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Germination and storage behaviour of seeds of the subtropical evergreen tree *Daphniphyllum glaucescens* (Daphniphyllaceae)

Ching-Te Chien[A], *Jerry M. Baskin*[B], *Carol C. Baskin*[B,C] *and Shun-Ying Chen*[A,D]

[A]Division of Silviculture, Taiwan Forestry Research Institute, 53 Nan-Hai Road, Taipei 10066, Taiwan.
[B]Department of Biology, University of Kentucky, Lexington, Kentucky 40506-0225, USA.
[C]Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky 40546-0312, USA.
[D]Corresponding author. Email: sychen@tfri.gov.tw

Abstract. *Daphniphyllum glaucescens* Blume ssp. *oldhamii* (Hemsl.) Huang is an important subtropical evergreen tree in Taiwan. Seeds of *D. glaucescens* have non-deep, simple, epicotyl morphophysiological dormancy, and a minimum of 10–12 weeks is required for the first step of seedling production, i.e. hypocotyl emergence. It is not known how to decrease the time for seedling production and how to store seeds for retention of viability. We determined the effects of (i) gibberellic acid and cold-stratification on germination (hypocotyl emergence) and (ii) storage temperature and seed moisture content (MC) on germinability. Exogenous application of GA_3 and of GA_4 promoted germination and increased the germination rate. Moist cold-stratification at 5°C also promoted germination; the longer the stratification period, the faster the rate of germination. More than 70% of seeds (fresh seeds, MC = 37.6%) dried to an MC of 6.4%, 8.5% and 25.5% (fresh weight basis) retained germinability after a 1-month storage at 5°C, whereas germination percentage decreased to 0–2% after a 12-month storage at the same temperature. Germination percentage of seeds dried to the same MC and stored at 15°C decreased to 0% after 8 months, whereas seeds stored at −20°C did not germinate even after just 1 month of storage. The present evidence suggests that seeds of *D. glaucescens* have intermediate rather than orthodox or recalcitrant storage behaviour.

Introduction

The Daphniphyllaceae contains only the genus *Daphniphyllum*, which consists of 10 species in eastern Asia, Indomalaysia and northern (tropical) Australia (Mabberley 1997). The two species that occur in Taiwan are *D. glaucescens* ssp. *oldhamii* and *D. himalaense* ssp. *macropodum*, and they are overstorey trees in natural evergreen forests throughout the whole island. *D. glaucescens* ssp. *oldhamii* is an important component of the *Cyclobalanopsis stenophylloides–D. glaucescens* ssp. *oldhamii* and *Machilus zuihoensis–D. glaucescens* ssp. *oldhamii* associations in evergreen broadleaved forests at elevations <1500 m. *D. himalaense* ssp. *macropodum* occurs at altitudes of 1000–2200 m in broadleaved forests at lower elevations and in coniferous forests, e.g. with *Tsuga formosana* or *Picea morrisonicola*, at higher elevations (Liou *et al*. 2006).

Daphniphyllum glaucescens ssp. *oldhamii* has the following three varieties: *oldhamii*, *kengii* and *lanyuense*, with the last one being endemic to Taiwan (Huang 1993). *D. glaucescens* Blume ssp. *oldhamii* (Hemsl.) Huang var. *oldhamii* (Hemsl.) Huang (hereafter *D. glaucescens*, the subject of the present study) is an evergreen, dioecious tree, distributed from Taiwan to southern China, Cambodia and Vietnam.

The mature fruit of *D. glaucescens* is a diaspore and technically refers to the anatomical seeds plus the attached (indehiscent) hard tissue (endocarp) and external fleshy fruit part (exo- and mesocarp) (Wang 2000; Baskin *et al*. 2009). Fruits (hereafter called seeds) of *D. glaucescens* have non-deep, simple, epicotyl morphophysiological dormancy, i.e. a small, linear, underdeveloped embryo that must grow inside the seed before the hypocotyl emerges (later on, when the emerged hypocotyl is ~1 cm long, the radicle emerges from it). Seeds also have a dormant epicotyl that requires a 12–14-week incubation (after the hypocotyl/radicle emerges) to emerge from the seed (Baskin *et al*. 2009).

Although seeds of this potentially important medicinal species (see below) have been germinated in laboratory of the University of Kentucky, USA, it takes 24–38 weeks for them to produce a seedling (Baskin *et al*. 2009). Thus, information on how to decrease the time required for seedling production is needed. Further, seed storage behaviour is not known.

