# REPRODUCTIVE BIOLOGY OF MEDICINAL WOODLAND HERBS INDIGENOUS TO THE APPALACHIANS

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Matthew A. Albrecht

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by

MATTHEW A. ALBRECHT

has been approved for

the Department of Environmental and Plant Biology

and the College of Arts and Sciences by

Brian C. McCarthy

Professor of Environmental and Plant Biology

Benjamin M. Ogles

Dean, College of Arts and Sciences

# Abstract

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REPRODUCTIVE BIOLOGY OF MEDICINAL WOODLAND HERBS INDIGENOUS TO THE APPALACHIANS (138 pp.)

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Eastern deciduous forests include a remarkable number of plants that are utilized widely for their medicinal properties. However, the long-term sustainable use of medicinal forest plants requires that methods be developed for restoring and cultivating them in natural and semi-natural settings. This study examined the seed regeneration biology of several perennial forest herbs that are internationally traded in the increasingly lucrative botanical medicines industry. Specific objectives were to: (1) determine how environmental cues (temperature, light, substrate, and burial) regulate dormancy-break and carry-over in seed populations of *Actaea racemosa* (black cohosh), *Hydrastis canadensis* (goldenseal), and *Sanguinaria canadensis* (bloodroot) ; (2) experimentally evaluate seedling recruitment probabilities of *A. racemosa*, *H. canadensis*, *S. canadensis*, and *Panax quinquefolius* in different forest microenvironments defined by varying levels of leaf litter and opposing topographic positions; (3) classify seed dormancy and determine optimum germination temperatures for *Collinsonia canadensis* (stoneroot) and *Dioscorea villosa* (wild yam); and (4) quantify survival rates and dormancy-levels of *A. racemosa* and *H. canadensis* seed populations stored in artificial conditions. Results from these studies showed that: (1) germination probabilities can vary widely when seeds experience different environmental cues, although temperature is the primary factor regulating dormancy-break and germination; (2) some perennial forest herbs can form

persistent seed banks and spread germination across time; (3) leaf litter and topographic position can act as ecological filters during early life-history stages; and (4) storing seed populations prior to outplanting reduces viability in *H. canadensis* seeds and deepens dormancy levels in *A. racemosa* seed. Recommendations are made for cultivating, managing, and restoring these species in the eastern deciduous forest landscape.

Approved:

Brian C. McCarthy

Professor of Environmental and Plant Biology

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## Introduction

Seed dormancy, defined as a seed characteristic that determines what environmental conditions are required for germination (Vleeshouwers et al. 1995), plays a central role in plant life-histories because seeds represent an essential link for species persistence across space and time (Harper 1977; Rees 1997). In temperate latitudes, dormancy serves two primary functions that provide a means for plants to cope with environmental heterogeneity (Angevine and Chabot 1979; Jurado and Flores 2005). First, dormancy prevents emergence during seasons when the probability of seedling survival and growth are low (Vleeshouwers et al. 1995; Allen and Meyer 1998; Fenner and Thompson 2005). Second, dormancy can spread germination over time by allowing a fraction of the seed crop to 'carry-over,' even when conditions are favorable for germination and survival (Allen and Meyer 1998; Schütz 2002). This ensures the availability of seeds to buffer against stochastic demographic events, such as reproductive and establishment failures (Hille Ris Lambers et al. 2005) or, alternatively, as a hedge against unpredictable germination cues (Venable and Brown 1988).

In mesic deciduous forests of eastern North America, seeds of many perennial woodland herbs, including the focal species of this study, are morphophysiologically dormant (MPD) at maturity (Baskin and Baskin 1988, 2001). In seeds with MPD, underdeveloped embryos are physiologically dormant at the time of dispersal (Baskin and Baskin 2001; 2004b). Although temperature is the primary factor regulating dormancy-break in species with MPD, the response of seeds to light, temperature variation,

substrate conditions, and burial varies enormously across taxa (Baskin and Baskin 2001). In this study, I examined the environmental cues of seed regeneration in perennial woodland herbs – black cohosh (*Actaea* [*Cimicifuga*] *racemosa* L.), goldenseal (*Hydrastis canadensis* L.), and bloodroot (*Sanguinaria canadensis* L.) – that are commonly harvested from the wild or "cultivated" in woodlands for international trade in the botanical medicines industry. These species are experiencing population decline throughout their natural range due to habitat alteration, over-harvesting, and excessive deer browse pressure (Greller et al. 1990; Sanders and McGraw 2002; Ruhren and Handel 2003; Mulligan and Gorchov 2005). Developing recovery and conservation plans requires a comprehensive understanding of how environmental factors define the seed regeneration niche of declining and threatened species (Schemske et al. 1994).

