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A comprehensive view of epicotyl dormancy in *Viburnum furcatum*: combining field studies with laboratory studies using temperature sequences

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Abstract

Seeds with epicotyl dormancy reside in soil up to 15 months (or longer), being exposed to a sequence of temperatures, before seedlings completely emerge (i.e. with both roots and shoots). Heretofore, few studies have examined precise temperatures, especially in sequences, for promotion of radicle and cotyledon emergence and how they relate to environmental cues in nature. *Viburnum* is the best known genus to exhibit epicotyl dormancy and, as such, we investigated the Japanese *V. furcatum*, hypothesizing a similar kind and level of dormancy. The underdeveloped embryos in mature seeds in October were spatulate shaped, unlike those in other *Viburnum* species, and they elongated from late June to August of the following year. Radicles emerged after embryo growth until mid-October, followed by cotyledons from mid-April to mid-May. Temperatures required for embryo growth, radicle and cotyledon emergence in the laboratory approximated closely those in the field. Embryo elongation and radicle emergence occurred at warm temperature regimes, and gibberellic acid (GA_3) did not substitute for this warm temperature requirements. Following a 120-d cold stratification of seeds with an emerged radicle, shoots emerged from seeds at 10, 15, 15/5, 20/10 and 25/15°C. We identified that seeds of *V. furcatum* have deep simple epicotyl morphophysiological dormancy like the majority of other *Viburnum* species. For propagation of the species from seeds, the nearly 2-year period for seedling emergence could be shortened to 8 months: start fresh seeds at 25/15°C (60 d) and then move them through a sequence of 15/5°C (30 d) → 0°C (120 d) → 20/10°C (30 d).

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Introduction

Most kinds of dormancy in seeds that readily imbibe water are broken by one type of treatment – usually warm stratification (after-ripening) or cold stratification – and radicles and cotyledons emerge simultaneously (Baskin and Baskin, 1998). Epicotyl dormancy is an exception. In this dormancy, the radicle emerges in response to a dormancy-breaking environmental cue but the epicotyl remains dormant for a few to several weeks until it is exposed to another dormancy-breaking environmental cue. In the deep, simple epicotyl morphophysiological dormancy (MPD), warm stratification is required for the underdeveloped embryo to elongate inside the seed for radicle emergence, and then cold stratification is needed for cotyledon emergence from seeds with an emerged radicle. In contrast, in seeds with non-deep, simple epicotyl MPD, cotyledons require either a period of warm stratification (Baskin *et al.*, 2008) or a very short period of cold stratification (Dhyani *et al.*, 2013) to emerge from seeds with an emerged radicle. Epicotyl dormancy is also known to occur in seeds with fully developed embryos, which do not need to grow inside the seed before radicle emergence (Jayasuriya *et al.*, 2010, 2012).

Regardless of whether the embryo is under- or fully developed, seeds with epicotyl dormancy reside in the soil for a relatively long time before seedlings emerge completely (i.e. with both roots and shoots). For example, complete seedling emergence takes 7 months for *Cimicifuga racemosa* or 11 months for *Gagea lutea* with late summer or late spring seed dispersal, respectively (Baskin and Baskin, 1985; Kondo *et al.*, 2004),

and 15 months or longer for *Viburnum acerifolium* or *V. opulus* with late autumn dispersal (Hidayati *et al.*, 2005; Walck *et al.*, 2012). During this time, seeds are exposed to a natural sequence of temperatures. Previous studies that have investigated the requirements to overcome epicotyl dormancy: (1) incubated seeds over a set (often narrow) of single constant or alternating temperatures; (2) stratified seeds at one temperature and then incubated them at a different temperature; and/or (3) used a move-along experiment (Takagi, 2001; Adams *et al.*, 2003; Mondoni *et al.*, 2009; Copete *et al.*, 2011; Mattana *et al.*, 2012). In a move-along experiment, a cohort of seeds is moved through a sequence that simulates the duration and temperatures of seasons from dispersal until radicles and then cotyledons emerge. This type of experiment has become a standard practice among biologists studying epicotyl dormancy, especially since it approximates the phenology of embryo growth, and radicle and cotyledon emergence, in nature.

A couple of studies on epicotyl dormancy have varied the temperature sequences (albeit to a limited amount) to better understand precisely which temperatures in an appropriate manner promote emergence of root or cotyledon. Kondo *et al.* (2005) showed that radicle emergence in *Corydalis ambigua* (Fumariaceae) was earlier and higher in seeds moved (→) through a sequence of 25/15 → 15/5 → 0°C than a sequence of 10 → 5°C. Baskin *et al.* (2009a) found that hypocotyl (which eventually produced a root) and cotyledon emergence of *Daphniphyllum glaucescens* (Daphniphyllaceae) was much earlier and slightly greater for seeds given a sequence of 15/6 → 20/10 → 25/15 → 20/10°C than 5/1 → 15/6 → 20/10 → 25/15°C or 25/15 → 20/10 → 15/6 → 20/10°C. Thus, responses of seeds vary depending on the temperature sequence.

Among plant genera, *Viburnum* (Adoxaceae; APG III, 2009) is the best known to exhibit epicotyl dormancy, with seeds of most species having deep, simple epicotyl MPD (Baskin *et al.*, 2009b; Moura and Silva, 2010; Chien *et al.*, 2011; Walck *et al.*, 2012). Moreover, members of this genus are important components of various types of vegetation in the temperate zone around the world and are in high demand in horticulture and restoration programmes. Yet, we know very little about the specifics of temperatures, especially sequences of temperatures, for complete seedling emergence in members of this genus. A comprehensive study of embryo growth, radicle emergence and cotyledon emergence in *Viburnum*, combining field studies on phenology with laboratory studies using a move-along experiment and using various temperature sequences, has not been done.