Two well known ways to overcome the dormancy and promote the germination of seeds are exogenous application of gibberellins and cold-stratification (moist-chilling) (Bewley and Black 1994; Baskin and Baskin 1998). Gibberellins such as GA_1, GA_3 (gibberellic acid), GA_4 and GA_7 are plant hormones that promote the germination of seeds of many species of both angiosperms and gymnosperms (Kucera *et al*. 2005), and they can sometimes partially or completely replace cold-stratification for induction of seed germination (Bewley and Black 1994; Baskin and Baskin 1998).

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Seeds can be divided into the following three categories on the basis of storage behaviour: orthodox, recalcitrant and intermediate (Roberts 1973; Ellis *et al*. 1990, 1991). Orthodox seeds generally have a low MC following dispersal and can be dried to 5%, or even lower, and stored at subzero temperatures (optimum = −18°C) for long periods of time without a loss of viability, whereas recalcitrant seeds are shed at a high MC and lose viability rapidly if they are dried to a MC of less than ~20–30%. Further, they cannot be stored at temperatures below 0°C. Seeds with intermediate storage behaviour survive a MC of ~6–12% and can be stored at cool (>0°C) temperatures; however, subzero temperatures rapidly reduce the viability of these seed. With further studies on seeds of an increasingly wide range of species, especially on those from the tropics and subtropics, it has become apparent that there is a continuum of storage behaviours rather than discrete categories (Farrant *et al*. 1988; Berjak and Pammenter 2002).

Stems, leaves, fruits and roots of *D. glaucescens* and other *Daphniphyllum* spp. contain a diversity of alkaloids with complex polycyclic skeletons (Mu *et al*. 2006; Wang *et al*. 2007; Li *et al*. 2008; Morita and Kobayashi 2008; Yahata *et al*. 2009), and some of them exhibit cytotoxic and antioxidant activities (Gamez *et al*. 1998; Kobayashi *et al*. 2003; Morita and Kobayashi 2003, 2008; Morita *et al*. 2006; Mu *et al*. 2007). Plants of this genus have been used in traditional Chinese medicine to treat asthma, snake bites, rheumatism and cough (The Editorial Committee of the Administration Bureau of Traditional Chinese Medicine 1998), and seeds also have been used for oil extraction (Wang 2000). Thus, being able to store seeds of *D. glaucescens* and to propagate the taxon from seeds may be of future benefit from an economic as well as conservation perspective. Thus, the present study had the following two primary objectives: (1) to determine whether GA and cold-stratification could shorten the first period of seedling production, i.e. hypocotyl emergence; and (2) to determine the effect of storage temperature and seed MC on germinability, which have previously not been reported for *Daphniphyllum*.

Materials and methods

Seed harvesting and handling

Mature fruits (drupes) of *D. glaucescens* with a black–purple colour were harvested from a montane subtropical evergreen forest in a monsoon climate at Yang-Ming-Shan, northern Taiwan (25°10′N, 121°32′E, 600 m asl) on 5 October 2006, 29 October 2007 and 17 October 2008. Fruits were placed on a mesh (2.83 mm^2) stainless steel pan and kneaded by hand in water to remove the pulp (exocarp + mesocarp). The seeds plus the attached endocarp were air-dried at room temperature for 1 day and then temporarily stored in a sealable polyethylene bag at 5°C. Fresh *D. glaucescens* seeds collected in 2008 were 7.94 ± 0.39 mm (mean ± s.e.) long, 5.36 ± 0.22 mm wide and 5.35 ± 0.22 mm thick (n = 20), and the number of seeds per litre was ~4680. The seed consists of a small embryo (embryo length : endosperm length = 0.16 ± 0.03, n = 15), surrounded by a large endosperm, a thin seed coat and an endocarp with considerable variation in thickness (0.4 ± 0.28 mm, n = 10). Hereafter, the term seed refers to the intact seed with endocarp, which is the germination unit in *Daphniphyllum*. The criterion for seed germination used in the present study was hypocotyl emergence (see Introduction).

