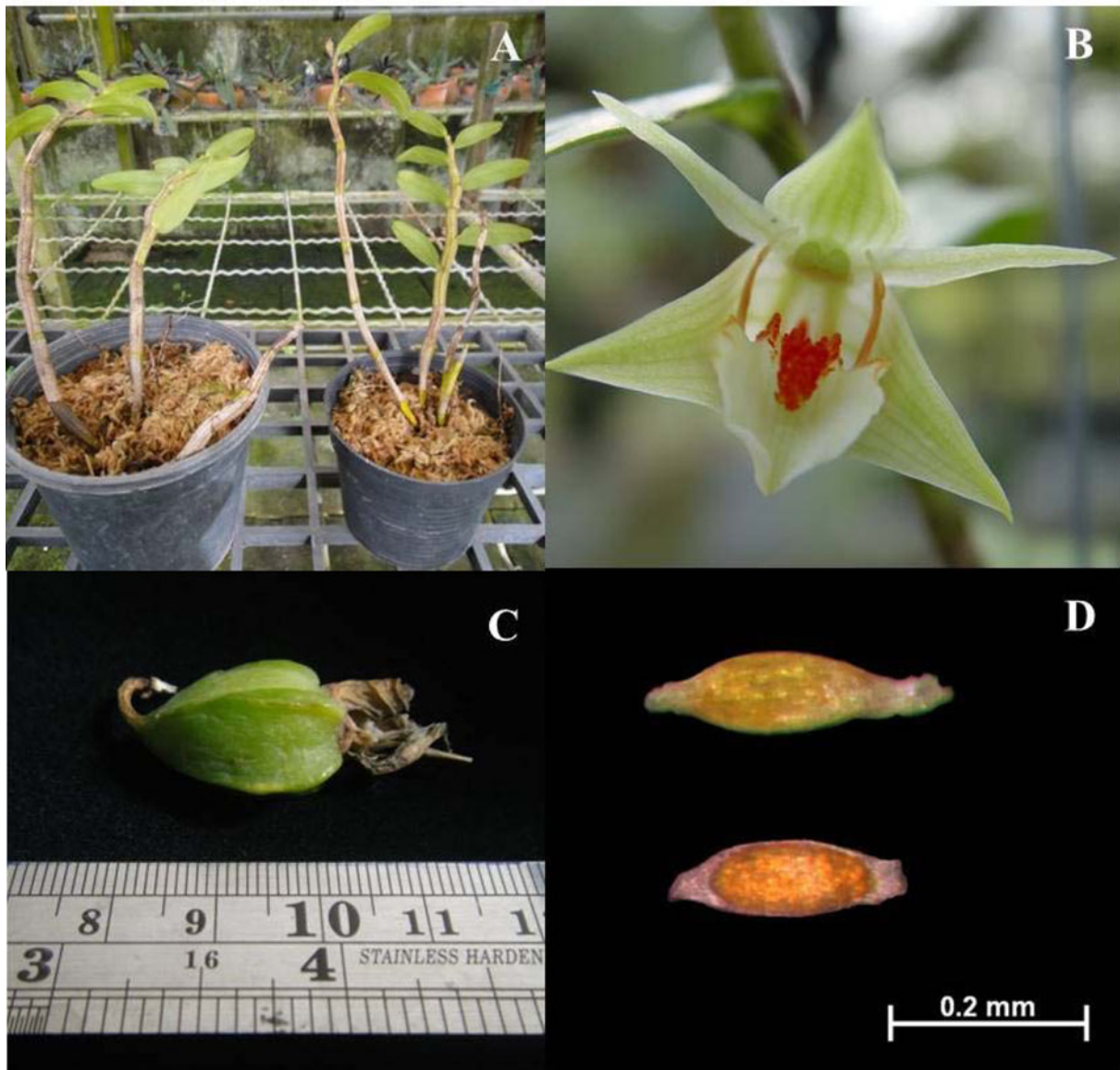
Fig. 1. Plant, fruit and seed characteristics of *D. cruentum*. (A) Plants cultivated in the green house. (B) Flowering stage. (C) Maturing capsule. (D) Dry-stored seeds (lot 1) before (upper) and after vital staining with tetrazolium chloride, showing orange-red stained embryo (lower).

dimension varied from 162 to 172 μm long and 48 to 54 μm wide (Table 1). Based on the seed:embryo volume ratios, the air space did not vary between seed lots ($P > 0.05$), being around 15–17%; meaning that the embryo occupied about 83–85% of the seed volume (Table 1). The frequency distribution for seed air space across 30 *Dendrobium* species, including *D. cruentum* (this study), indicated that two-thirds had embryo volumes that were >60% of the seed volume (Fig. 3).

Seed embryos were orange-yellow in colour, and viable embryos were turned orange-red when exposed to tetrazolium chloride (Fig. 1). When seeds were incubated *ex vitro* (i.e. without disinfection and when sown on plain agar), the embryos doubled in volume in the first 7 d. This applied to both seed embryos that were alive and turning green or dead and turning brown (Fig. 4; Table 2). For germination to the spherical protocorm stage, there was a sevenfold increase in embryo volume by 28 d. In contrast, seeds disinfected and sown *in vitro*, in the presence of

macroelements and sucrose, achieved a 28-fold increase in embryo volume by 28 d and formed a top-shaped protocorm (Fig. 4; Table 2). The volume increases through each stage of germination (imbibition, spherical and top-shaped protocorm formation) were significantly different ($P < 0.05$).

