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Seed germination ecophysiology of two western North American species of *Sambucus* (Caprifoliaceae sensu lato), and comparisons with eastern North American and European congeners

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Abstract

Seeds of the western North American (wNA) species *Sambucus caerulea* in the *Sambucus nigra* species complex and *Sambucus callicarpa* in the *Sambucus racemosa* complex contain embryos that are 70–75% fully developed when freshly matured. These embryos must elongate approximately 15–25% before radicle emergence occurs. Embryos in seeds of both species grew better at 5°C than at 25/15°C. Dormancy was broken by cold stratification at 5°C, which was also the optimal temperature for germination. Gibberellic acid substituted for cold stratification in both species. Thus, seeds of both species have intermediate complex morphophysiological dormancy (MPD). The seed dormancy characteristics of both species differ from those of congeners in eastern North America (eNA). In contrast, dormancy break in *S. callicarpa* is similar to its European congeners, but is unclear in *S. caerulea*. Our study represents the first to compare seed dormancy among wNA, eNA and European congeners. In North America, the geographic pattern of dormancy break for *Sambucus* is similar to that of *Osmorhiza* (Apiaceae) and *Erythronium* (Liliaceae): cold stratification is required in wNA taxa and warm + cold stratification is required in eNA taxa. Moreover, the pattern in *S. callicarpa* is similar to that in some species of *Sanicula* (Apiaceae): cold stratification in Europe and warm + cold stratification in eNA. The evolution of seed dormancy in these groups might have occurred under similar environmental circumstances, resulting in the sharing of dormancy-breaking mechanisms within geographical regions.

Keywords: Caprifoliaceae, dormancy evolution, morphophysiological seed dormancy, stasis, western North America.

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Introduction

Intercontinental disjunctions coupled with phylogenies have served an important role in advancing our understanding of the evolution of seed dormancy (Wen *et al*. 2002). Disjunction in the ranges of congeneric species provide a unique system for understanding the origin and evolution of allopatric taxa (Wen *et al*. 1996) and their physiological traits, such as seed dormancy and germination. Similarity in the traits of taxa from disjunct regions implies that very little change has occurred since their divergence (i.e. ecophysiological stasis), whereas differences in traits suggest that change has occurred probably in response to selection (Walck *et al*. 2002). Moreover, mapping seed traits on independent phylogenetic trees of genera with intercontinental disjunctions has allowed ancestral versus derived states to be determined and let hypotheses on evolutionary trends in seed dormancy to be developed (Wen *et al*. 2002).

Examination of the biology of disjunct species is best done within a broad biogeographic context. Many species

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showing the classical disjunction between eastern North America (eNA) and eastern Asia (eA), also have congeneric members in western North America (wNA) or Europe (Thorne 1972). Contrary to morphological comparisons, molecular data suggest closer evolutionary links between species in eNA and wNA than between those in North America and Asia (Wen 1999). In the eNA–wNA–eA biogeographic pattern, seed dormancy was similar between wNA and eA congeners in *Osmorhiza* (Baskin & Baskin 1984, 1991; Baskin *et al*. 1995; Walck *et al*. 2002; Walck & Hidayati 2004), but it was similar between eNA and eA in *Aristolochia* (Adams *et al*. 2005) and in *Erythronium* (Baskin & Baskin 1985, 1998; Baskin *et al*. 1995; Kondo *et al*. 2002).

Many species in temperate regions of the Northern Hemisphere have seeds, when freshly ripened, with embryos that must elongate before the radicle emerges (Nikolaeva 1977; Baskin & Baskin 1998). If their embryo grows and the radicle emerges in approximately 30 days at suitable environmental conditions, then the seeds are classified as having morphological dormancy (MD). In contrast, the embryo might require a treatment such as exposure to low (0–10°C) and/or high (≥15/6°C) temperatures to overcome a physiological block for embryo growth and radicle emergence. In this case, the seeds are morphophysiologically dormant (MPD). Several levels of MPD have been described and distinguished based on: (i) the temperatures required for embryo growth and for dormancy release; and (ii) whether gibberellic acid (GA_3) overcomes dormancy (Baskin & Baskin 1998; Baskin *et al*. 2008). In seeds of many species with MD or MPD, the embryo is considerably less than 50% the length of the seed (Baskin & Baskin 2007). However, in other species, such as those in the genus *Sambucus*, the embryo is occasionally >50% the length of the seed (Hidayati *et al*. 2000).

In the present study, we examined the seed dormancy pattern in a genus with a here-to-fore unstudied geographic pattern: eNA–wNA–Europe. The genus *Sambucus* (Caprifoliaceae sensu lato, Viburnaceae, Adoxaceae or Sambucaceae; Benko-Iseppon & Morawetz 2000) occurs in Africa, the Asian–Malesian Region, Australia, the Caribbean Islands, Europe, and from North America to South America, but is primarily a genus of the Northern Hemisphere (Ferguson 1966; Bolli 1994). Prior to the present study, requirements for overcoming seed dormancy and for germination were investigated for members of the genus growing in eNA and Europe. Seeds of eNA *S. canadensis* (*S. nigra* ssp. *canadensis*) have deep simple MPD, that is, warm followed by cold stratification is needed to break dormancy, embryos grow at warm temperatures, and GA_3 substitutes for warm, but not cold stratification (Hidayati *et al*. 2000). In contrast, seeds of *S. racemosa* from Europe (*S. racemosa* ssp. *racemosa* var. *racemosa*; Gleason & Cronquist 1991) have intermediate complex MPD (i.e. dormancy break and embryo growth occur during cold stratification, and GA_3 overcomes dormancy) and those from eNA (*S. racemosa* ssp. *pubens* var. *pubens*) had deep simple MPD.