To fill this gap in knowledge, we selected as our study species *V. furcatum* Blume ex Maxim. This species is a deciduous broadleaved shrub found commonly in the understorey of *Fagus crenata* climax forests on the mountains of Japan (Hukusima *et al.*, 1995) and it regenerates by vigorous sprouting from buds at the base of the stem (Yamanaka and Tamai, 1986; Hara, 1990). Plants of this species growing in closed-canopy conditions have greatly reduced fruit production compared with those in gaps (Hara *et al.*, 1991). Fruits of *V. furcatum* are an important component in the diet of Japanese black bears (*Selenarctos thibetanus japonicus*) during summer months (Nozaki *et al.*, 1983). Therefore, regeneration of this species along forest edges and in gaps should be encouraged. However, to our knowledge, no information is available on how to regenerate this species from seeds, especially the requirements for seed dormancy break and germination. Thus, knowledge of seed germination ecology is necessary, if the species is to be used for restoration programmes.

In this study, we first documented the timing of embryo growth and of radicle and cotyledon emergence outdoors. To this end, we correlated these phenological events to recorded temperatures and observation on snow accumulation throughout the 21-month field study. Next, we performed a series of laboratory experiments. We conducted a move-along experiment to follow a cohort of seeds through an 'annual' temperature sequence starting in at a summer regime. We hypothesized that, like the majority of other *Viburnum* species, seeds of *V. furcatum* may also require a warm summer temperature for embryo growth and radicle emergence after seed dispersal in autumn, and a cold winter temperature for shoot emergence. We then examined the specific temperature requirements for embryo growth, radicle emergence and cotyledon emergence by using single constant and alternating temperature regimes, and by using sequences of 2–5 temperatures varying in duration. Two other experiments were done to determine: (1) the effects of gibberellic acid (GA_3) on radicle emergence; and (2) the duration of cold stratification required to promote cotyledon emergence. Using this information, we could also determine whether seeds of this species have non-deep or deep, simple epicotyl MPD. Finally, results of this study provided information for understanding methods of propagating the species from seeds for restoration programmes, as many native species are not used in restoration programmes due to the presence of dormancy and the lack of understanding of how to overcome it.

Materials and methods

Plant material

Viburnum furcatum is widely distributed from Japan to the Korean Peninsula, the Sakhalin Oblast (Russia) and

Taiwan (GBIF, 2011; USDA-ARS, 2011). In Japan, it grows in mesic woods and along edges of forests in mountainous regions (Ohwi, 1965). Mature black fruits were collected on 1 and 6 October 2007 from plants growing in woods near Sapporo, Hokkaido, Japan. Following collection of fruits, all seeds (true seed plus endocarp) were separated from the pulp (exocarp and mesocarp) and stored dry at ambient room conditions (*c*. 25°C) for 4–7 d before studies were initiated. Only visibly filled seeds were used in the study.

Field phenology study

The phenology of embryo growth and of radicle and cotyledon emergence was monitored, starting on 10 October 2007, in a non-temperature-controlled metal framehouse located outdoors on the campus of Hokkaido University (Sapporo, Japan). The framehouse was covered with shade cloth from mid-June until the last week of October, to simulate the approximate environment in woods, and it was not covered during winter and spring. Soil (1:1 v/v mixture of vermiculite and leaf mould) in the trays (length 42 cm × width 26 cm × depth 8 cm) and pots (diameter 18 cm × depth 15 cm) in which the seeds were buried (described later) was watered as needed to keep it moist throughout the study. Temperatures at the soil surface were measured in three places every 15 min throughout the study using thermo data loggers (RT-30S, Espec Mic, Aichi, Japan). Daily mean, maximum and minimum temperatures were calculated from these data. Snowfall covered the ground from 21 November 2007 through 3 April 2008 and from 24 November 2008 through 1 April 2009.

Phenology of embryo growth

Ten seeds, imbibed overnight, were cut into thin sections (40 μm) using an automicrotome. The embryo length of each seed was measured using a dissecting microscope equipped with a micrometer. On the same day, 15 other seeds were placed in each of 12 fine-mesh polyester bags (length 9.5 cm × width 7.0 cm) and buried at a soil depth of 3 cm in a tray in the framehouse. Thereafter, 10 seeds were removed at random from one bag about every 30 d until 9 June 2008 and at 15- to 21-d intervals until 28 August 2008, and embryo length was measured. On 28 August 2008, lengths of fully elongated embryos ($N = 10$, mean ± SE $= 4.62 \pm 0.23$ mm), i.e. embryo length just prior to radicle emergence, were recorded. The lengths of embryos during the experiment are expressed as percentages based on the lengths of fully elongated embryos.

Phenology of radicle emergence

Fifty seeds were placed in each of four fine-mesh polyester bags and buried 3 cm deep in soil in a tray in the framehouse. Seeds in the bags were examined for radicle emergence at 15-d intervals until 20 August 2008 and at 4- to 9-d intervals thereafter. Seeds with an emerged radicle were counted and removed from the bags, and then the others were reburied.

Phenology of cotyledon emergence

Fifty seeds were sown on the soil surface in each of four pots in the framehouse and covered with about 1 cm of sieved soil. Immediately after observing the first radicle emergence in the above experiment ('Phenology of radicle emergence') cotyledon emergence was monitored about every 15 d until snowfall. During winter, cotyledon emergence was not monitored under snow. Beginning immediately after snow melt, on 1 April 2009 (after two winters from seed sowing), cotyledon emergence was monitored at 3- to 5-d intervals.

Laboratory experiments

For all laboratory experiments, seeds were placed in plastic Petri dishes (diameter 90 mm × depth 10 mm) on three sheets of Whatman No. 1 filter paper moistened with distilled water. Petri dishes were sealed with Parafilm to reduce water loss during incubation. The daily photoperiod was 12 h in both constant and daily alternating temperature regimes. In the alternating temperature regimes, high temperature was given for 12 h in light each day and low temperature for 12 h in darkness. Seeds incubated at 0°C were kept in constant darkness. The light source, cool white fluorescent tubes, gave a photon irradiance (400–700 nm) at seed level of 15–20 $\mu mol\, m^{-2}\, s^{-1}$. Seeds were examined initially at intervals of 15–20 d for mould infection on their surface. If fungal infection occurred, seeds were washed thoroughly in sieves, first by running tap water and afterwards with distilled water, and then placed on a new set of filter papers. At each observation, germinated seeds (emerged radicle) were removed from the dishes after they were recorded. Water was added to dishes as needed to keep the filter paper moist. Except where noted, all experiments started on 10 October 2007. In all simulated temperature sequences for laboratory experiments, alternating maximum/minimum temperatures of 25/15°C for summer, 15/5°C for spring and autumn, and constant 0°C for winter were used, based on historical average monthly air-temperatures at Sapporo, Hokkaido, recorded over 30 years (1981–2010) (JMA, 2014). Although the maximum spring temperature at the soil surface in our outdoor experiment was about 5°C higher than data from the Japan Meteorological Agency, minimum and mean temperatures were almost the same. Since seeds were buried at a depth of 1–3 cm in soil,

the experienced temperatures in spring were assumed to be 15/5°C.