In the light, temperature ($F = 54.8$; $P < 0.001$) had a significant effect on germination (spherical protocorm formation), but medium (*ex vitro* or *in vitro*) did not ($F = 3.4$; $P > 0.07$) for fresh seeds (Fig. 5). Both temperature ($F = 30.5$; $P < 0.001$) and the medium used ($F = 21.9$; $P < 0.001$) had treatment effects in dry-stored seeds, with *ex vitro* germination consistently higher than *in vitro* germination (Fig. 5). The range of temperature over which germination was recorded was slightly wider *ex vitro* (around 15–30°C) than *in vitro* (around 20–25°C), with 25°C clearly effective for both seed lots (Fig. 5). Total germination was, however, about twice as high for fresh seeds, lot 2, compared to dry-stored seeds, lot 1 (around 60 *vs* 30% maximum).
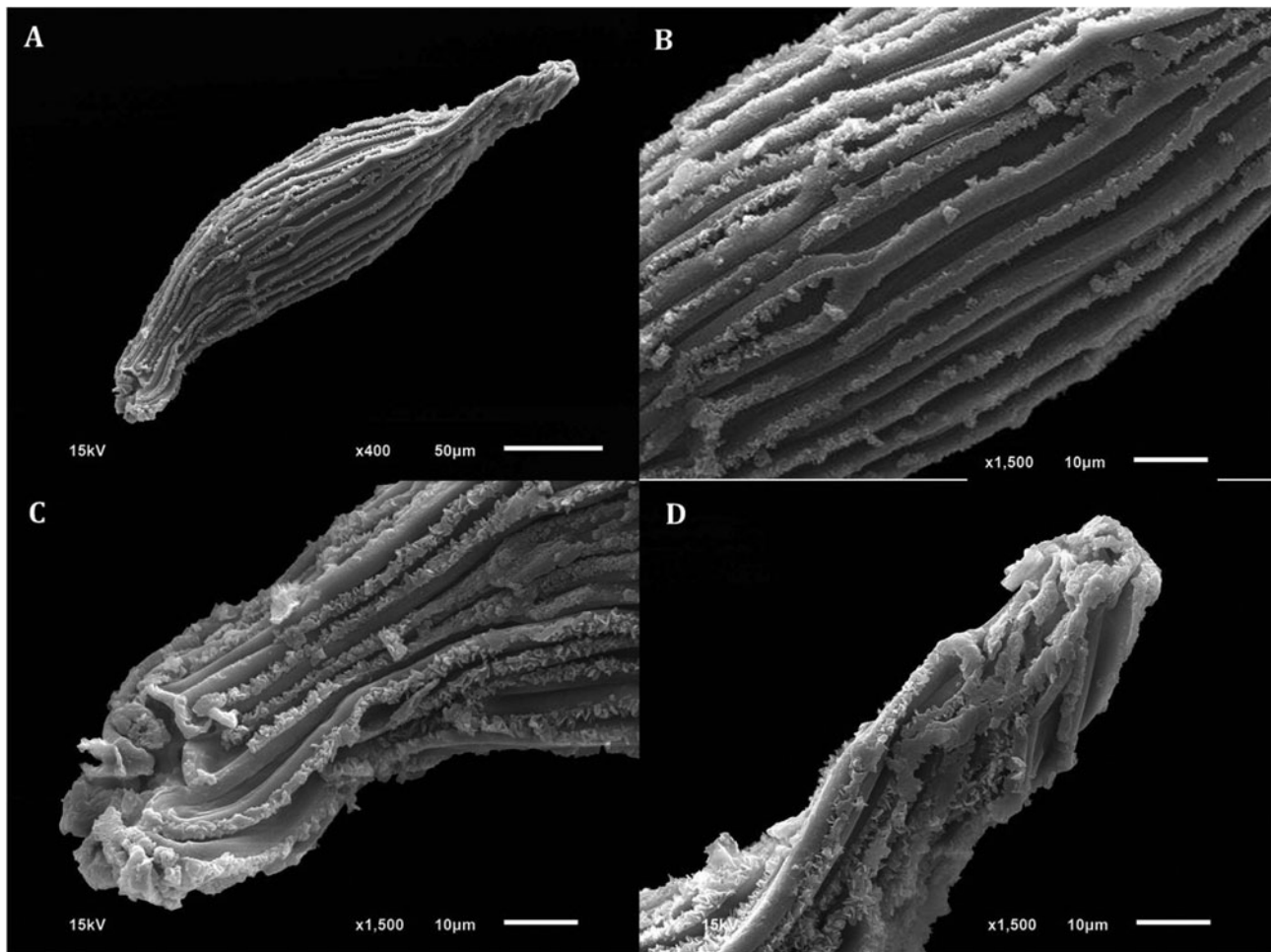
Fig. 2. Scanning electron microscopy (SEM) of *D. cruentum* seeds. (A) Whole seed. (B) Elongated testa cells with thickened walls. (C) Close-up of micropyle opening. (D) Close-up of chalazal end and cell wall thickenings with frilled edges.