We selected two *Sambucus* species from wNA to fill the gap in our understanding of seed dormancy in this genus: *S. caerulea* Raf. and *S. callicarpa* Greene. *Sambucus caerulea* (or *S. cerulea*) occurs from British Columbia (Canada) to western Montana (USA), and south to California, Arizona and New Mexico (Cronquist *et al*. 1984). Although some taxonomic treatments consider *S. caerulea* a distinct species (Cronquist *et al*. 1984; Pojar & MacKinnon 1994), Bolli (1994) designated it as a subspecies of the near-cosmopolitan *S. nigra* complex (*S. nigra* L. ssp. *cerulea* [Raf.] R. Bolli). The geographic range of *S. callicarpa* is mostly along the Pacific Coast from Alaska to California (Hitchcock & Cronquist 1973; Brayshaw 1996). Although some authors recognize the plant as a distinct species (Farrar 1995), others place it as an infraspecific taxon of *S. racemosa* (*S. racemosa* var. *racemosa*, *S. racemosa* ssp. *pubens* var. *arborescens*; Hitchcock & Cronquist 1973; Bolli 1994). Based on morphological and molecular data, taxa within the *S. nigra* complex, with the exception of *S. caerulea*, and within the *S. racemosa* complex form a clade (Eriksson & Donoghue 1997).

Previous work on seeds of *S. caerulea* and *S. callicarpa* suggests that they are dormant at maturity and that cold or warm + cold stratification overcomes it (Brinkman 1974; Clancy & Maguire 1979; Norton 1986; Taylor 2002). Given that embryos in the genus, although relatively longer than classical underdeveloped embryos, must elongate before radicle emergence (Martin 1946; Brinkman 1974; Tylkowski 1982; Hidayati *et al*. 2000), we hypothesized that seeds of *S. caerulea* and *S. callicarpa* would have MPD. Thus, the first purpose of the present study was to confirm whether seeds of these two species have MPD and, if so, classify its level (sensu Baskin & Baskin 1998). Specifically, the temperature requirements for dormancy break and germination; conditions under which embryo growth, if any, occurs prior to radicle emergence; and whether GA_3 overcomes dormancy were compared between species and/or collection sites. The phenology of germination was examined for *S. callicarpa*. The second purpose of the present study was to compare the seed biology of these two wNA species of *Sambucus* to their eNA and European congeners.

Materials and methods

Fruit collection, cleaning and storage

Unless otherwise stated, ripe fruits of *S. caerulea* were collected near St Helens in Columbia County, Oregon, USA, on 12 August 2000 and near Port Townsend in Jef-

ferson County, Washington, USA on 3 October 2005, and those of *S. callicarpa* were collected near Tillamook in Tillamook County, Oregon, on 11 August 2000 and near Port Townsend in Jefferson County, Washington on 8 July 2004. When collected, the pulp of the fruit was soft and entirely blue (*S. caerulea*) or red (*S. callicarpa*). The exocarp and mesocarp were removed from the fruits of both species, and then the seeds (true seed plus endocarp, hereafter referred to as seeds) were stored dry in glass jars under laboratory conditions (21°C) for up to 3 weeks until studies were initiated. Seeds in all lots were placed into a beaker of water and seeds that floated were removed because they contained no embryo; those that sank to the bottom of the beaker contained embryos and were used in the experiments.

Laboratory set-up and general procedures

Five temperature-controlled and light-controlled incubators and two refrigerators equipped with a light and time clock were used. The incubators were set at 12 h/12 h daily alternating thermoperiods of 15/6, 20/10, 25/15, 30/15 and 35/20°C and the refrigerators were set at a constant 1 or 5°C. Temperatures in the incubators approximated mean daily maximum and minimum monthly air temperatures in the seed collection areas: winter, 5; early spring and late autumn, 15/6; late spring and early autumn, 20/10 and summer, 25/15°C (Peterson & Vose 1997). Cool white, 20-W fluorescent tubes, which produced a photon flux density (400–700 nm) at seed level of approximately 50–70 $\mu mol/m^2/s$, were used as the light source in the incubators; 15-W tubes producing approximately 10 $\mu mol/m^2/s$ were used in the refrigerators. The daily photoperiod was 14 h in the incubators and refrigerators. At the alternating temperatures, the photoperiod extended from 1 h before the beginning of the high temperature period to 1 h after the beginning of the low temperature period.

Seeds were placed in plastic Petri dishes (diameter 6.0 cm) on white quartz sand for germination studies or in plastic (or glass) Petri dishes (diameter 10.0 cm) on two sheets of Whatman Number 1 filter paper for embryo growth and GA_3 studies. Both the sand and filter papers were moistened with distilled water before the seeds were placed on them. All dishes were wrapped with plastic film to restrict water loss during incubation and stratification, and water was added to the dishes as needed. Three replicates of 25 seeds per dish were used in each treatment for the germination studies, and one dish of 25 seeds was used each time measurements were made in the embryo-growth studies.

Emergence of the radicle was the criterion for germination. The viability of ungerminated seeds was determined by pinching them with forceps under a dissecting microscope to see if they contained firm, white (viable) embryos or soft, light brown (non-viable) ones. Tetrazolium tests (Grabe 1970) confirmed that white embryos were viable and that brown ones were not. Germination data were transformed to percentages on the basis of the number of viable seeds. For the embryo-growth studies, embryos were excised from seeds with a razor blade and their lengths were measured under a dissecting microscope equipped with a micrometer.

Dormancy break and germination requirements

Three experiments were carried out to examine the type(s) of stratification needed for dormancy break and the temperature regimes required for germination. The 25/15°C thermoperiod was used for warm stratification and 5°C was used for cold stratification because these temperatures are near optimal for breaking dormancy of many species whose seeds require warm or cold temperatures, respectively, to come out of dormancy (Stokes 1965). In preliminary studies, 1°C did not promote dormancy break and/or germination in seeds of *S. caerulea* and *S. callicarpa* (S. N. Hidayati & J. L. Walck, unpubl. data, 2001).