Effects of an 'annual' temperature sequence ('move along') on embryo growth, and radicle and cotyledon emergence

To monitor embryo growth, five Petri dishes containing 30 seeds each were placed in the following temperature sequence that simulates an 'annual' seasonal cycle beginning with warm temperature: summer (25/15°C, 60 d) → autumn (15/5°C, 60 d) → winter (0°C, 120 d) → spring (15/5°C, 60 d) → summer (25/15°C, 60 d). Two seeds were removed at random from each of the five dishes at 30-d intervals, and lengths of the 10 embryos measured as described previously. For radicle emergence, a set of four-replicate dishes (30 seeds each) were followed through the same temperature sequence. Seeds with an emerged radicle were counted and removed from the dishes at 2- to 3-d intervals, and then they were used for observations on cotyledon emergence. For observations on cotyledon emergence, four polyethylene containers (length 8 cm × width 8 cm × depth 5 cm), each with four 5-mm-diameter drainage holes in the bottom, were filled with soil (1:1 v/v mixture of vermiculite and leaf mould). The four containers corresponded to the four Petri dishes in which radicle emergence was recorded, i.e. all seeds with an emerged radicle from one Petri dish went into the same container. Seeds in Petri dishes with an emerged radicle were buried about 1 cm deep in soil in each container. Containers were watered from the bottom and covered with a transparent polythene bag with small puncture holes to reduce evaporation of water but to allow gas exchange. Cotyledon emergence was monitored weekly until winter temperature periods were finished and, thereafter, at daily intervals.

Temperature requirements for embryo growth

Five Petri dishes containing 100 seeds each were placed at three constant temperatures of 0, 15 and 25°C and at three sequences of temperature regimes: 15/5°C (60 d) → 0°C (30 d), 15/5°C (60 d) → 25/15°C (30 d) and 25/15°C (60 d) → 15/5°C (30 d). Two seeds were removed at random from each of the five dishes in each temperature treatment at 30-d intervals, and lengths of the 10 embryos measured as described previously. Embryos elongated fully within 90 d of incubation at the temperature sequence of 25/15°C (60 d) → 15/5°C (30 d), and constant 15°C; therefore, the experiment was terminated 90 d after the seeds were sown.

Effects of single temperature regimes on radicle emergence

Four replicates of 30 seeds were incubated at three constant (0, 15 and 25°C) and four alternating (15/5, 20/10, 25/15 and 30/20°C) temperature regimes. Observations on radicle emergence were made at weekly intervals. The experiment was terminated 300 d after sowing the seeds, at which time no additional radicles had emerged for 2 weeks.

Effects of various temperature sequences on radicle emergence

Four replicates of 30 seeds were subjected to each of the temperature sequences shown in Fig. 5, beginning with moderate autumn/spring (15/5°C), winter (0°C), or summer (25/15°C) temperatures. Seeds with an emerged radicle were counted at 3- to 4-d intervals. The experiment was terminated 330 d after seed sowing, at which time no additional radicles had emerged for 2 weeks.

Effects of various durations for simulated summer and autumn temperatures on radicle emergence

Four replicates of 30 seeds each were incubated at the following temperature sequences: (a) 25/15°C (0, 30 or 60 d) → 15/5°C (60 d) → 0°C (60, 30 or 0 d) (three summer periods); and (b) 25/15°C (60 d) → 15/5°C (0, 30 or 60 d) → 0°C (60, 30 or 0 d) (three autumn periods). The seeds were monitored for radicle emergence at 2- to 7-d intervals. The experiment was terminated 120 d after seed sowing, when radicles had emerged from approximately 80% of the seeds in treatment (b).

Effects of GA_3 on radicle emergence

Since 90 d of warm stratification at a temperature sequence of 25/15°C (60 d) → 15/5°C (30 d) was adequate for high radicle emergence, three replicates of 30 seeds were incubated at 0°C (60 d) + GA_3 (0, 100 or 1000 ppm) → 15/5°C (30 d) for 90 d to determine the ability of GA_3 to substitute (or not) for a warm stratification requirement in the dormancy-breaking process. The initial 0°C temperature was used because this temperature regimen is too low to be effective for warm stratification (Stokes, 1965). Filter paper in 90-mm-diameter Petri dishes was moistened with either distilled water (control, 0 ppm) or a solution of 100 or 1000 ppm GA_3, dissolved in distilled water by adding 2–3 drops of ethyl alcohol. The number of seeds with an emerged radicle was monitored at weekly intervals.

Effects of various durations of cold stratification on cotyledon emergence from radicle-emerged seeds

On 14 December 2007, four replicates of 80 seeds, each with an emerged radicle (1–11 mm long), were removed from the warm incubation treatments of two laboratory experiments (i.e. 'Effects of single temperature regimes on radicle emergence' and 'Effects of various temperature sequences on radicle emergence', see above). These radicle-emerged seeds (which would have been discarded) were placed in each of four fine-mesh polyester bags and buried 3 cm deep in soil

(1:1 v/v mixture of vermiculite and leaf mould) in a tray in the framehouse under ≥ 30 cm of snow for 30, 60, 90 or 120 d. In mid-January 2008, February 2008, March 2008 and April 2008, one bag was exhumed at random and the tray reburied under the same depth of snow. Exhumed seeds were rinsed with distilled water and then four replicates of 20 seeds each were buried in the same soil type at about 1 cm depth in polyethylene containers (length 5 cm × width 15 cm × depth 5 cm) and incubated in the laboratory at a simulated spring temperature of 15/5°C for 60 d. Cotyledon emergence was recorded at weekly intervals until cotyledon emergence was first observed and then at 2- to 4-d intervals until mid-June 2008.