Effect of GA_3 and of GA_4 on radicle emergence

Fresh seeds harvested in 2008 were soaked in concentrations of 0 (water control), 26, 260 and 2600 μM GA_3 (95% purity, Sigma, St Louis, MO, USA) and GA_4 (>90% purity, from Professor Lewis N. Mander, Australian National University) for 20 h at room temperature, and then mixed with moist sphagnum moss for incubation at an alternating temperature of 20/10°C in a 12-h daily photoperiod (~100 μmol m^{-2} s^{-1}, 400–700 nm, cool white fluorescent light). Eighteen dishes (N) of 50 seeds each harvested in 2007 were incubated at 30/20°C (n = 6), 25/15°C (n = 3), 20/10°C (n = 3) and 25°C (n = 6) for 18 weeks on water-moistened sphagnum moss. Then, seeds from three dishes at 30/20°C and at 25°C each were soaked in a solution of 2600 μM GA_3 for 1 day, after which they were returned to the dishes and their original incubation temperature for another 14 weeks. The other three replications at 30/20°C and at 25°C without a GA treatment served as controls for the effect of GA_3 on hypocotyl emergence. Seeds at 25/15°C and 20/10°C continued to be incubated in sphagnum moss moistened with water for the remainder of the 32-week experiment. Results are expressed as hypocotyl emergence (germination) and as mean germination time (MGT) in days. $MGT = (\Sigma n_i t_i)/N$, where n_i is the number of seeds germinated in t_i days from the beginning of the test, and N is the total number of germinated seeds at the end of the test (Naylor 1981). MGT is a measure of the rate of germination and of the sharpness of the germination peak.

Effect of cold-stratification on hypocotyl emergence

Fresh seeds were mixed with moist sphagnum moss (water content ~400% of its dry mass) in sealed polyethylene bags as described above and cold-stratified at 5°C for 4, 8, 12, 16 and 20 weeks. After the treatment, the seeds were incubated at 20/10°C for 32 weeks. Each treatment consisted of three replications of 50 seeds each.

Drying treatment and storage conditions

The moisture content of fresh seeds was 37.6 ± 0.8%. Fresh seeds were dried at three relative humidities (RHs) maintained with saturated salt solutions of $MgCl_2$ (RH 33%), $Ca(NO_3)_2$ (RH 56%) and KNO_3 (RH 92.5%) in closed containers (15 L) at 15°C for 21 days. MC of fresh seeds and of those taken from containers was measured following ISTA (1999) procedures. Thus, four replications of five seeds each were cut into small pieces ≤4 mm in length and width, before oven-drying for 17 h at 103°C. Seeds at RHs maintained by the saturated salt solutions reached MCs of 6.4%, 8.5% and 25.5%, respectively. They were sealed quickly in laminated aluminium foil and stored at 15°C, 5°C and −20°C each for 1, 4, 8 and 12 months. Thereafter, seeds from each of the storage temperatures and MCs were mixed with moist sphagnum moss in sealed polyethylene bags and incubated at 15/6°C at a 12-h photoperiod (as described above) for 32 weeks, during which germination was recorded weekly. Three replications of 40 seeds for each seed treatment were used. At the end of the 32 weeks, ungerminated seeds were cut and embryos were observed carefully for viability. For an embryo to be judged

viable, it had to be firm, white and non-shriveled. Results are expressed as hypocotyl emergence percentage and as MGT in days.

Statistical analysis

Germination percentages (mean ± s.e.) were calculated on the basis of the number of treated seeds. Means were compared by analysis of variance (ANOVA) and by the least significant difference (l.s.d.) test at the 5% level of significance, by using SAS and Microsoft Office Excel 2003. Percentage data were arcsine square-root transformed before the analysis.

Results

Effect of GA_3 and of GA_4 on hypocotyl emergence

GA_3 increased germination from 40% (water control) to 74% (2600 µM) and decreased MGT from 115 to 77 days, whereas GA_4 increased germination from 40% (water control) to 99.3% (2600 µM) and decreased MGT from 115 to 42 days (Table 1). GA_4 concentrations of 26 and 260 µM also very effectively stimulated seeds to germinate at high percentages and rates, whereas these same concentrations of GA_3 did not. GA_3 also promoted germination when seeds were treated following 18 weeks of incubation, i.e. GA_3-treated seeds germinated at 64.0% and 74.7% at 30/20°C and 25°C, respectively (Fig. 1), whereas the water control maintained at these temperatures resulted in germination percentage of ≤1% (data not shown in Fig. 1).