Seeds of both species collected in 2000 were incubated in the light at 5, 15/6, 20/10, 25/15, 30/15 and 35/20°C for 2 weeks. The fresh seeds remained at these thermoperiods for a total of 26 weeks, and were examined at 2-week intervals and the germinated seeds were counted and removed. As some seeds of both species are dispersed in late summer, they could experience several weeks of warm stratification before they are exposed to cold-stratifying temperatures. Thus, seeds were: (i) warm stratified in light for 6 and 12 weeks; (ii) cold stratified in light for 6 and 12 weeks; and (iii) given the following warm + cold combinations in light: 6-week warm + 6-week cold, 6-week warm + 12-week cold, 12-week warm + 6-week cold and 12-week warm + 12-week cold. Following all stratification treatments for each species, seeds were incubated in light at 15/6, 20/10, 25/15, 30/15 and 35/20°C for 2 weeks.

For the second experiment, seeds of *S. caerulea* collected in 2005 were placed in light at 5, 15/6, 20/10, 25/15, 30/15 and 35/20°C, and those of *S. callicarpa* collected in 2004 were incubated at 5, 15/6, 20/10 and 25/15°C. At 2-week intervals, they were examined and seedlings were counted and removed for a total of 48 weeks.

In the third experiment, *S. caerulea* seeds collected in 2005 and *S. callicarpa* seeds collected in 2004 were placed for 4, 8 and 12 weeks at 25/15°C ('pretreatment') in light followed by 36 weeks at 5°C in light and the seeds were monitored for germination every 2 weeks. In addition, seeds that acted as controls were also maintained at 5 and 25/15°C in the light for 36 weeks, and scored for germi-

nation at 2-week intervals. Seedlings, when present, were counted and removed from the dishes.

Effects of GA_3 on dormancy break and germination

Seeds of *S. caerulea* collected in 2005 ($n = 25$ per dish) and *S. callicarpa* collected in 2004 ($n = 50$) were placed on filter paper moistened either with distilled water (control) or with a solution of 10, 100 or 1000 mg/L of GA_3 (K-GA_3) dissolved in distilled water in glass Petri dishes. Three replicates (Petri dishes) were used per treatment. To test whether GA_3 could substitute for warm or cold stratification, 25/15°C was used because it is too high to be effective for cold stratification and 5°C was used because it is too low for warm stratification (Stokes 1965). Seeds were incubated in light on distilled water or the GA_3 solutions at 5 or 25/15°C for 36 weeks. Germination was monitored at 4-week intervals.

Requirements for embryo growth

Two experiments were conducted to examine the conditions required for embryo growth. At the start of each experiment, the lengths of the seeds and embryos were determined for 25 seeds of each species 24 h after they had been placed on moist filter paper at room temperature. For the first experiment, 25 seeds of each species, collected in 2000, were placed in each of two Petri dishes and one dish was incubated at 5°C and the other at 25/15°C. Embryos were measured after 24 weeks at these two temperatures. In the second experiment, *S. caerulea* seeds collected in 2005 ($n = 25$ per dish) and *S. callicarpa* seeds collected in 2004 ($n = 25$) were placed in each of five Petri dishes. One dish each was incubated at 5 and 25/15°C for 20 weeks. The other three dishes were placed at 25/15°C and after 4, 8 and 12 weeks of incubation ('pretreatment') were moved to 5°C for 20 weeks and then measured. For both experiments, if a seed had germinated during the study, embryo length was recorded as the critical length required for germination (i.e. when embryos had grown enough to split the seed coat and endocarp).

Phenology of germination

Seeds of *S. callicarpa*, collected in July 2004 near Seattle in King County, Washington, were depulped and then placed into water to separate seeds that were empty (i.e. seeds that floated in water) from those that contained endosperm and embryo (i.e. seeds that sunk). Three hundred seeds each were sown on and lightly covered with soil and leaf litter collected from beneath *S. callicarpa* plants in two plastic Anderson propagation flats (36.8 cm [width] × 45.7 cm [length] × 6.4 cm [depth]; Anderson Die and Manufacturing Inc., Portland, Oregon) on 20 July 2004. The trays were placed out-of-doors in a cold frame (at the Seattle Parks and Recreation Department facilities, Seattle, Washington), which was covered with plastic roll-up covers for ventilation, and the soil was kept moist. At weekly intervals all germinated seeds were counted and removed from the trays until 24 May 2005. Mean maximum and minimum daily air temperatures for each week of the study were calculated from data collected at the Seattle–Tacoma International Airport, approximately 9 km from the study site (National Weather Service 2009).

Statistical analyses

Means and standard errors were calculated for germination percentages and embryo lengths. Germination percentages were arcsine square-root transformed for the statistical analyses. In the first germination experiment, ANOVAs tested whether responses to treatments (condition [fresh, control and eight combinations of warm + cold stratification], temperature regime) differed between species (3-way ANOVA) or within each species (2-way ANOVAs) (SPSS 2000). As germination in the second and third experiments and in the GA_3 experiment was followed over incubation time (within-subject factor), means were compared using repeated-measure (RM) ANOVAs and Greenhouse–Geisser corrected probabilities are reported. Between-subject factors in the RMANOVAs were: temperature regime for the second experiment and condition (0 [control at 5°C or at 25/15°C], 4, 8 or 12 week pretreatment) for the third experiment and concentration and temperature regime for the GA_3 experiment. If the experimental set-ups were identical for the two species, the term species was also included as a between-subject factor in the RMANOVA. Protected least significant tests (PLSDs, $P = 0.05$) followed all analyses, and *t*-tests compared sizes of seeds and of embryos between species and between sites or of embryos at the beginning and at the end of the treatments.