Determination of optimum temperature for cotyledon emergence from radicle-emerged seeds

The previous experiment showed that radicle-emerged seeds required about 120 d of cold stratification at 0°C for a high percentage of cotyledon emergence. On 19 December 2007, approximately 315 radicle-emerged seeds (which would have been discarded), from three previous laboratory experiments (i.e. 'Effects of single temperature regimes on radicle emergence', 'Effects of various temperature sequences on radicle emergence' and 'Effects of various durations for simulated summer and autumn temperatures on radicle emergence') were collected and placed in six steel Petri dishes (diameter 90 mm × depth 15 mm) on four moist filter papers, and covered by four moist filter papers, for cold stratification at 0°C. After 120 d, the radicle-emerged seeds were rinsed with distilled water and buried about 1 cm deep in soil, as described previously. Three containers with 15 seeds each were transferred to 5, 10, 15, 15/5, 20/10, 25/15 and 30/20°C. Cotyledon emergence was monitored at 2- to 3-d intervals for 60 d.

Statistical analyses

Means and standard errors were calculated for percentage values for embryo length and for radicle and cotyledon emergence, which were based on the number of viable seeds (< 5% seeds rotted) and on the number of seeds with an emerged radicle, respectively. Data for embryo length and for radicle and cotyledon emergence were log or arcsine square-root transformed for analyses, but non-transformed data are shown in the figures. Levene's test of homogeneity revealed that transformations were unsuccessful in correcting heteroscedasticity for some experimental results. Thus, means were compared either by one-way analyses of variance (ANOVA, if data met assumption of homogeneity of variance) or by Welch's *F* test (Welch's ANOVA, if data violated the assumption of homogeneity of variance) followed either by the Tukey post-hoc tests (if equal variances assumed) or the Games–Howell post-hoc tests (if equal variances not assumed) ($P = 0.05$) using SPSS 16.0.2 (SPSS Inc., Chicago, Illinois, USA).

Results

Field phenology study

Embryos in mature seeds at the time of dispersal on 10 October 2007 were 1.1 ± 0.07 mm long, which was only 23.8 ± 1.5% of the length of the fully elongated embryo (4.6 ± 0.2 mm). Thus, embryo length increased about 320% between seed dispersal and radicle emergence (Fig. 1). At winter and spring temperatures, embryos did not grow. They grew rapidly (to 100.3 ± 5.2%) between 2 July 2008 and 28 August 2008, when the average maximum, minimum and mean daily temperatures were 25.2, 17.2 and 19.9°C, respectively (Figs 1 and 2). Radicles began to emerge immediately after embryo growth and by 10 October 2008 radicles had emerged from 91% of the seeds. During the period of radicle emergence (28 August–10 October), mean maximum, minimum and mean weekly temperatures were 23.1, 15.3 and 18.3°C, respectively (Fig. 2). Cotyledon emergence was observed from only 45% of the seeds during the second spring from mid-April to mid-May 2009, when mean maximum, minimum and mean weekly temperatures were 22.7, 4.0 and 10.1°C, respectively (Fig. 2). The low percentage (45%) of cotyledon emergence was caused by dense growth of *Marchantia polymorpha* L., which covered the soil surface and restricted easy emergence of cotyledons under field conditions.

Effects of an 'annual' temperature sequence ('move along') on embryo growth, and radicle and cotyledon emergence

Embryos grew rapidly to 93% of that of the fully elongated embryo during the first 60 d of incubation at summer (25/15°C) temperature and thereafter grew little (to 97%) when shifted to autumn (15/5°C) temperature of the sequence (Fig. 3). Radicle emergence began immediately after embryo elongation at summer temperature and occurred from 90% of the seeds by 91 d at autumn temperature. Cotyledon emergence was delayed until after winter temperature (0°C) and it occurred from 74% of the seeds at spring temperature (15/5°C) of the sequence.

Temperature requirements for embryo growth

During 90 d of incubation, embryos hardly grew at 0°C and they grew slightly, up to about 50% of their full