The possible promoting or inhibitory effect of light on *ex vitro* seed germination was assessed at one constant and one alternating temperature. Light level had no significant ($P > 0.05$) effect on germination of fresh seeds (lot 2) at 25°C when comparing light ($12\,\mathrm{h\,d^{-1}}$) *versus* dark (weekly unwrapping dishes for scoring) *versus* double dark (unwrapped after 28 d) (Table 3). For dry-stored seed, the effect of light was variable, with germination in double dark > light > dark at 25°C (Table 3). However, among temperature treatments (constant *vs* alternating) for dry-stored seeds, the effect of alternating temperature (30/20°C) was to reduce germination significantly ($P < 0.05$) at comparable light levels, except for the dark treatment (Table 3).

Finally, the effect of nitrate concentration on the *ex vitro* germination of the two seed lots was assessed at 25°C in the light and dark (Fig. 6). At each KNO_3 concentration, there was no effect of light conditions on the germination of fresh ($F = 0.007$; $P = 0.93$) or dry-stored seed ($F = 0.109$; $P = 0.75$). The effect of nitrate on germination was just significant in fresh seeds ($F = 2.941$; $P = 0.05$) and highly significant in dry-stored seeds ($F = 4.32$; $P = 0.01$), mainly as a result of an inhibitory effect on germination in the light and the dark at 30 mmol KNO_3 (Fig. 6). There was no interaction between the factors of light and nitrate ($F = 0.386$; $P = 0.82$ for fresh seed and $F = 1.17$; $P = 0.36$ for dry-stored seed).

Discussion

Seed micromorphology

Orchid seed micromorphometric characteristics are important in elucidating taxonomic, phylogenetic and phytogeographic relationships (Verma et al., 2014), including in *Dendrobium* (Chattopadhyay et al., 2010). While orchid seeds are often <1 mm long, and can be considered to be DUST-like, they are of different shapes, including ellipsoid, oblongoid, ovoid, globose, trigonous, tetragonous and fusiform in the Epidendreae (Dressler, 1993; Molvary and Kores, 1995; Molvary and Chase, 1999). In the case of *D. cruentum*, the seeds are fusiform, with thickened testa cell walls (Figs 1–3), quite typical of *Dendrobium* species (Chaudhary et al., 2014). Moreover, *D. cruentum* testa cell thickenings have a cottony-white substance, previously observed in sub-tropical *D. parishii* Rchb.f. and *D. williamsonii* Day & Rchb.f. (Chaudhary et al., 2014).

Comparative studies on 32 threatened Western Himalayan orchids reveal that most of the seed space is air, with only 10 species having embryos that exceeded 50% of the seed volume (Verma et al., 2014). In contrast, in *Dendrobium* species studied thus far, the seed embryo is relatively large and often (two-thirds of cases) occupies >60% of the seed volume (Fig. 3). Our previous comparative study on nine *Dendrobium* species revealed that the

Table 1. Micromorphometric data of the seed and embryo ($n = 30$) of *D. cruentum*

Seed lot	Seed dimensions			Embryo dimensions			
	Length (mm)	Width (mm)	Volume (mm³)	Length (mm)	Width (mm)	Volume (mm³)	Air space (%)
Dry-stored seeds (lot 1)	0.2852 ± 0.0221[a]	0.0625 ± 0.0058[a]	0.000295 ± 5.918E-05[a]	0.1619 ± 0.0162[b]	0.0538 ± 0.0042[a]	0.000247 ± 4.675E-05[a]	15.4 ± 9.2[a]
Fresh seeds (lot 2)	0.2857 ± 0.0171[a]	0.0580 ± 0.0046[b]	0.000254 ± 4.753E-05[b]	0.1721 ± 0.0101[a]	0.0482 ± 0.0049[b]	0.000212 ± 4.726E-05[b]	17.0 ± 5.87[a]

Data for fresh and stored seeds are significantly different ($P < 0.05$) when followed by different letters.
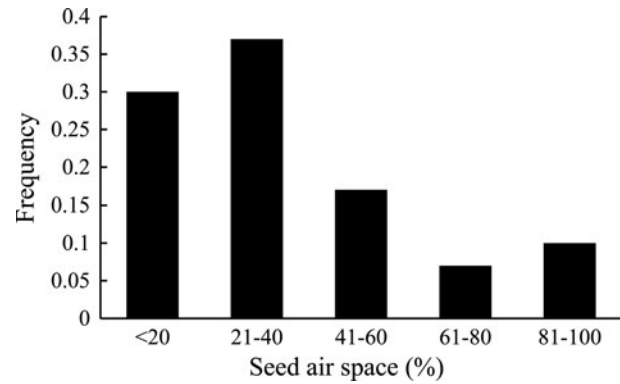