Results

Dormancy break and germination requirements

In the first experiment, germination responses significantly differed between species and among conditions and temperature regimes within each species (all factors and interactions, $P < 0.001$). No fresh (non-stratified) seeds from either species germinated during 2 weeks of incubation at 5–35/20°C (Table 1). In contrast, 32 and 67% of *S. caerulea* and *S. callicarpa* seeds, respectively, germinated during 26 weeks of incubation at 5°C and only 0–3% did so at 15/6–35/20°C. Seeds of *S. caerulea* germinated up to 8%, and those of *S. callicarpa* to 28%, at

Table 1 Effects of a warm (25/15°C), a cold (5°C) and a warm followed by a cold stratification period on germination percentages (mean ± standard error) of seeds from two species of *Sambucus* collected in Oregon

Weeks at		Incubation temperature regimes (°C)					
25/15°C	5°C	5	15/6	20/10	25/15	30/15	35/20
S. caerulea							
0	0 (fresh)	0	0	0	0	0	0
0	0 (control)	32 ± 4	0	0	0	0	0
6	0	—†	0	0	0	0	0
12	0	—	0	0	0	0	0
0	6	—	0	0	1 ± 1	0	3 ± 1
0	12	—	3 ± 1	1 ± 1	3 ± 1	5 ± 4	3 ± 3
6	6	—	0	1 ± 1	0	0	1 ± 1
6	12	—	0	3 ± 3	4 ± 2	0	8 ± 2
12	6	—	0	3 ± 1	0	0	0
12	12	—	4 ± 2	4 ± 2	5 ± 1	5 ± 1	7 ± 3
S. callicarpa							
0	0 (fresh)	0	0	0	0	0	0
0	0 (control)	67 ± 5	1 ± 1	0	3 ± 3	0	0
6	0	—	0	0	0	0	0
12	0	—	0	0	0	0	0
0	6	—	0	8 ± 0	5 ± 3	5 ± 3	3 ± 1
0	12	—	9 ± 4	17 ± 5	13 ± 3	24 ± 2	4 ± 2
6	6	—	0	1 ± 1	0	1 ± 1	1 ± 1
6	12	—	4 ± 2	4 ± 2	4 ± 2	3 ± 1	3 ± 1
12	6	—	0	1 ± 1	3 ± 1	3 ± 1	1 ± 1
12	12	—	19 ± 5	28 ± 6	15 ± 1	28 ± 8	1 ± 1

†, warm-stratified and/or cold-stratified seeds were not incubated at 5°C. Incubation of freshly matured and stratified seeds lasted for 2 weeks, but incubation of control (non-stratified) seeds lasted for 26 weeks. All stratification and incubation treatments were done in the light. Differences of ≥4% or ≥7% between any means within *S. caerulea* or *S. callicarpa* data, respectively, are significantly dissimilar (protected least significant tests, $P = 0.05$).

15/6–35/20°C following warm, cold and warm + cold stratification.

In the second experiment, the germination responses of both species significantly differed among temperature regimes over time (time, temperature, time × temperature, $P < 0.001$). Seeds of *S. caerulea* germinated up to 36% at 5°C during 48 weeks of incubation, whereas those of *S. callicarpa* germinated to 49% (Fig. 1). No seeds from either species germinated at temperatures ≥15/6°C.

In the third experiment, germination responses varied significantly among pretreatments over time (both factors and time × condition, $P \leq 0.003$), but they were similar for both species (species × condition, species × time × condition, $P \geq 0.073$). Seeds of *S. caerulea* and of *S. callicarpa* germinated to (mean ± standard error [SE]) 31 ± 4% and 64 ± 5%, respectively, during 36 weeks of incubation at 5°C, but none germinated at 25/15°C (data not shown). Seeds of *S. caerulea* given 4–12 weeks of a 25/15°C pretreatment and then kept at 5°C for 36 weeks germinated from 0 to 1 ± 1%. In contrast, seeds of *S. callicarpa* given 4 and 8 weeks of a 25/15°C pretreatment germinated to 27 ± 11% and 24 ± 14%, respectively, during 36 weeks of incubation at 5°C, whereas those given a 12-week pretreatment germinated to only 1 ± 1%.

Effects of GA_3 on dormancy break and germination

Germination responses to various concentrations of GA_3 and to various temperature regimes varied significantly over time and were similar for both species only among concentrations (all factors and interactions, $P \leq 0.029$, except species × concentration, $P = 0.500$). Seeds of *S. caerulea* germinated up to 41 and 88% on 0–100 and 1000 mg/L GA_3, respectively, during 36 weeks of incubation at 5°C and up to 24 and 96%, respectively, at 25/15°C (Fig. 2). *Sambucus callicarpa* seeds germinated up to 26 and 53% on 0–100 and 1000 mg/L GA_3, respectively, during 36 weeks of incubation at 5°C and up to 42 and 94%, respectively, at 25/15°C.

Requirements for embryo growth

Seeds of *S. caerulea* and of *S. callicarpa* from Oregon were similar in mean (± SE) length (3.34 mm ± 0.04 *vs* 3.32 ± 0.05, *t*-test, $P = 0.753$), but those from Washington differed (3.39 mm ± 0.07 *vs* 3.08 ± 0.05, $P = 0.001$). Embryos in *S. caerulea* seeds were longer than those in *S. callicarpa* seeds in Oregon (2.50 mm ± 0.04 *vs* 2.30 ± 0.06, $P = 0.005$), but they were the same length in Washington