Despite their longstanding cultural and economic value, seeds of these medicinal woodland herbs are notoriously problematic to germinate, and the available prescriptions for germinating seeds are sometimes conflicting or based on anecdotal observation (Hus 1907; Harding 1936; Deno 1993; Sinclair and Catling 2001; Cech 2002; Persons and Davis 2005). Prior germination phenology studies in an unheated glasshouse concluded that *A. racemosa* and *H. canadensis* seeds have deep simple epicotyl MPD (Baskin and Baskin 1985a) and deep simple MPD (Baskin and Baskin 2001), respectively, although the role of other environmental cues on germination remains unknown. Relying on Barton's (1944) study of *S. canadensis* seed collected in central New York, Baskin and Baskin (2001) concluded that approximately half the seed population exhibited deep simple epicotyl MPD whereas the other half exhibited deep simple double MPD (or "double dormancy"). The experimental temperatures applied to seeds, however, did not

mimic the temperature sequences that *S. canadensis* seeds experience following dispersal, suggesting that seed germination patterns could differ in the field. Elaiosome removal may also function as a germination cue in ant-dispersed plants such as *S. canadensis* (Lobstein and Rockwood 1993), although this has been relatively unexplored in field conditions and in combination with other environmental cues.

In conjunction with a field burial study, I used a factorial experimental design to subject seed populations of medicinal woodland herbs to combinations of temperature and photoperiod regimes in the laboratory to fully characterize dormancy-break and germination. Laboratory studies can clarify the environmental factors that cue dormancy-break and germination in field conditions (Thompson and Grime 1979; Baskin and Baskin 2001), and provide germination recommendations to enhance the development of outplanting material needed in restoration, reintroduction, and cultivation efforts. Here, I asked the following questions: (1) Is there concordance between post-dispersal temperature regimes and dormancy-break? (2) Do cues such as photoperiod, burial, and elaiosome removal play a role in dormancy-break and/or germination? (3) Do these species form persistent seed banks in field conditions? (4) Is the length of dormancy be shortened to facilitate conservation efforts with medicinal plants?

## Materials and Methods

### *Seed collection and processing*

During the summers of 2002 and 2003, seeds of the three species were collected from two wild populations (each > 500 ramets) in second-growth (> 80 yr) Appalachian hardwood forests in southeast Ohio. Seeds were gathered from populations growing in

similar habitats (e.g., slope aspect, forest type, and microenvironmental conditions). Fruits were collected from multiple ramets spread throughout the population in order to maximize genetic diversity of seeds. Seeds were brought back to the laboratory where they were thoroughly mixed prior to the experiments and then stored in brown paper bags until germination studies were initiated. A tetrazolium stain test on a random sub–sample of 50 ripened seeds indicated that ≥ 92% of seeds were viable in all seed populations.

Immediately following collection, *H. canadensis* seeds were extracted from berries according to a two-step process (Cech 2002). First, in order to loosen the fleshy pericarp, freshly collected fruits were placed into a bucket of water and allowed to soak for 24 h. Seeds were then manually removed from the pulp and thoroughly cleaned. Only seeds that sank in water were used for germination, as seeds that float are nonviable. *A. racemosa* fruits were allowed to dry at room temperature for 5 d prior to germination studies. Seeds were then removed from follicles by gently tapping the infructescence against the side of a paper bag. Only brown, firm seeds were used in germination studies. For *S. canadensis*, whole capsules were collected from populations when they began to show signs of splitting open along their sutures. Capsules were allowed to ripen and disperse into paper bags in the laboratory, which occurred within 3-5 d of collection. In 2002, I experimentally created two seed samples by removing the elaiosome on half of the seeds (hereafter: – elaiosome and + elaiosome). Effort was made to replicate Lobstein and Rockwood's (1993) methods by carefully removing the elaiosome with forceps.