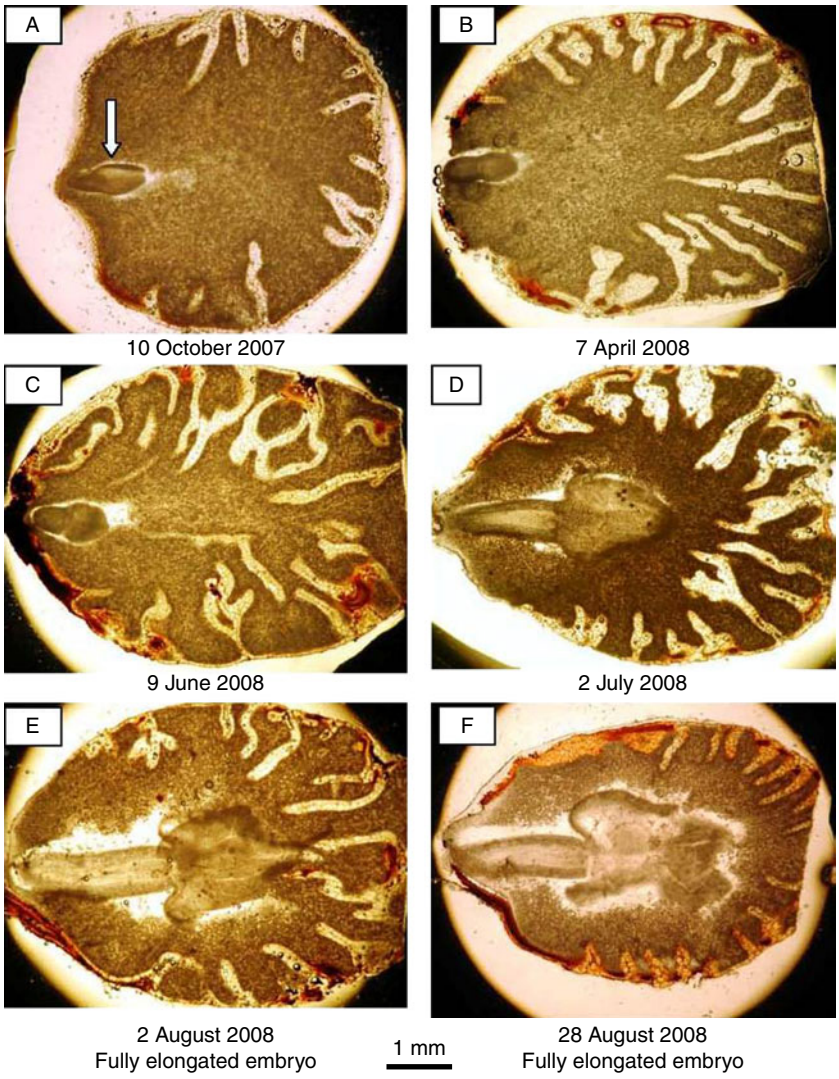

Figure 1. (colour online) Light micrographs of unstained embryo (arrow) in seeds of *Viburnum furcatum* kept outdoors in a framehouse in Sapporo, Japan. Seeds were buried in soil (1:1 v/v mixture of vermiculate and leaf mould) in trays.

length, by 90 d at temperature regimes of 25°C, 15/5°C (60 d) → 0°C (30 d) and 15/5°C (60 d) → 25/15°C (30 d) (Fig. 4A). However, by 60 d of incubation at temperature regimes of 15°C and 25/15°C (60 d) → 15/15°C (30 d), embryo growth (≥92%) was significantly higher (Welch's $F_{5,23.1} = 44.4$, $P < 0.001$) than at all other temperature regimes.

Effects of single temperature regimes on radicle emergence

During 60 d of incubation, radicles emerged from 50–55% of the seeds at 15, 20/10 and 25/15°C (Fig. 4B). However, radicles emerged from ≥75% of the seeds incubated for 300 d at 15, 20/10 and 25/15°C; from 32–41% of the seeds at 15/5 and 30/20°C; and from 11% at 25°C. Radicle emergence at 15, 20/10 and 25/15°C on 300 d of incubation was significantly higher ($F_{5,18} = 51.1$, $P < 0.001$) than it was at other temperature regimes. No radicles emerged from seeds incubated at 5°C (treatment excluded from statistical analyses).

Effects of various temperature sequences on radicle emergence

There was no significant ($F_{4,15} = 1.02$, $P = 0.430$) effect of sequences of temperatures on final percentage of radicle emergence (Fig. 5). During 300 d of incubation, radicles emerged from ≥85% of the seeds at all temperature sequences; however, 90 d of incubation were adequate for high (84%) radicle emergence at 25/15°C (60 d) → 15/5°C (60 d) → 0°C (120 d) → 15/5°C(60 d) → 25/5°C.

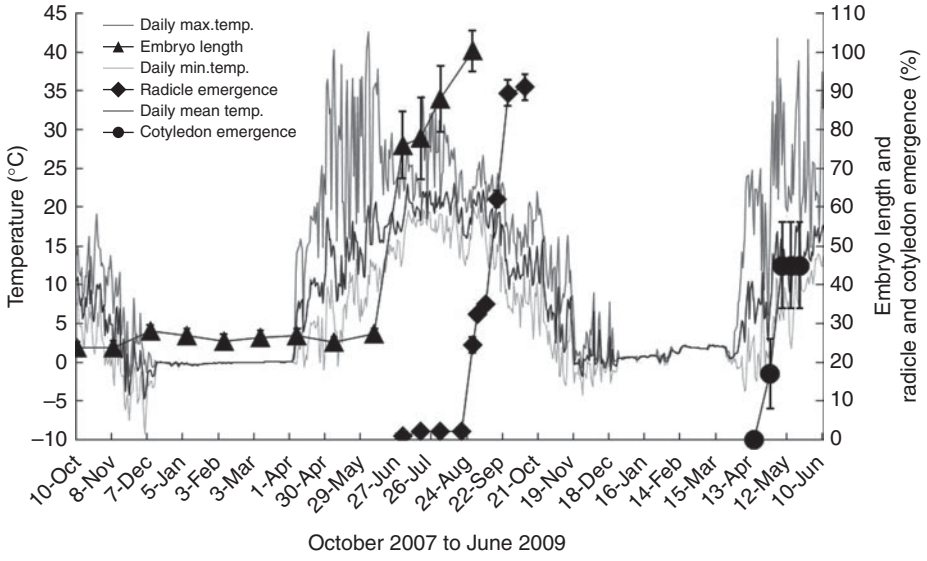

Figure 2. Daily maximum, minimum and mean temperatures and phenology of embryo growth, and of radicle and cotyledon emergence, in *Viburnum furcatum* seeds grown outdoors in a framehouse in Sapporo, Japan. Seeds were buried in soil (1:1 v/v mixture of vermiculate and leaf mould) in trays. Embryo length is given as a percentage of that of fully elongated embryos. Bars are ±1 SE. A lower percentage of cotyledon emergence was observed in the field experiment due to dense growth of *Marchantia polymorpha* L. on the soil surface in pots.

Effects of various durations for simulated summer and autumn temperatures on radicle emergence

During 120 d of incubation, only 2% of radicles emerged when seeds were not exposed to summer (25/15°C) temperature. However, seeds exposed to summer temperature for 30 or 60 d had significantly ($F_{2,9} = 115.7$, $P < 0.001$) higher (≥79%) radicle emergence in the temperature sequence 25/15°C (30 or 60d) → 15/5°C (60d) → 0°C than those not exposed to 25/15°C within the same temperature sequence (Fig. 6A). There was no significant ($F_{2,9} = 2.69$, $P = 0.121$) effect of duration of simulated autumn on radicle emergence (73 to 87%) in the temperature sequence 25/15°C (60 d) → 15/5°C (0, 30 or 60 d) → 0°C (Fig. 6B).

Effects of GA_3 on radicle emergence

Radicles did not emerge from any seeds at temperature sequence of 0°C (60 d) → 15/5°C (30 d) within 90 d on incubation at any GA_3 concentration (data not shown).