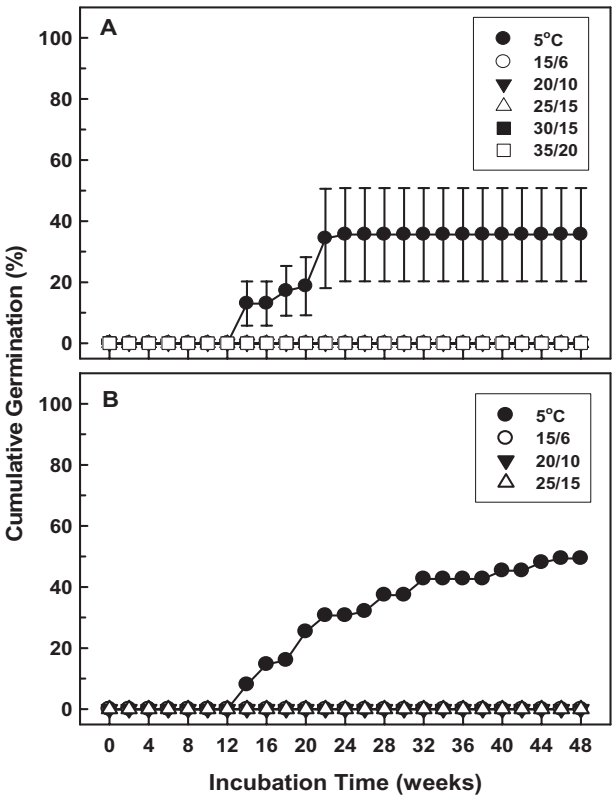

Fig. 1 Cumulative germination percentages (mean ± standard error [SE], SE shown if ≥5%) for seeds of (A) *Sambucus caerulea* from Washington kept in light at six temperature regimes and of (B) *Sambucus callicarpa* from Washington at four temperatures during a 48-week incubation period. For both species, germination percentages at 5°C were significantly higher than those at the other temperatures at week 48 (protected least significant tests, $P = 0.05$).

(2.40 mm ± 0.07 *vs* 2.30 ± 0.04, $P = 0.217$). *Sambucus caerulea* embryos in Oregon and in Washington were approximately 3.05 and 2.92-mm long, respectively, when they had grown enough to start splitting the seed coat/endocarp and *S. callicarpa* embryos were approximately 2.65 and 2.84-mm long, respectively. Embryos in the seeds of both species grew at 5°C and very little at 25/15°C during 20–24 weeks of incubation (Fig. 3). When given a warm period prior to incubation at 5°C, embryos of *S. caerulea* grew only with a 4-week pretreatment, whereas those of *S. callicarpa* did so with 4–12 weeks of pretreatment.

Phenology of germination

Seeds of *S. callicarpa* sown in July 2004 began germinating between 9 and 15 February 2005, when mean weekly maximum and minimum temperatures were 8.0 and –1.0°C (mean = 3.5°C), respectively, after receiving an approximately 16-week period (26 October 2004 to 8 February 2005) when temperatures were 9.0 and 3.4°C (mean = 6.2°C), respectively (Fig. 4). Most germination occurred between 9 February 2005 and 15 March 2005, when temperatures were 12.6 and 3.4°C (mean = 8.0°C), respectively, and very little germination occurred after 15 March 2005 until the study ended on 24 May 2005, during which time the temperatures were 15.8 and 7.6°C (mean = 11.7°C), respectively.

Discussion

At maturity, seeds of *S. caerulea* and of *S. callicarpa* contain embryos that occupy approximately 70–75% of the seed length, depending on the location of the population. These embryos are relatively large when compared with the measurements in Baskin and Baskin (2007) for consideration of underdeveloped embryos (≤50%) or those of other *Sambucus* species (60%, Hidayati *et al*. 2000; 67–69%, Tylkowski 1982). Between the size at initial stages of growth (i.e. in freshly matured seeds) and at the time when embryos had grown enough to start splitting the seed coat and endocarp, embryo length in seeds of *S. caerulea* and of *S. callicarpa* increased by approximately 15–25%. Thus, we consider the embryos in these two species, which have distinguishable cotyledons and radicle, to be 'underdeveloped' because growth, albeit relatively little, occurred before the radicle emerged and to be consistent with the dormancy classification in Hidayati *et al*. (2000) for comparative purposes. Moreover, Tylkowski (1982) considered *S. nigra* and *S. racemosa* '. . . with not fully developed embryos'. In contrast, Nikolaeva *et al*. (1985) classified seeds from members of the genus *Sambucus* as having physiological dormancy ('B_1' or 'B_3' cf. Baskin & Baskin 2008) and not MD or MPD, which suggests that they considered the embryos to be fully developed in the genus. Freshly matured seeds of *S. caerulea* and of *S. callicarpa* did not germinate during 2–4 weeks of incubation, indicating that their embryos were dormant, but seeds germinated following cold stratification (Table 1; Fig. 1). Thus, seeds of these two species have MD (i.e. underdeveloped embryos) and a physiological block to germination. They would be classified as having MPD at maturity.

Levels of MPD are initially divided into simple and complex on the basis of temperature at the time of embryo growth (Baskin & Baskin 1998). Embryos in seeds with simple MPD need relatively high temperatures (≥15°C) for growth, whereas those with complex MPD require low temperatures (0–10°C). Because embryo growth in seeds of both *Sambucus* species occurred at low temperatures (Fig. 3), they have a complex type of MPD. Further classification of the complex type of MPD into non-deep, intermediate, or deep depends on conditions conducive