Fig. 3. Frequency distribution for seed air space in 30 *Dendrobium* species. Species names follow the original publications and are thought to be synonyms for three species based on plants of the World Online (https://powo.science.kew.org/). Species: *D. chrysanthum*, *D. chrysotoxum*, *D. clavatum* (synonym of *D. denneanum*), *D. crepidatum*, *D. cruentum*, *D. cunninghamii*, *D. densiflorum*, *D. devonianum*, *D. draconis*, *D. farmeri*, *D. fimbriatum*, *D. findlayanum*, *D. formosum*, *D. hercoglossum*, *D. heterocarpum*, *D. hookerianum*, *D. insigne*, *D. lineale*, *D. lituiflorum*, *D. moschatum*, *D. nobile*, *D. ochriatum*, *D. parishii*, *D. primulinum* (synonym of *D. polyanthum*), *D. pulchellum*, *D. schildhaueri* (synonym of *D. kontumense*), *D. strebloceras*, *D. transparens*, *D. wardianum*, *D. williamsonii*. Information sources: Arditti and Ghani (2000); Chaudhary et al. (2014); Prasongsom et al. (2017); Diantina et al. (2020a) and Tongbram et al. (2012).

embryo occupied 64–87% of the seed volume (Prasongsom et al., 2017). Moreover, these 'large' embryo seeds generally germinated as well *ex vitro* as *in vitro* (Prasongsom et al., 2017). Similarly, the air space in *D. cruentum* seeds is only around 13–15% (Table 2) and germination *ex vitro* is possible (Figs 4–6).

The determination of embryo size, including relative to the seed or endosperm, is a valuable way of morphologically categorizing seeds (Martin, 1946; Baskin and Baskin, 2014), and embryo size data have been used to study the evolution of dormancy (Forbis et al., 2002; Finch-Savage and Leubner-Metzger, 2006; Linkis et al., 2010). For example, reconstructions for embryo to seed ratio (E:S) using family means for 179 families show that E:S increases in derived angiosperms compared with ancestral angiosperms (Forbis et al., 2002). Moreover, morphological (MD) and morphophysiological (MPD) dormancy is often present in seeds with differentiated embryos and a low E:S ratio. In *Ribes* (Grossulariaceae) species, E:S needs to increase from approximately 0.2–0.3 to 0.6–0.8 before germination can be completed, and among species, the greater the initial embryo length the lower the dormancy level, which is also co-associated with warmer mean annual temperatures (Mattana et al., 2013). Habitat is also an evolutionary driver for embryo size in Apiaceae, with a large relative embryo length associated with more rapid germination of species in dry and open habitats (Vandelook et al., 2012). Similarly, in *Dendrobium* species, the seed air space is more dependent on climatic regions than phylogeny. For example in 20 species of eastern North India, average embryo volumes increase from 62 to 76% of the seed volume in species adapted to temperate *versus* tropical habitats (Chaudhary et al., 2014). In tropical *D. cruentum*, the DUST seeds have a relatively large embryo (approximately 84% of the seed volume; Table 1) that does not need to extend in length before germination occurs. Such a strategy in this orchid potentially reduces the distribution of germination times and improves competitiveness in its epiphytic niche.

Taken with our earlier studies on 12 *Dendrobium* species (Prasongsom et al., 2017; Diantina et al., 2020a), the results for
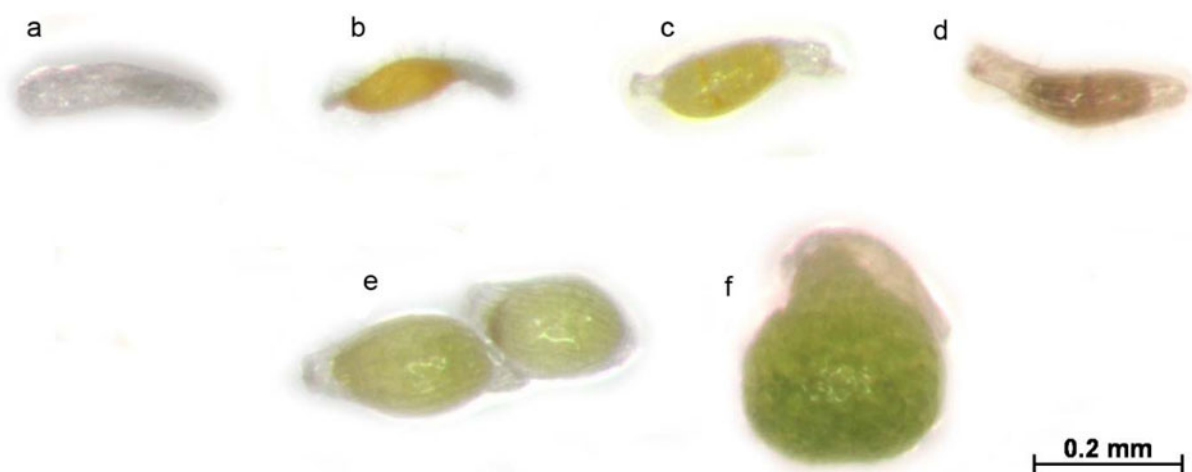
Fig. 4. Stages of *D. cruentum* Rchb. f. seed embryo growth *ex vitro* (a–e) and in vitro (f). (a) Embryoless seed. (b) Full seed, with the embryo occupying about 87% of the seed volume. (c) Seed turning green after 7 d imbibition at the start of embryo growth. (d) Full, dead seed, in which the embryo failed to achieve germination and shows signs of oxidative stress (browning). (e) Spherical embryos (protocorm) of germinating seeds after 28 d *ex vitro*. (f) Top-shaped protocorm formation from germination seeds after 28 d *in vitro*.