Effect of cold-stratification on hypocotyl emergence

Cold-stratification of fresh seeds at 5°C greatly increased germination percentages and rates for seeds during incubation at 20/10°C (Fig. 2). Stratification at 5°C for 4 weeks significantly increased germination to 50%. The germination percentage of seeds cold-stratified for 8 or 12 weeks was 82–85%, and time to first hypocotyl emergence decreased from 7–10 weeks of fresh seeds to 2–4 weeks. MGT at 20/10°C decreased with increasing length of cold-stratification time (data not shown).

Table 1. Effects of gibberellins on the percentage and rate (mean germination time, MGT) of germination of *Daphniphyllum glaucescens* seeds incubated at 20/10°C in light for 32 weeks

Means ($n = 3$) in the table for percentage germination followed by the same letter are not significantly different from each other (l.s.d., $P = 0.05$)

Treatment and parameter	GA (µM)			
	0 (water)	26	260	2600
GA_3				
Germination (%)	40.0 ± 5.7c	50.7 ± 6.6c	51.3 ± 10.9c	74.0 ± 4.3b
MGT (days)	115 ± 21	109 ± 7	102 ± 6	77 ± 4
GA_4				
Germination (%)		95.3 ± 2.5a	98.0 ± 2.8a	99.3 ± 0.9a
MGT (days)		57 ± 2	49 ± 1	42 ± 1

Drying treatment and storage conditions

Germination of seeds with MCs of 6.4%, 8.5% and 25.5% stored at 15°C decreased from 92% in fresh seeds to 0% after 8 months, and of those stored at 5°C it decreased to 2.1%, 1.3% and 0%, respectively, after 12 months (Table 2). No seeds stored at −20°C germinated even after 1 month of storage.

Discussion

Gibberellins enhanced the seed germination percentage and rate in *D. glaucescens.* This group of plant hormones is well known for its ability to break seed dormancy in taxonomically and biogeographically diverse groups of species. Germination

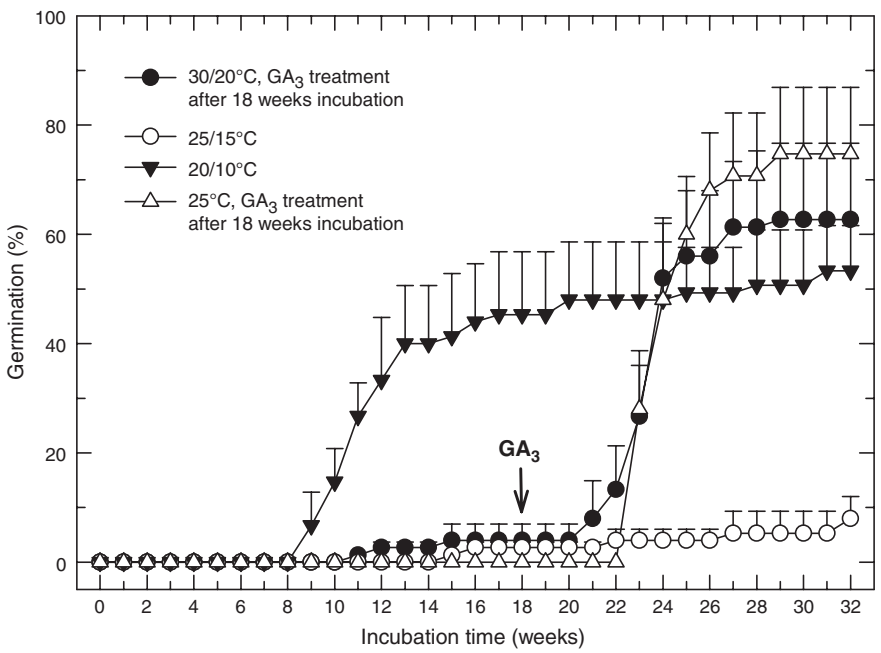

Fig. 1. Effect of GA_3 on the germination (mean ± s.e.) of *Daphniphyllum glaucescens* seeds harvested in 2007. Seeds incubated at 30/20°C and at 25°C were treated with 2600 µM GA_3 for 24 h at the end of the 18th week of incubation and then returned to the original temperatures for germination.