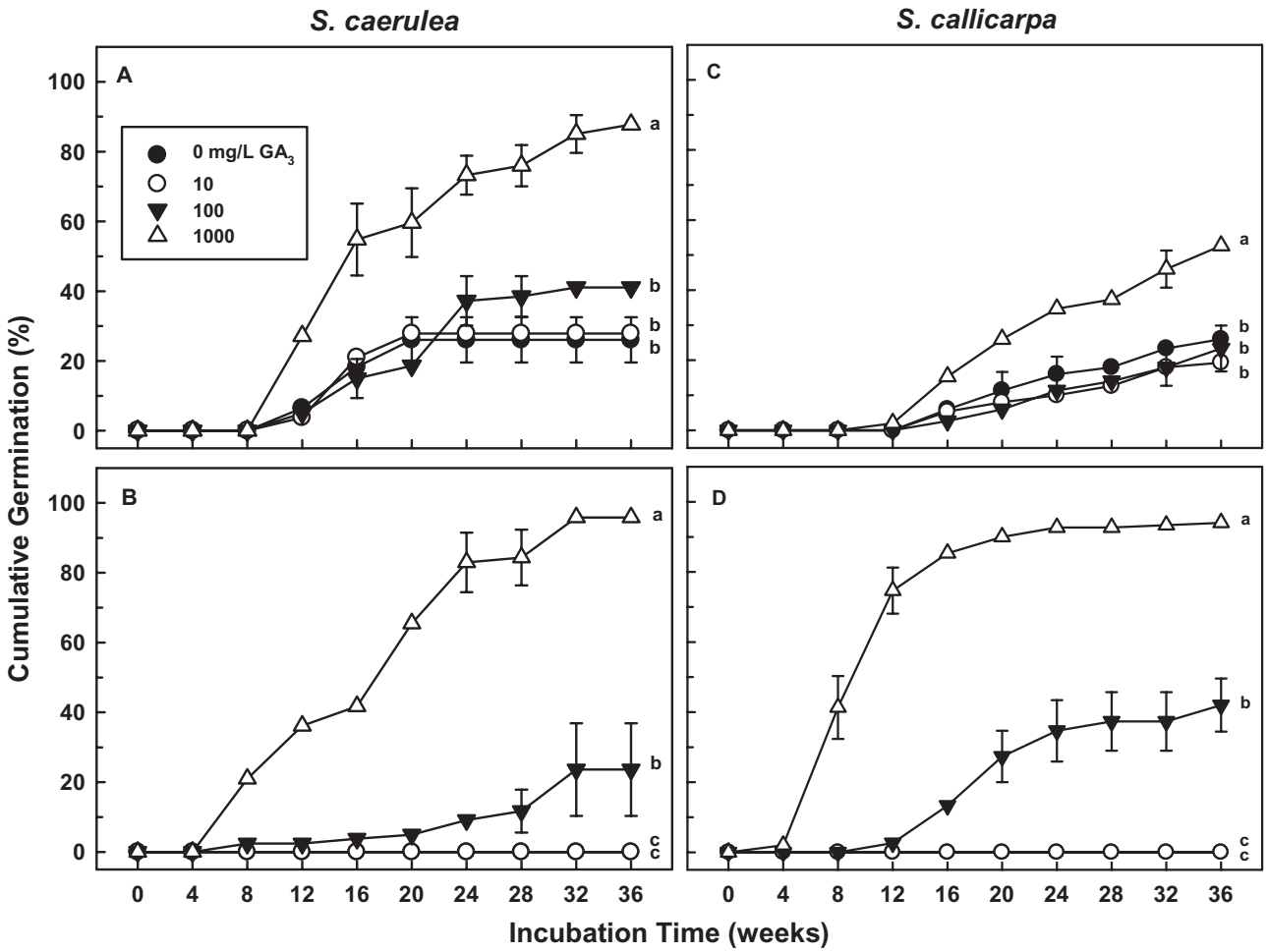

Fig. 2 Effect of three concentrations of gibberellic acid (10–1000 mg/L GA_3) and of distilled water (0 mg/L GA_3) on cumulative germination percentages (mean ± standard error [SE], SE shown if ≥5%) of *Sambucus caerulea* and of *Sambucus callicarpa*. Seeds were incubated in light at (A,C) 5°C or at (B,D) 25/15°C for 36 weeks. Means at week 36 with dissimilar lowercase letters are significantly different (protected least significant tests, $P = 0.05$). In figure parts (B) and (D), the data points for 0 and for 10 mg/L GA_3 overlap.

for dormancy release: temperature requirements and GA_3. Non-deep complex MPD is broken by a warm + cold stratification, and GA_3 substitutes for warm stratification but, not for cold stratification. In contrast, intermediate complex and deep complex MPDs are broken by cold stratification and GA_3 overcomes dormancy only in seeds with intermediate complex MPD (Baskin & Baskin 1998). For both species, GA_3 effectively overcame the cold stratification requirement, resulting in high percentages of seeds germinating at warm temperatures (Fig. 2). Other studies (Brinkman 1974; Clancy & Maguire 1979; Norton 1986) on *S. caerulea* have also noted the beneficial effects of cold stratification and GA_3 for overcoming dormancy. Accordingly, seeds of *S. caerulea* and of *S. callicarpa* have intermediate complex MPD.

Seeds of *S. caerulea* and of *S. callicarpa* germinated to higher percentages, and embryos elongated more, with cold stratification only than with a warm-temperature pretreatment given prior to cold stratification (Table 1; Fig. 3). Whereas *S. caerulea* seeds mostly did not respond to a warm pretreatment, *S. callicarpa* seeds germinated up to 28% and embryos grew. Brinkman (1974) reported 44% germination of *S. callicarpa* seeds during 4–6 weeks of incubation at 20/16°C following 4 weeks at room temperature (~21°C) + 13 weeks at 3°C. Taylor (2002) found that a warm stratification period (18–21°C) of 6 weeks to 2 months prior to a cold stratification period of 3–4 months stimulated 75–80% and 15–50% germination in *S. callicarpa* (as *S. racemosa*) and in *S. caerulea* seeds, respectively. Making comparisons between the results of our study and those of Brinkman (1974) and Taylor (2002) are difficult because much information is not given in the latter two studies, for example, incubation temperatures or length (and conditions) of storage before initiation of the germi-