Effects of various durations of cold stratification on cotyledon emergence from radicle-emerged seeds

The radicle-emerged seeds had to be exposed to cold stratification (at 0°C, under snow) for cotyledon emergence. No cotyledon emerged from any seeds when cold stratified for 30 d (treatment excluded from statistical analyses). After 60 d of cold stratification, cotyledons emerged from only 3% of seeds. As the duration of cold stratification period increased from 90 to 120 d, cotyledon emergence increased significantly (Welch's $F_{2,4.8} = 82.3$, $P < 0.001$) from 25 to 74% (data not shown).

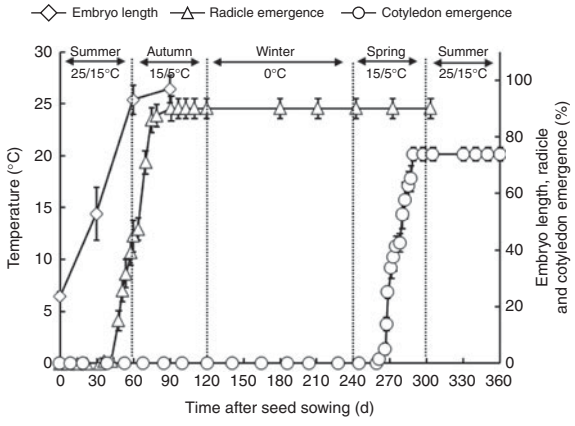

Figure 3. Effects of a simulated 'annual' temperature sequence on embryo growth and radicle and cotyledon emergence of *Viburnum furcatum*. Seeds were placed in sealed plastic Petri dishes and incubated with a 12-h daily photoperiod (except 0°C) at the temperature sequence: 25/15°C [day/night] (60 d) → 15/5°C(60 d) → 0°C(120 d) → 15/5°C(60 d) → 25/15°C(60 d) for 360 d. Seeds with an emerged radicle were buried in soil in a tray for cotyledon emergence. Embryo length is given as explained for Fig. 2. Bars are ±1 SE.

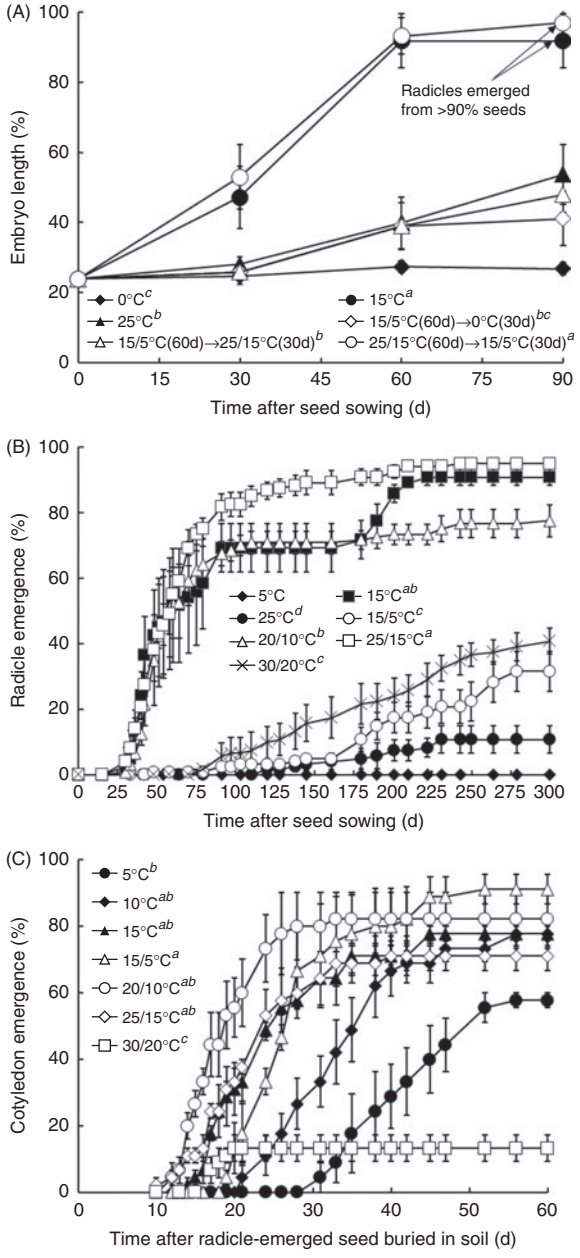

Figure 4. Effects of different constant and alternating temperatures and/or temperature sequences on (A) embryo growth, (B) radicle emergence and (C) cotyledon emergence of *Viburnum furcatum* seeds. Embryo length is given as explained for Fig. 2. Bars are ±1 SE. Temperature regimes followed by the same letter are not significantly different [(A) Welch's *F* test followed by the Games–Howell post-hoc test, and (B, C) one-way ANOVA followed by Tukey's post-hoc test, $P < 0.001$].

Determination of optimum temperature for cotyledon emergence from radicle-emerged seeds

During 60 d of incubation, only 13% of cotyledons emerged from seeds with an emerged radicle at 30/20°C (Fig. 4C). Cotyledon emergence was initially slow at 5°C, but it increased to 58% within 60 d of incubation. After 30 d of incubation at 15/5° and 20/10°C, cotyledons had emerged from ≥71% of seeds, and at 15° and 25/15°C from 64%. By 60 d of incubation, the final percentage of cotyledon emergence at 5° and 30/20°C was significantly lower ($F_{6,14} = 31.7$, $P < 0.001$) than at all other temperature regimes.