Table 2. Changes in embryo dimensions of *D. cruentum* dry-stored seed (lot 1) during germination *ex vitro* and *in vitro*

| Growth stage | Embryo dimensions* | | | |
	Length (mm)	Width (mm)	Volume (mm³)	Volume increase cf. unimbibed seed
Unimbibed	0.1630 ± 0.0110^c	0.0553 ± 0.0197^d	$0.0002780 \pm 0.0001141^d$	–
Imbibed *ex vitro*, 7 d	0.1606 ± 0.0359^c	0.0823 ± 0.0135^c	$0.0005981 \pm 0.0002625^c$	×2.15
Imbibed *ex vitro*, 7 d, dead seed	0.1603 ± 0.0267^c	0.0833 ± 0.0062^c	$0.0005899 \pm 0.0001292^c$	×2.12
Spherical protocorm size 28 d *ex vitro*	0.1950 ± 0.0250^b	0.136 ± 0.0103^b	$0.0019090 \pm 0.0003770^b$	×6.87
Top-shaped protocorm size after 28 d *in vitro*	0.2423 ± 0.0364^a	0.2323 ± 0.0542^a	$0.0076450 \pm 0.0047790^a$	×27.5

*30 embryos assessed per growth stage. Mean values within columns followed by different letters are significantly different ($P < 0.05$, *t*-test).

D. cruentum emphasize the importance of not limiting considerations of the ecology, evolutionary and phylogenetic trajectory of orchids to the seeds' external (testa) morphology (Parthibhan et al., 2012; Barthlott et al., 2014). Rather, embryo size (and volume) determinations in relation to the seed appear to provide new perspectives on the ecophysiology and evolution of orchid seed germination and dormancy in the way that the E:S ratio has done for angiosperms as a whole (Linkis et al., 2010; Vandelook et al., 2012; Willis et al., 2014).

Nitrate, temperature, light and seed germination

Many orchid seed culture media include two forms of nitrate (KNO_3 and NH_4NO_3), and a concentration of about 10 mmol is known to increase seed germination and seedling development in *Dendrobium aqueum* Lindl. (Parthibhan et al., 2012). In contrast, increasing the concentration of NO_3^- ions to approximately 5 mmol slightly decreases dark germination in the temperate terrestrial species *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. (Rasmussen, 1995), indicating the potential for a species-specific effect of nitrate on germination. We exposed seeds of *D. cruentum* seeds to a 30-fold range of KNO_3 (1–30 mmol), within the known range of physiological activity for nitrate when applied as KNO_3, that is, generally