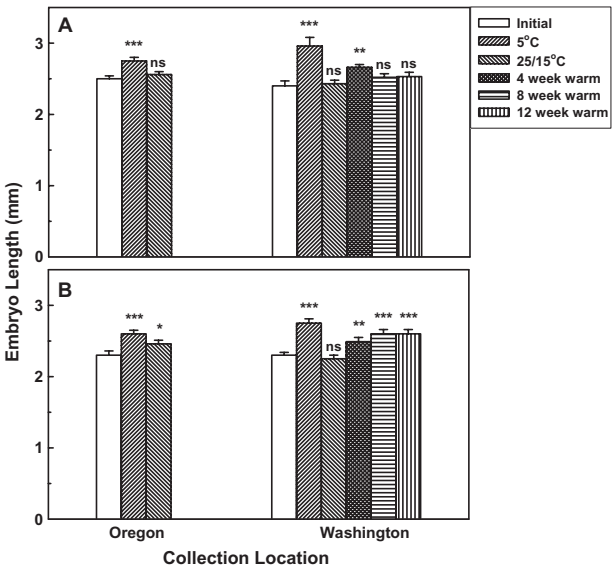

Fig. 3 Embryo lengths (mean ± standard error) in seeds of (A) *Sambucus caerulea* and (B) *Sambucus callicarpa* collected at two locations. Seeds from Oregon were incubated for 24 weeks at 5°C or at 25/15°C, and those from Washington for 20 weeks at 5°C or at 25/15°C or placed for 4–12 weeks at 25/15°C and then incubated for 20 weeks at 5°C. Asterisks above bars indicate a significant difference between lengths at the beginning of the experiment (initial) and at the end of the treatment (*t*-test; ns, not significant, *$0.05 \geq P > 0.01$, **$0.01 \geq P > 0.001$, ***$P \leq 0.001$).

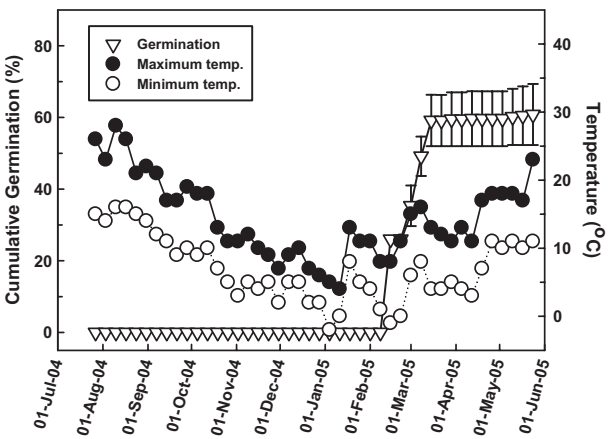

Fig. 4 Cumulative germination percentages (mean ± standard error [SE]; SE shown if ≥5%) of *Sambucus callicarpa* seeds collected and sown on soil in July 2004 and placed in a cold frame. Mean weekly maximum and minimum temperatures (temp.) are shown from 20 July 2004 to 24 May 2005.

nation tests. However, seeds in the Taylor (2002) study might have been stored dry in glass jars in a refrigerator before the experiments were initiated (J. Taylor, pers. comm, 2009). We also found that a warm + cold stratification treatment broke dormancy in 28% of *S. caerulea* seeds (Table 1; Fig. 3) and in both species following 2 years of dry storage (in glass jars) at ambient laboratory conditions (~21°C) (S. N. Hidayati & J. L. Walck, unpubl. data, 2002). Seeds of *S. caerulea* germinated to 51% during 36 weeks at 5°C, but to 77–91% during 32–24 weeks at 5°C following a 4–12 week warm stratification period at 25/15°C, and those of *S. callicarpa* germinated to 12% at 5°C, but to 39–47% at 5°C following warm stratification. However, our germination results on fresh seeds were consistent among populations in Oregon and in Washington for both of these two species (i.e. cold stratification without a warm pretreatment was optimal for breaking dormancy).

A similar inconsistency in seed dormancy break and germination requirements occurs between studies on the European *S. racemosa* and *S. nigra*. Tylkowski (1982) reports that seed germination of both of these species required a warm pretreatment of 15, 20 or 25°C for at least 3 weeks before cold stratification at 3°C. In contrast, only cold stratification was needed for seed germination of *S. racemosa* and of *S. nigra* as reported by Hidayati *et al*. (2000) and Nikolaeva *et al*. (1985), respectively. *Sambucus racemosa* seeds in Tylkowski's (1982) study were stored in tightly sealed bottles at –3°C for 5 months, and *S. nigra* seeds for 16 months, before germination tests were started. The storage length for seeds of *S. nigra* is unknown for Nikolaeva *et al*. (1985), but storage of seeds of *S. racemosa* was only for 14 days in Hidayati *et al*. (2000) during which time they were mailed from Sweden to the USA. Thus, differences among these studies reporting cold stratification versus warm + cold stratification may be connected with the length and/or conditions of storage.

The optimum temperature for embryo growth and for germination was 5°C for *S. caerulea* and *S. callicarpa* seeds, and no or little germination took place over the range of test temperatures (15/6–35/20°C) even following warm, cold or warm + cold stratification (Table 1; Fig. 1). Likewise, the optimum temperature for seed germination of the wNA species of *Osmorhiza*, which have deep complex MPD, was at 1, 5 or 5/1°C coinciding with the temperatures needed for cold stratification (Baskin *et al*. 1995; Walck & Hidayati 2004). In contrast, non-dormant seeds of the eNA *S. canadensis* and *S. pubens* and of the European *S. racemosa* germinated to high percentages over a broad range of temperatures (15/6–35/20°C) (Hidayati *et al*. 2000) and those of the European *S. nigra* and *S. racemosa* to moderate and high percentages at constant 3 and 15°C (Tylkowski 1982).