*General laboratory experiments*

Laboratory studies were conducted in temperature and light controlled environmental chambers set at a 12L:12D alternating photoperiod illuminated by Cool White Fluorescent© tubes (high R:FR ratio). Three of the chambers were set at daily fluctuating temperature regimes of 15/6, 20/10, and 30/15 ºC, respectively, whereas the fourth germinator was set at a constant temperature of 5 ºC. These thermoperiods are recommended for use in germination studies with eastern deciduous forest herbs (Baskin and Baskin 2004a), and simulate mean daily maximum and minimum seasonal temperatures in mid- to low-elevation forests of the central Appalachian region: 30/15 ºC (summer), July, August, and September; 20/10 ºC (early fall/late spring), October, May and June; 15/6ºC (late fall/early spring), November, March, and April; and 5 ºC (winter), December, January, and February.

Each treatment level combination consisted of four replicates comprising 30 seeds per dish for *H. canadensis* and *S. canadensis* and 50 seeds per dish for *A. racemosa* (hereafter, a 'replicate' refers to a set of four Petri dishes). In a pilot study, I found that fresh seeds of these species were susceptible to rotting when germinated on moistened filter paper in Petri dishes wrapped with Parafilm, even after sterilization with a light bleach solution. Thus, seeds were placed in glass Petri dishes (9 cm diameter) on top of a 1-2 cm mix (1:1:1) consisting of peat moss, sand, and field soil collected from the sites where seeds were collected. Field soil was sieved prior the experiments in order to remove propagules from the resident seed bank. Seeds remained constantly imbibed throughout the study by adding distilled water to Petri dishes as needed. Petri dishes were checked every 7 d for germinants until the completion of each laboratory experiment,

when all nongerminated seeds were subsequently dissected and examined beneath a microscope; seeds with firm and fleshy embryos were scored as viable. Germination fractions were then adjusted to reflect the fraction of viable seeds that germinated in each dish. Hereafter, germination refers to seeds in which the radicle emerged whereas seedling emergence refers to seeds in which both the radicle (primary root) and cotyledons emerged.

*Seasonal temperature sequence and dormancy-break*

I used a modification of the double germination phenology template (sensu Baskin and Baskin 2004a) to clarify what types of temperature sequences break dormancy in each of the medicinal woodland herbs. These sequences simulate different temperature regimes seeds experience in the field following dispersal. Replicate sets of newly dispersed seed (2002) of each species were started at either summer (30/15 ºC), autumn (20/10ºC), or winter (5 ºC). Seeds started in the summer treatment were moved through the following sequence: summer (12 wk at 30/15 ºC) → early autumn (4 wk at 20/10 ºC) → late autumn (4 wk at 15/6 ºC) → winter (12 wk at 5 ºC) → early spring (4 wk at the 15/6 ºC) → late spring (4 wk at 20/10 ºC). Seeds started in the autumn treatment were moved through the following sequence: early autumn (4 wk at 20/10 ºC) → late autumn (4 wk at 15/6 ºC) → winter (12 wk at 5 ºC) → early spring (4 wk at the 15/6 ºC) → late spring (4 wk at 20/10 ºC) → summer (12 wk at 30/15 ºC). Seeds started in the winter treatment were moved through the following sequence: winter (12 wk at 5 ºC) → early spring (4 wk at the 15/6 ºC) → late spring (4 wk at 20/10 ºC) → summer (12 wk at 30/15 ºC) → early autumn (4 wk at 20/10 ºC) → late autumn (4 wk at 15/6 ºC).

Seeds were moved through each temperature sequence twice. Controls were nonstratified seeds kept continuously moist and placed in light for 180 d at each of the four thermoperiods. Replicate sets of nonstratified seeds were also placed in the dark at each of the four thermoperiods and checked for germination at the end of 30 d.

Germination was also tested in light and darkness and on different substrates in the summer temperature sequence described previously. To test for the effects of light (12 h/12h photoperiod) and darkness on germination, replicate sets of newly dispersed seed (collected in 2003) of each species were stratified and incubated in light and darkness. To determine how different stratification substrates influenced germination, replicate sets of newly dispersed seeds were also placed on field soil, peat moss, sand, and a 1:1:1 mix of the three substrates.