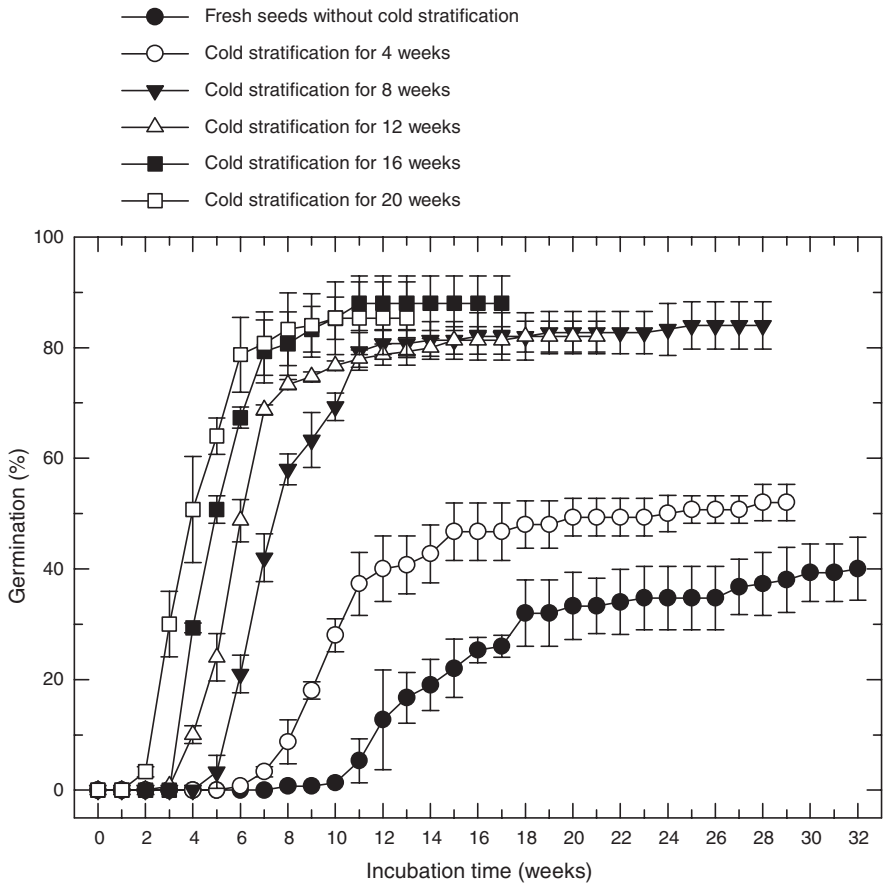

Fig. 2. Effect of cold-stratification at 5°C on germination (mean ± s.e.) of *Daphniphyllum glaucescens* seeds. Seeds were harvested in 2008 and incubated at 20/10°C in light for 32 weeks after cold-stratification.

Table 2. Effects of moisture content and storage temperature on seed germination percentages of *Daphniphyllum glaucescens*

Seeds were harvested in 2006 and incubated at 15/6°C for 32 weeks after storage for 1, 4, 8 and 12 months. Final germination percentage of fresh seeds (MC = 37.6 ± 0.8%) at 15/6°C was 92%. The ungerminated seeds were dead after 32 weeks of incubation at 15/6°C. Any means (n = 3) in the table followed by the same letter are not significantly different from each other (l.s.d., P = 0.05)

MC (%)	Temp. (°C)	Storage period (months) 1	4	8	12
6.4	15	52.0 ± 3.3c	15.3 ± 7.7ef	0	0
	5	70.7 ± 5.0ab	58.0 ± 7.5bc	22.0 ± 10.2de	2.0g
	–20	0	0	0	0
8.5	15	73.3 ± 5.2ab	8.0 ± 1.6f	0	0
	5	72.0 ± 5.7ab	62.7 ± 0.9bc	24.7 ± 3.8de	1.3g
	–20	0	0	0	0
25.5	15	69.3 ± 7.7ab	28.7 ± 6.6d	0	0
	5	78.0 ± 4.3a	58.0 ± 17.7bc	51.3 ± 4.1c	0
	–20	0	0	0	0

of dormant seeds of *Santalum acuminatum* from Australia was enhanced by GA_3 and GA_4, with GA_4 being considerably more effective than GA_3 (Loveys and Jusaitis 1994). Exogenous application of GA_3 broke dormancy of *Fagus sylvatica* seeds from Europe and substituted for cold-stratification (Nicolás *et al*. 1996). Dormancy in seeds of the New World tropical rainforest tree *Omphalea oleifera* was broken by 250 ppm GA_3 (Sánchez-Coronado *et al*. 2007). Germination of several tree and shrub species in subtropical Taiwan, with different taxonomic placements than those listed above, also was enhanced by GA, e.g. *Phellodendron amurense* var. *wilsonii* (Chien *et al*. 2006), *Prunus campanulata* (Chen *et al*. 2007), *Myrica rubra* (Chen *et al*. 2008) and *Taxus mairei* (Chien *et al*. 1998). All of these species are angiosperms, except *T. mairei*, which is a gymnosperm.