Discussion

Phenology of seedling emergence

Like the majority of *Viburnum* species (Giersbach, 1937; Barton, 1958; Hidayati *et al.*, 2005; Walck *et al.*, 2012), *V. furcatum* seeds required a long period (21 months) for complete seed germination (shoot emergence) under field conditions in Hokkaido, Japan. Embryos in freshly matured seeds of this species in autumn were underdeveloped and failed to grow during the following winter and spring temperatures. However, they elongated fully during mid-summer at an average maximum/minimum temperature of about 25/17°C (Figs 1 and 2). Similarly, in laboratory experiments, embryos elongated fully within 60 d at a continuous temperature of 25/15°C or 15°C (Fig. 4A). Immediately after embryo growth, radicles began to emerge during late summer and continued to do so until mid-October, at an average maximum/minimum temperature of 23/15°C. In laboratory conditions, rapid and high emergence of radicles took place when seeds were

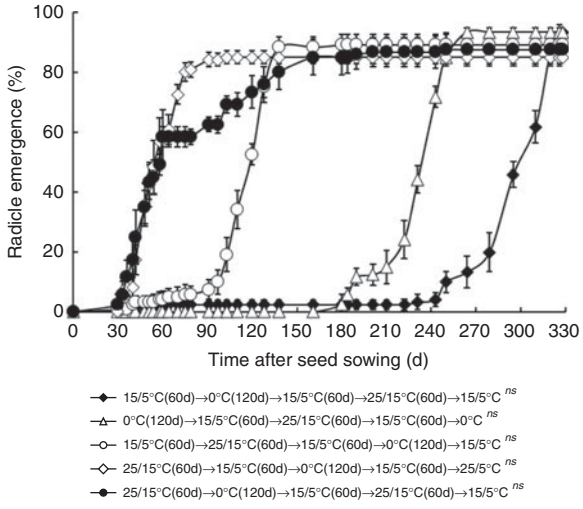

Figure 5. Effects of various temperature sequences on radicle emergence in seeds of *Viburnum furcatum*. Seeds were incubated on moist filter paper in sealed plastic Petri dishes. Temperature regimes are given as day/night temperature (number of days), as explained for Fig. 3. Bars are ±1 SE. Temperature regimes followed by 'ns' indicates not significant (one-way ANOVA, $P = 0.43$).

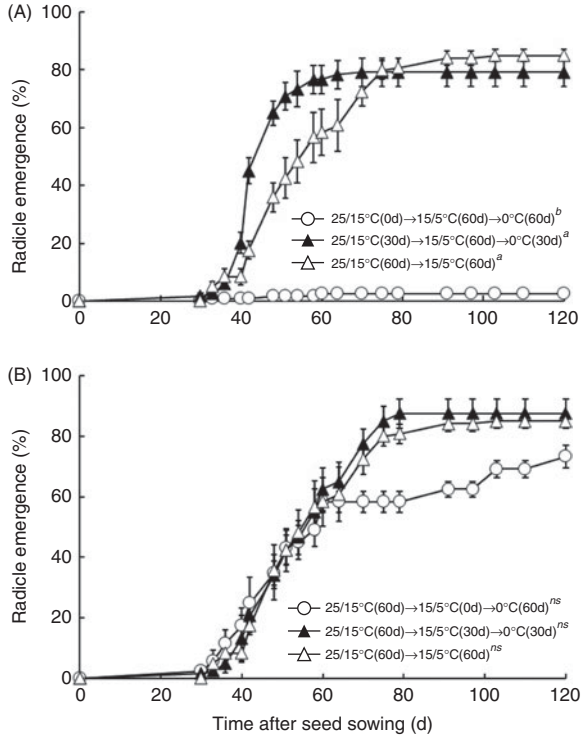

Figure 6. Effects of duration of simulated (A) summer (for 0, 30 and 60 d) and (B) autumn (for 0, 30 and 60 d) temperature sequences on radicle emergence of *Viburnum furcatum* seeds. Seeds were incubated on moist filter paper in sealed plastic Petri dishes. Temperature regimes are given as day/night temperature (number of days), as explained for Fig. 3. Bars are ±1 SE. Temperature regimes followed (A) by a different letter are significantly different (one-way ANOVA followed by Tukey's post-hoc test, $P < 0.001$), and followed (B) by 'ns' indicates not significant (one-way ANOVA, $P = 0.121$).

incubated continuously at 25/15°C or 15°C or in a temperature sequence that included 25/15 → 15/5°C (Figs 4B, 5 and 6). Cotyledon emergence from *V. furcatum* seeds was delayed for 8 months after radicle emergence. Under field conditions, emergence of cotyledons was observed from mid-April to mid-May after snowmelt of the second spring after seed dispersal; average maximum/minimum temperature was about 23/4°C (Fig. 2). In the laboratory, cotyledons emerged most rapidly at 20/10°C and highest at 15/5°C (Fig. 4C). Further, exposure to cold stratification was required for cotyledon emergence, with 120 d being optimum in the durations tested. Thus, temperatures in our laboratory experiments related to embryo growth, radicle emergence and cotyledon emergence approximated closely those in the field.

Phenology of radicle and cotyledon emergence with maximum/minimum temperatures is available for only one other species of *Viburnum* (Hidayati *et al.*, 2005). Radicles from seeds of *V. acerifolium* planted in a non-heated greenhouse (Kentucky, USA) in November 1998 emerged in October 1999, when average maximum/minimum temperature was 19/9°C. Peak cotyledon emergence from this cohort of seeds occurred in early January 2000, when the average temperature was 10/5°C. Temperatures associated with radicle and cotyledon emergences under near-natural conditions were higher for *V. furcatum* seeds than for *V. acerifolium* seeds. This allowed radicle emergence to be earlier in autumn and cotyledon emergence to be later in spring for *V. furcatum* as compared to *V. acerifolium*.

Specific temperature requirements

Elongation of embryos (to their full extent) and radicle emergence occurred fastest and most frequently at 15, 20/10 and 25/15°C, averaging within the range of 15–20°C (Figs 4 and 5). Temperatures of 0, 5, 15/5 (i.e. ≤ 10°C) or 25°C and 30/20°C (i.e. warm temperature with a mean of 25°C), did not effectively promote embryo growth or radicle emergence. Moreover, embryos fully elongated and high radicle emergence occurred at the sequence 25/15°C (60 d) → 15/5°C (30 d) as compared to 15/5°C (60 d) → 25/15°C (30 d). In fact, both processes were not promoted until seeds were given 25/15°C, for at least 30 d, whatever the order of temperatures before or after 25/15°C (Figs 5 and 6). Thus, there is a relatively narrow temperature range during which embryos grow and radicles emerge. At the time of dispersal in mid- to late-autumn, temperatures are too low to promote embryo growth and radicle emergence (Fig. 2). Both of these processes were observed in the field when the daily mean habitat temperature was consistently ≥15°C, which started in June. In contrast, radicle emergence in the Japanese *Corydalis ambigua*, which has deep simple epicotyl MPD, occurred at 0 and 5°C, regardless of whether the sequence started at 10°C or at 25/15 → 15/5°C (Kondo *et al.*, 2005). With this low temperature requirement, radicle emergence in this species occurs in March–April in Hokkaido, Japan and much earlier than that in *V. furcatum*.