*Effects of cold stratification on seedling emergence*

Seedling emergence was evaluated in a 4 × 3 factorial experiment by allowing warm–stratified seed to experience varying lengths of simulated winter temperature (5 ºC) and then incubating the warm → cold–stratified seed in thermoperiods that simulate spring temperatures. Replicate sets of newly dispersed seeds (2003) of *A. racemosa* and *H. canadensis* were transferred through the same seasonal temperature sequences described previously. After 0 (control), 4 (short stratification), 8 (intermediate stratification), and 12 (long stratification) wks at 5 ºC, replicate sets of dishes for each species were removed and incubated at 15/6 ºC, 20/10 ºC, and 30/15 ºC, where seedling emergence was monitored over a 12 wk period. Seedling emergence was calculated as the fraction of seeds with emerged radicles that produced cotyledons.

*Effects of gibberellic acid ($GA_3$)*

A 4 × 3 factorial design was used to determine if gibberellic acid ($GA_3$) could break dormancy in fresh seeds of each species. In each of 48 petri dishes, 25 seeds were placed on top of two pieces of Whatman #1 filter paper. Replicate sets (i.e., four dishes) of seeds (2003) were moistened to saturation either with distilled water (controls), 100 mg/L, or a 1000 mg/L solution of gibberellic acid ($GA_3$) solution, prepared by dissolving the appropriate amounts of potassium salt in distilled water. Replicate sets ($n$ = 4 dishes) were placed at each thermoperiod where seed germination was monitored over a 60 d period.

*Seed burial*

Seed persistence under field conditions was evaluated by burying seeds of each species in 'seed packets' and then retrieving seeds periodically to test for germination and viability. Burial experiments commenced immediately following collection of freshly dispersed seed in 2002: 7–June for *S. canadensis*, 3–July for *H. canadensis,* and 19–September for *A. racemosa*. For each species, seed quantities approximating natural deposition rates were placed into each 'seed packet,' constructed of 10 × 10 cm nylon mesh squares sewn closed on three sides and stapled closed at the open end (*A. racemosa*: $n$ = 40 packets, 50 seeds/packet; *H. canadensis*: $n$ = 24 packets, 30 seeds/packet; *S. canadensis: n* = 32 packets, 15 seeds/packet). Each bag was tied to a metal stake with nylon string to facilitate recovery. In 2002, twelve 4 m transects were established in a ravine that ran parallel to the contour of an E–SE facing slope at the Ridges Land Lab (39º19′30″ N, 82 º07′ 05″ W), a second–growth hardwood forest in Athens County, Ohio,

USA. At every 0.5 m on each transect, a seed packet was buried at a depth of approximately 2 cm. Every 90 d (over a 720 d period) from the initial burial date of each species, seeds packets (*A. racemosa*: $n = 5$ packets; *H. canadensis*: $n = 3$ packets; *S. canadensis*: $n = 4$ packets) were retrieved by selecting at random one packet from each transect. On each retrieval date, seed packets were placed into plastic bags and brought back to the laboratory for analysis. Seeds with emerged radicles were scored as germinates. Nongerminated seeds that were firm when pinched were placed into Petri dishes (up to 30 seeds per dish) and tested for germination in incubators representing the optimum thermoperiods (12L:12D) for each species. Every 7 d, germination was scored; after 30 d, all nongerminated seeds were split and scored as viable if embryos were white, firm, and, fleshy.

*Statistical analyses*

For laboratory studies, germination fractions (number of seeds that germinated per Petri dish/number of total viable seeds per Petri dish) were analyzed using a generalized linear model (PROC GENMOD; SAS Institute 2001), with a logit link function and poison error distribution. Because the variance is generally greater than the mean with count data test statistics may be overestimated, increasing the probability of committing a Type I error (Littell et al. 2002). This overdispersion was accounted for by dividing the likelihood ratio $\chi^2$ by the Pearson $\chi^2$ /df (PSCALE option in SAS), resulting in an analogous ANOVA *F*-test (Littell et al. 2002). Post-hoc pairwise comparisons were made using the least–squares means procedure when main effects were significant.