0.1–50 mmol (Duermeyer et al., 2018). To seeds, nitrate has a dual function; as a nutrient and as a signal, discrimination between the two is not often attempted. To complicate matters, nitrate as a signal appears to contribute to the dormancy level, including through seed development, and to stimulating germination (Toorop, 2015; Duermeyer et al., 2018). The efficacy of nitrate can relate to an increase in population sensitivity to alternating temperature, for example, *Ranunculus sceleratus* L. (Probert et al., 1987), to a decrease in ABA, for example, in *Arabidopsis* (Ali-rachedi et al., 2004), or to light responsiveness via the active form of phytochrome (Pfr) (Probert et al., 1987; Baskin and Baskin, 2014). In reviewing evidence for 20 species, it was found that half of the nitrate-related responses in seeds were associated with light; for example, nitrate reduces the light requirement for germination (three species) or improved germination in the dark (five species) (Duermeyer et al., 2018). In contrast to seeds of the orchid *Bletilla striata* (Thunb.) Rchb.f. that are stimulated to germinate by low concentrations of nitrate (Ichihashi and Yamashita, 1977), nitrate seems to promote germination in *D. cruentum* seed only slightly (around 20% increase) at 3 mmol for fresh (lot 2) seed and at 10 mmol for dry-stored (lot 1) seed (Fig. 6). This response is not dependent on light conditions (light *vs* dark), however. More typically, seeds receptive to nitrate are much more sensitive, for example, 20 mmol KNO_3
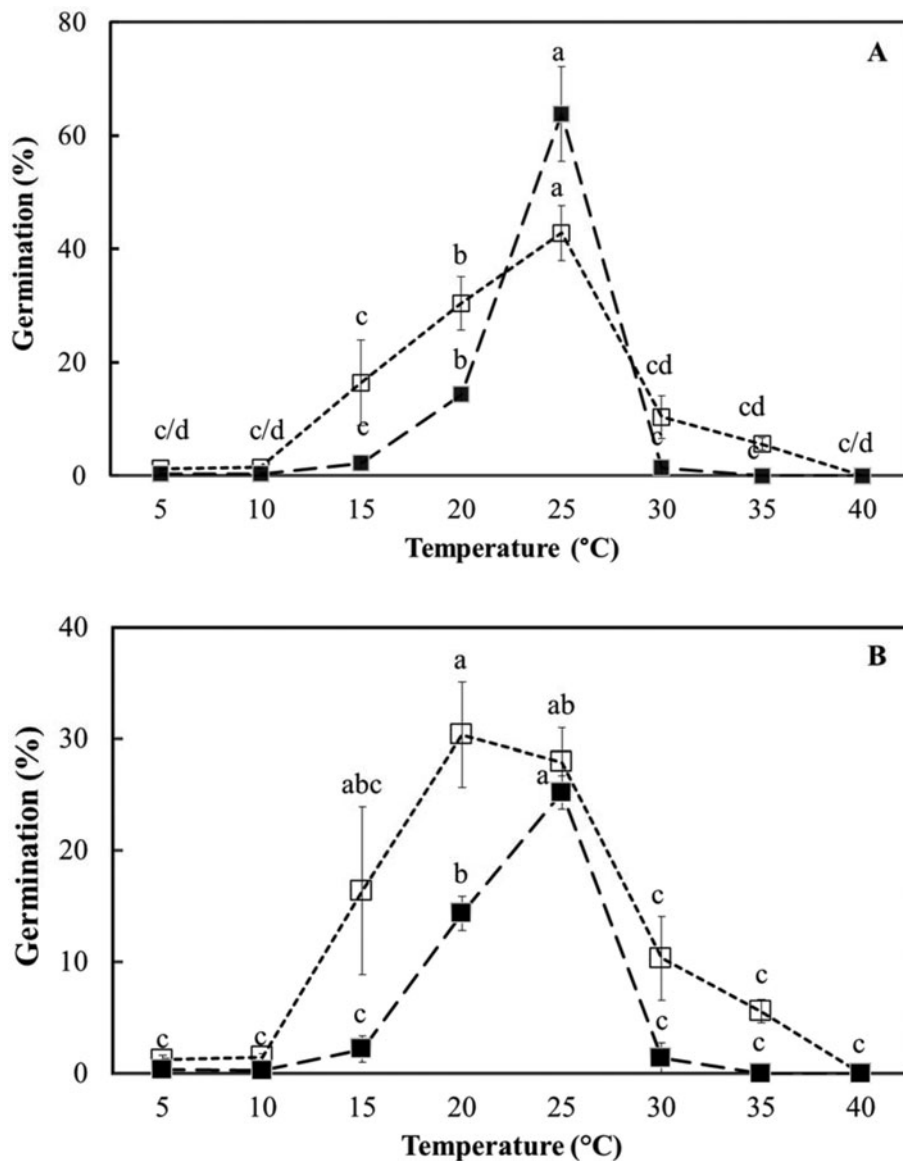
Fig. 5. Effect of temperature on *D. cruentum* seed germination (spherical protocorm production) *ex vitro*, on plain agar without disinfection (□) and *in vitro*, on ½ MS medium with disinfection (■). (A) Fresh seeds (lot 2) and (B) dry-stored seed (lot 1) were assessed for germination after 28 d under a 12 h photoperiod. For each temperature, significant germination differences ($P < 0.05$) between sowing methods are indicated by different letters based on triplicate sowings of 100–150 full seeds.

Table 3. Effect of temperature (25 and 30/20°C) and light (12/12 h light, dark and double dark condition) on the *ex vitro* germination (spherical protocorm formation) of fresh (lot 2) and dry-stored (lot 1) *D. cruentum* seeds after 28 d

Seed lot	Temperature (°C)	Conditions	Germination (%)*
Fresh seed (lot 2)	25	Light	42.8 ± 4.8^a
		Dark	37.9 ± 7.7^a
		Double Dark	35.3 ± 3.1^a
Dry-stored (lot 1)	25	Light	$20.9 \pm 0.8^{a,A}$
		Dark	$11.7 \pm 1.5^{b,A}$
		Double Dark	$27.9 \pm 2.2^{a,A}$
	30/20	Light	$3.9 \pm 3.3^{b,B}$
		Dark	$11.1 \pm 1.7^{a,A}$
		Double Dark	$15.9 \pm 2.4^{a,B}$

*Within a single temperature treatment, the results are significantly different with the lighting condition when the data are followed by a different lower case letter. For dry-stored seed only, data are significantly different for specific light conditions across temperature treatments when followed by difference upper case letters.