In the present study, seeds given incubation pre-treatments at 5°C (i.e. cold-stratification) germinated at higher percentages (and rates) at 20/10°C than did those not stratified. However, one must consider that seeds given a cold-stratification period of 0–20 weeks were incubated at 20/10°C for the same length of time (32 weeks). Thus, the total incubation time of cold-stratified seeds (time at 5°C + time at 20/10°C) was longer than that of non-stratified controls (0 time at 5°C + 32 weeks at 20/10°C). Did cold-stratification at 5°C cause an increase in germination percentage, or were higher germination percentages in seeds pre-treated at 5°C due to longer periods of incubation? First, considering the data in Fig. 2, after 32 weeks of incubation at 20/10°C, the germination percentage for the control (0 weeks at

5°C) was 40%, whereas that for seeds receiving 4, 8, 12, 16 and 20 weeks at 5°C, followed by incubation at 20/10°C for the remainder of a 32-week period was 52%, 84%, 82%, 88% and 86%, respectively. Second, whereas the germination percentage for the control was only 40% in 32 weeks, seeds cold-stratified at 5°C for 8, 12, 16 and 20 weeks reached 80% germination in 19, 24, 22 and 27 weeks, respectively. To sum this up, if $x + y$ = number of weeks to reach 80% germination, x = number of weeks of cold-stratification (or incubation) at 5°C and y = number of weeks of incubation at 20/10°C, then $x + y = 19$, 24, 22 and 27 weeks for seeds cold-stratified for 8, 12, 16 and 20 weeks, respectively. We can conclude, then, that stratification at 5°C, and not just the sum of the length of incubation at 5°C (cold-stratification) and 20/10°C, had a very positive effect on germination at 20/10°C of this lot of seeds collected in 2008. However, the germination percentage of seeds of *D. glaucescens* collected from the same locality in 2006 was ~80% after 20–24 weeks at 20/10°C, i.e. no cold-stratification pre-treatment (Baskin *et al*. 2009). We do not know why there were year-to-year differences in germination, although the seeds were treated in the same way in both years, except that those in 2006 were incubated on wet sand whereas those in 2008 were incubated on moist sphagnum moss.

By following the protocol of Hong and Ellis (1996) as a general guideline, we classified the seeds of *D. glaucescens* as having intermediate storage behaviour. Freshly harvested seeds of *D. glaucescens* germinated at a high percentage (92) after 32 weeks of incubation at 15/6°C, indicating that they were germinable. MC of fresh seeds was high (37.6%), and drying the seeds to a MC of 6.4% did not result in them losing viability. Thus, the seeds of *D. glaucescens* are not recalcitrant. The germination percentage of seeds with a MC of 6.4% was 58% after storage for 4 months at 5°C, whereas no seeds stored at −20°C for even 1 month germinated. Thus, the seeds probably are intermediate in storage behaviour. If the seeds with a MC of 6.4% had survived storage at −20°C for 3 months or longer, they could be classified as orthodox (Hong and Ellis 1996).

In the natural forest, the fruits of *D. glaucescens* are rapidly dispersed after they mature in October. The shed fruits are dormant, i.e. they exhibit epicotyl morphophysiological dormancy, and hence do not germinate until the next spring. We speculate that root emergence may occur in spring and shoot emergence in early summer. A phenological study of embryo growth and germination of this species in its natural habitat is in progress.

Conclusions and recommendations

Fresh seeds of *D. glaucescens* are dormant, and moist-chilling (cold-stratification) at 5°C or exogenous application of the gibberellins GA_3 or GA_4 can break the dormancy. Thus, we recommend that fresh seeds be cold-stratified or gibberellin-treated and then sown for germination and seedling production, which will require several months. The seeds appear to exhibit intermediate storage behaviour. If seeds cannot be sown immediately after collection, they can be stored dry (at MC < 10%) at 5°C for 4 months or less, without a significant loss of germinability. However, the best storage condition is on a moist substrate at 5°C. Under this condition, seeds can be stored for at least 20 months, which not only extends the storage time but also increases the percentage and rate of germination (Fig. 2).

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