Seeds of *S. callicarpa* sown out-of-doors in July 2004 did not germinate until mid-February to mid-March 2005 (Fig. 4). As the requirements for dormancy break, embryo growth and germination for seeds of this species are similar to those of *S. caerulea*, we suggest that their germination phenology would also be the same. Dormancy would prevent germination following dispersal in late

Table 2 Summary of the temperatures required for embryo growth and for dormancy break, germination responses to gibberellic acid (GA_3) and the level of morphophysiological dormancy (MPD, sensu Baskin & Baskin 1998) for seeds of seven *Sambucus* taxa (sensu Cronquist *et al*. 1984; Bolli 1994) in eastern North America (eNA), western North America (eNA) and Europe

Species	Range	Temperature required: Embryo growth	Temperature required: Dormancy release	GA_3 substitutes for stratification: Warm	GA_3 substitutes for stratification: Cold	Level of MPD	Reference
S. nigra complex							
ssp. *nigra*	Europe	?	W + C	?	?	?	Tylkowski (1982)
		C	C	n/a	?	Intermediate or deep complex†	Nikolaeva *et al*. (1985)
ssp. *canadensis*	eNA	W	W + C	+	–	Deep simple	Hidayati *et al*. (2000)
ssp. *caerulea*	wNA	C	C	n/a	+	Intermediate complex	Present study
S. racemosa complex							
ssp. *racemosa* s. *str*.	Europe	C	C	n/a	+	Intermediate complex†	Hidayati *et al*. (2000)
ssp. *pubens*							
var. *pubens*	eNA	W	W + C	+	–	Deep simple	Hidayati *et al*. (2000)
var. *arborescens* (*S. callicarpa*)	wNA	C	C	n/a	+	Intermediate complex	Present study
var. *microbotrys*	wNA	C	C	n/a	(+)	Probably intermediate complex	McDonough (1969), Conrad & McDonough (1972)

† Seeds were classified as having 'deep physiological dormancy' by Nikolaeva *et al*. (1985) (cf. Baskin & Baskin 2008). C, cold stratification; W, warm stratification; +, yes; –, no; ?, not tested; (+), probably yes; n/a, not applicable because seeds require only cold stratification.

summer and it would be broken by cold stratification over winter. By late winter, seeds would be non-dormant and capable of germinating. The low temperature requirement for seeds of both species ensures that germination occurs in a relatively narrow window of opportunity in late winter. Thus, seedling emergence of *S. callicarpa* occurred when temperatures averaged 6–8°C and ceased once temperatures were >12°C.

Fruits of *S. caerulea* and of *S. callicarpa* ripen from July to September, and when fully ripe are dispersed by a variety of animals (Brinkman 1974). Seed passage of *S. racemosa* (probably *S. callicarpa*) through vertebrate frugivores (birds, bears) enhances germination (Traveset & Willson 1997). A total of 31, 14 and 19% of the seeds ingested by varied thrushes (*Ixoreus naevius*), black bears (*Ursus americanus*) and American robins (*Tardus migratorius*), respectively, germinated; controls germinated to approximately 6%. These percentages are low compared with our results for *S. callicarpa*. Thus, passage through the digestive tracts of animals does not curtail the need for additional treatments to overcome dormancy. Likewise, Clergeau (1992) found that seeds of *S. nigra* germinated to 36 and 43% when defecated or regurgitated, respectively, from starlings (*Sturnus vulgaris*) and blackbirds (*Turdus merula*), but they germinated to 62% when depulped and incubated under simulated natural temperatures.

The second objective of the present study was to determine if wNA species of *Sambucus* have the same or a different level of MPD than their congeners in eNA and Europe. Within the *S. nigra* species complex, the wNA taxon *caerulea* has intermediate complex MPD and the eNA taxon *canadensis* has deep simple MPD requiring cold and warm + cold conditions, respectively, to overcome dormancy (Table 2). Although we cannot classify the level of MPD in the European taxon *nigra*, release from dormancy occurs either with cold stratification (Nikolaeva *et al*. 1985) or with warm + cold stratification (Tylkowski 1982). In contrast, in the *S. racemosa* complex wNA taxon *arborescens* (= *S. callicarpa*) shares the same level of MPD as the European *racemosa*, but the MPD level in both of these taxa differs from the eNA *pubens* (Table 2). Another wNA taxon, *microbotrys*, apparently requires only cold stratification to overcome dormancy in its seeds and GA_3 improves germination (McDonough 1969; Conrad & McDonough 1972), suggesting that seeds of this taxon have intermediate complex MPD. Thus, the geographic pattern of MPD in both complexes is similar in North America, but it may differ substantially compared with Europe.

Differences in dormancy have been reported between eNA and European herbaceous congeners in the genera *Chaerophyllum* (Baskin & Baskin 1990; Baskin *et al*. 2004; Vandelook *et al*. 2007) and *Sanicula* (Vandelook & Van Assche 2008; Hawkins *et al*. 2010). In *Sanicula*, the European polycarpic perennial *S. europaea* requires cold stratification to overcome dormancy and one eNA species, which is a monocarpic perennial, needs warm + cold strati-

fication. Thus, the geographic pattern of dormancy release in some herbaceous perennials of *Sanicula* is similar to that seen in shrubs of the *Sambucus racemosa* complex. Within North America, polycarpic perennials of the eNA species of *Osmorhiza* and *Erythronium* need warm + cold stratification (Baskin & Baskin 1984, 1985, 1991, 1998) and wNA congeners must have cold stratification (Baskin *et al*. 1995; Walck *et al*. 2002; Walck & Hidayati 2004) similar to the pattern seen in shrubs of *Sambucus nigra* and *S. racemosa* complexes. The evolution of seed dormancy in the groups of taxa in *Sambucus*, *Osmorhiza* and *Erythronium*, albeit with very different growth forms, might have occurred under similar environmental circumstances, resulting in the sharing of dormancy-breaking mechanisms within geographical areas.

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