increases *S. officinale* germination by around 60% by controlling testa rupture and seed water content (Toorop, 2015). We conclude that the promotive effect of nitrate on *D. cruentum* seed germination was modest and might relate more to its nutrient rather than signalling effect. In any case, there is no evidence of nitrate reducing any light requirement for *D. cruentum* seed germination *ex vitro*, as germination is similar in the dark. It is possible that this response is confounded by the possible saturation of any light requirement during the dark treatment, as the dishes were opened briefly for germination scoring once a week for the first 3 weeks. However, comparable germination levels in the double dark treatment (Table 3) indicate this is not the case. Overall, this is not surprising as unlike small seeds (<0.1 mg) of many angiosperms that are light requiring (Baskin and Baskin, 2014), microseeds of epiphytic orchids are able generally to germinate in both light and dark (Arditti, 1979); however, long periods in the dark will ensure that the developing protocorms remain white, as seen for *D. aqueum* (Parthibhan et al., 2012). Nitrate does, though, have the potential to reduce germination in orchids, for example, at 2 mg NO_3^- dm^{-3} on mature seeds of *Pseudorchis*
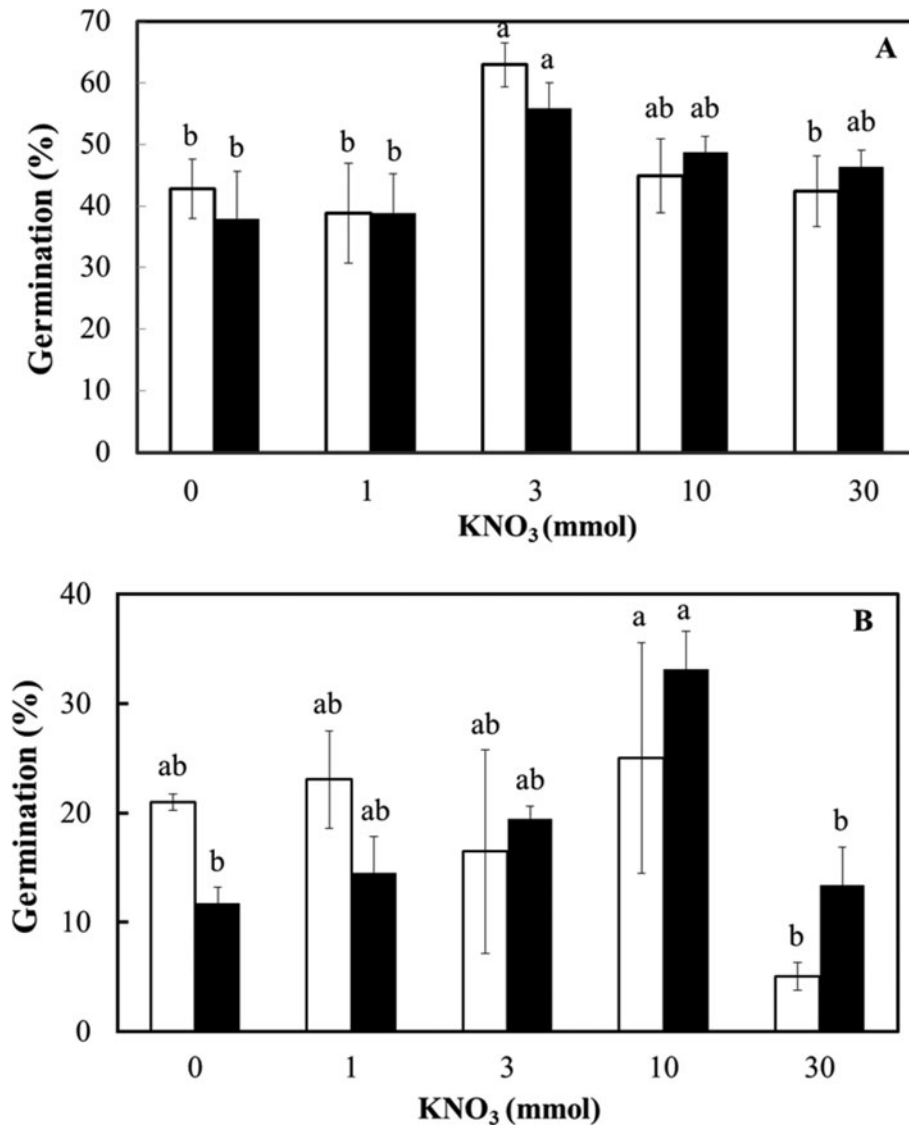
Fig. 6. *Ex vitro* germination of *D. cruentum* seeds in response to KNO_3 concentration (0, 1, 3, 10 and 30 mmol). Seeds were sown without disinfection on plain agar in the light (□) and dark (■) at 25°C. (A) Fresh seeds (lot 2) and (B) dry-stored seeds (lot 1) were assessed for germination after 28 d in the light (12 h photoperiod) or dark (occasional opening of wrapped dishes). For each light condition, significant germination differences ($P < 0.05$) between KNO_3 concentrations are indicated by different letters based on triplicate sowings of 100–150 full seeds.

albida (L.) Á.Löve & D.Löve (Ponert et al., 2013). Similarly, *D. cruentum* seeds exposed to 30 mmol KNO_3 had reduced germination *ex vitro* in the light and the dark (Fig. 6). Nitrate sensitivity during germination can affect orchid species' habitat preferences and distribution; orchids from oligotrophic habitats are much more sensitive than those of eutrophic habitats, which can be almost insensitive (Figura et al., 2020). Sensitivity to nitrate was also associated with soil parameters that indicated a higher nitrification rate.

Constant temperatures are clearly sufficient for *D. cruentum* seed germination (spherical protocorm formation; Fig. 3) *in vitro* and *ex vitro*. However, the temperature range for maximum germination (Fig. 5; Table 3) is quite narrow compared to many other species (Baskin and Baskin, 2014; Durr et al., 2015), although well adapted to the tropical conditions in Thailand. Apart from fresh seed (lot 2) at 25°C, the level of germination *in vitro* is lower than that observed *ex vitro*. This could arise if the concentration of nitrate in the medium (52.6 mmol KNO_3 plus ammonium nitrate) is supraoptimal and inhibitory. Regarding a possible interdependency of germination on light and alternating temperature, as in seeds of *R. sceleratus* (Probert

et al., 1987), *D. cruentum* seeds tend to germinate better under various light conditions when temperature is constant rather than alternating, with the same average temperature (Table 3). However, it is clear that the warm temperature phase (30°C) applied here is supraoptimal for germination (Fig. 5). While this has the potential to obscure temperature × light effects, it appears that *D. cruentum* seed germination does not need a complex and interacting set of environmental factors for effective germination. In fact, the evidence suggests that these DUST seeds are non-dormant.