Shoot emergence occurred over a broader range of temperatures than embryo growth and radicle emergence in *V. furcatum*. Following a 120-d cold stratification period, shoots emerged highest at 10, 15, 15/5, 20/10 and 25/15°C, as compared to 5 and 30/20°C, approximating a range of 10–20°C (Fig. 4C). Thus, shoot emergence in nature is restricted to the second spring following dispersal when habitat temperatures are ≥10°C (Fig. 2). Unlike *V. furcatum*, cotyledon emergence in the Taiwanese *D. glaucescens*, which has non-deep simple epicotyl MPD, did not require cold stratification and occurred at 15/6 and 20/10°C but not at 5/1 or 25/15°C (Baskin *et al.*, 2009a). Faster emergence of cotyledons occurred at a sequence of 15/6 → 20/10 → 25/15 → 20/10°C than if the

sequence started at 5/1 or 25/15°C. Although the cold stratification requirement differs between these two species, the temperatures for cotyledon emergence were similar. Further, in many species with morpho-physiologically dormant seeds, alternating temperatures are more effective for dormancy break and germination (Kondo *et al.*, 2004, 2005, 2006; Vandelook *et al.*, 2007; Phartyal *et al.*, 2009, 2012) than constant temperatures. An exception to this general situation is *Trillium camschatcense*, the seeds of which germinated better with constant temperatures (Kondo *et al.*, 2011). In *V. furcatum* both alternating and constant temperatures within the optimum temperature range were equally effective for dormancy break and germination (Fig. 4). This constant and alternating temperature response could be associated with the microclimate of the natural habitats of *V. furcatum*, since it grows both in gaps and under the closed canopy of *F. crenata* climax forests on the mountains of Japan (Hara *et al.*, 1991; Hukusima *et al.*, 1995).

Classification of dormancy and embryo morphology

The length of embryos in *V. furcatum* increased about 320% between seed dispersal and completion of embryo growth (prior to radicle emergence). This percentage falls within the range of that reported in other *Viburnum* species: 294% in *V. acerifolium* (Hidayati *et al.*, 2005), 300% in *V. odoratissimum* (Baskin *et al.*, 2008), 640% in *V. parvifolium* and 650% in *V. betulifolium* (Chien *et al.*, 2011) and 725% in *V. tinus* (Karlsson *et al.*, 2005). Thus, embryo elongation was required prior to radicle emergence, indicating that seeds of *V. furcatum* have morphological dormancy (MD). In addition, warm temperatures were required to break the first part of physiological dormancy (PD), thereby allowing embryo growth and subsequently radicle emergence, and GA_3 did not substitute for this temperature requirement. Cotyledon emergence from seeds with an emerged radicle was temporally separated, and required cold temperatures to overcome the second part of PD. Seeds containing an underdeveloped embryo with a warm + cold temperature requirement for radicle and cotyledon emergence are indicative of species with epicotyl MPD. Two levels of this MPD are recognized: deep simple epicotyl MPD and non-deep simple epicotyl MPD (Baskin *et al.*, 2009b). Given that GA_3 did not overcome dormancy and that a long period of cold stratification was required for cotyledon emergence, we conclude that seeds of *V. furcatum* have deep simple epicotyl MPD, like the majority of other *Viburnum* species.

The embryo morphology described for *Viburnum* species is 'linear', i.e. the embryo in freshly matured seeds is longer than wide with the cotyledons not expanded (Martin, 1946; Baskin *et al.*, 2009b; Moura and Silva, 2010; Chien *et al.*, 2011). In contrast, the embryo in *V. furcatum* seeds has expanded cotyledons that are wide relative to the radicle end. This shape is evident in fresh seeds on 10 October 2007 (Fig. 1A) and especially on 9 June 2008 (Fig. 1C); the cotyledons are not expanded in the embryo on 7 April 2008 but this might be due to the way in which the section was made. This type of embryo morphology would be classified as 'spatulate', though Martin (1946) illustrates that the size of the expanded cotyledons relative to the radicle stalk is more disproportionate (closely resembling a spoon) than what we observed in *V. furcatum*. To the best of our knowledge, this is the first report of spatulate embryos in *Viburnum*. Within Adoxaceae, seeds in the genus *Sambucus* also contain underdeveloped, spatulate-shaped embryos (Hidayati *et al.*, 2000). In contrast to *V. furcatum*, spatulate embryos in *Sambucus* species are *c*. 60% the length of the seed when freshly matured.

Practical implications

The results of our work have practical implications for propagating and cultivating this species (and probably other *Viburnum* species) in conservation and restoration programmes. The phenology study of *V. furcatum* outdoors revealed that seeds required about 1 year for embryo growth (first stage) and for radicle emergence (second stage), and about 1.9 years for cotyledon emergence (third stage) after seed dispersal (Fig. 2). Each of these three stages required specific temperature regimes. By using a modified move-along temperature sequence treatment in an incubator or greenhouse, this period of nearly 2 years could be shortened to approximately 8 months, with high emergence expected. We suggest starting fresh seeds at 25/15°C (for 60 d) and then moving them through a sequence of 15/5°C (30 d – see Fig. 4A) → 0°C (120 d) → 20/10°C (30 d – see Fig. 4C). The information on dormancy break and seed germination, both under controlled laboratory and *ex-situ* field conditions, will be of considerable value to seed biologists, restoration ecologists and forest managers who want to propagate the species from seeds.

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Conflicts of interest

None.

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