Dormancy concepts in orchids

Seed quality varied between the two seed lots of *D. cruentum*, with the dry-stored seed (lot 1) having lower germination than the fresh seed (lot 2) (Fig. 5). Undoubtedly, the processing time for the dry-stored *versus* fresh seed was longer, possibly resulting in seed ageing prior to cold storage. Orchid seed is reputed to be short-lived, with half lives of only about 2 weeks in humid conditions at about 40°C (Pritchard et al., 1999; Hay et al., 2010; Fileti et al., 2021). To counteract this risk, many of the experiments

were also run on fresh seed. In this instance, the seed came from a capsule that was just about to dehisce and used in the experiments soon after extraction. Such seed material might not have reached full maturity, thereby reducing the likelihood of the induction of maturation-related physiological dormancy. But this seems unlikely. Although the fresh seed (lot 2) dimensions are slightly narrower, the seed is not shorter than the dry-stored seed (Table 1). Moreover, the embryo occupancy of the seed space is not different between two seed lots (Table 1), indicating similar maturity.

Coat-imposed physical dormancy – a hindrance to water uptake through the testa, reinforced through high phenolic content – has been tentatively proposed for summer endemic members of the orchid genus *Disa* that inhabit high altitude grasslands with severe winter conditions to which the seeds are exposed (Thompson et al., 2001). Prolonged (4 h) chemical scarification with 1.75% NaOCl solution ensures maximum permeability of the testa and inner carapace, facilitating tetrazolium chloride permeation and conversion to formazan by the embryo. Similarly, surface disinfection with NaOCl bleach with 5% active chlorine both disinfects and scarifies seeds of *Limodorum trabutianum* Batt., an European terrestrial orchid, leading to much improved germination (Magrini and De Vitis, 2017). In contrast, seeds of the tropical epiphyte *D. cruentum* germinate (spherical protocorm) in 28 d on plain agar-water substrate without disinfection (Figs 4–6), indicating high permeability to water, no delay to the onset of germination and a lack of physical dormancy.

Undoubtedly physiological dormancy is present in the seeds of many orchid species from temperate regions, with germination benefiting from cold stratification and sowing *in vitro* on media composed of various elements and a carbon source (Rasmussen, 1995; Baskin and Baskin, 2014). When the seeds' dust-like form and undifferentiated embryo is factored in, it is not surprising that orchids are proposed to have a special kind of MD or MPD (Baskin and Baskin, 2014). But viewing orchid seed as an uniform propagule in form and function does not account for observed heterogeneities. In relation to form, tropical *Dendrobium* species tend to have relatively large embryos, occupying a considerable proportion of the seed volume (Figs 1, 3, 4, Table 1; Prasongsom et al., 2017; Diantina et al., 2020a). In contrast, the mean embryo and seed air space of ten temperate species in *Cephalanthera*, *Corallorhiza*, *Cypripedium*, *Epipactis*, *Paphiopedilum* and *Platanthera* is 12 and 88%, respectively (Arditti and Ghani, 2000). As noted earlier, 'dust' has no internal air spaces (Arditti and Ghani, 2000) and the term gives no hint of the variation in orchid seed internal micromorphology.

Furthermore, there are also reasons to reassess the wholesale assignment of orchid seeds to a DUST class of dormancy (Willis et al., 2014). Beyond the traditional five kinds of dormancy, dust seeds (DUST) are small in size (mostly ≤1.0 mm in length) and have undifferentiated embryos (Eriksson and Kainulainen, 2011). In the case of *D. cruentum* and other species assessed (Prasongsom et al., 2017), the embryos may be undifferentiated with few cells, but they are also relatively large compared to the size of the seed, and are able to germinate under a wide range of conditions immediately after dispersal without any dormancy-breaking treatments, that is, *ex vitro* (Figs 4–6). This type of response actually defines the dormancy class of ND (Baskin and Baskin, 2004, 2014). It seems, therefore, that seeds of some orchid species can be both DUST and ND. We concur with the view that for small embryos in dwarf seeds, the 'assignment of seeds to a dormancy class requires that studies be done to determine if embryos grow inside the seed before germination can occur' (Baskin and Baskin, 2005). In the case of *D. cruentum*, the large embryo does not need to grow substantially lengthwise within the seed before germination; rather spherical protocorm formation progresses and the testa splits (Fig. 4, Table 2).

Finally, it is thought that DUST seeds may have arisen from MPD seeds but not from MD seeds, and the ND state primarily evolved from PD but rarely from MPD (Willis et al., 2014). Analysis of the evolution of seed dormancy revealed no examples of a transition from DUST seeds to ND (Willis et al., 2014). Nonetheless, it seems that some *Dendrobium* species may disperse ND seeds.

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