Exponential decay functions were fitted to the fraction of seeds scored as dormant at each of the eight seasonal retrieval dates using the curve fitting function in NCSS (Hintze 2001). Seed survival is commonly modeled as an exponential loss of viability over time and tends to provide a reasonable fit to seed burial data (Murdoch and Ellis 2000). In order to determine whether laboratory simulations were a good predictor of seed carry-over in the field burial study, I used a generalized linear model to test for differences between the mean fractions of dormant seeds remaining in seed packets at the last retrieval date and in Petri dishes after two annual cycles (i.e., summer temperature sequence). Seed populations in these experiments would have experienced the equivalent of two annual temperature cycles, or two germination seasons. Separate tests were conducted for each species.

## Results

### *Effects of alternating temperatures*

Newly dispersed seeds of the three medicinal woodland herbs failed to germinate over a range of simulated thermoperiods in light and darkness during a 30 d incubation period, confirming that seeds were in primary dormancy (Baskin and Baskin 2001). All seeds remained dormant in light at summer (30/15 °C) and winter (5 °C) temperatures throughout the 48 wk germination test (Figure 1.1). Over time, however, some seeds lost dormancy when maintained in light at spring and autumn temperatures (Figure 1.1). After 48 wks at control thermoperiods, germination fractions for *A. racemosa* seeds were 0.75 ± 0.02 (mean ± se) and 0.49 ± 0.03 at the 15/6 °C and 20/10 °C thermoperiods, respectively. Germination fractions of *H. canadensis* seeds were 0.16 ± 0.05 after 48 wk

incubation at 15/6 °C. *S. canadensis* seeds with and without the elaiosome began germinating after 24 wks in the 20/10 °C thermoperiod. Elaiosome removal had no effect ($P = 0.18$) on mean germination fractions of *S. canadensis* seeds at the end of the 48 wk period.

*Seasonal temperature sequence and dormancy-break*

Temperature sequences significantly affected germination fractions in *S. canadensis* seeds ($F_{2, 9} = 8.15$, $P = 0.01$), but not in *H. canadensis* seeds ($F_{2, 9} = 2.44$, $P = 0.14$). *S. canadensis* seeds in the autumn sequence germinated to lower fractions compared to seeds in the summer (contrast summer vs. autumn: $P = 0.005$) and winter sequence (contrast winter vs. autumn: $P < 0.0001$). Germination fractions of *S. canadensis* seeds were similar among summer and winter temperature sequences (contrast summer vs. winter: $P = 0.27$). Seeds of *A. racemosa* germinated to lower fractions in the autumn sequence compared to the summer and winter sequence (Figure 1.2), although this was nearly significant ($F_{2, 9} = 3.34$, $P = 0.08$).

In the elaiosome removal experiments with *S. canadensis*, seeds germinated to similar fractions with (mean ± se; 0.56 ± 0.08) and without (0.45 ± 0.06) the elaiosome attached ($F_{1, 12} = 0.05$, $P = 0.83$). There was no significant interaction between elaiosome removal and seasonal temperature sequence on *S. canadensis* seed germination ($F_{1, 12} = 3.01$, $P = 0.11$).

Germination phenologies reflected the expected response of a strict spring germinator. In all species, seedlings (radicle and cotyledon) emerged at early spring temperatures (Figure 1.2). The optimum temperature for seedling emergence was 15/6 °C

for *A. racemosa* and *H. canadensis*, and 20/10 °C for *S. canadensis*. Radicle emergence in *A. racemosa* and *S. canadensis*, the two species with epicotyl MPD, was confined to autumn and winter temperatures (Figure 1.2). For all species, seeds placed initially in the winter sequence (5 °C) did not fully germinate until seeds experienced a summer → autumn → winter temperature sequence (Figure 1.2).

Species responded differentially to germination substrates (Table 1.1). Whereas subsrate had no effect on germination fractions of *A. racemosa* ($F_{3,12} = 2.52$, $P = 0.11$) and *H. canadensis* ($F_{3,12} = 0.81$, $P = 0.11$) seeds, germination fractions of *S. canadensis* seeds were significantly lower on sand compared to other substrates ($F_{3,12} = 11.89$, $P = 0.0007$; Table 1.1.1).

*Effects of photoperiod*

Germination fractions in light and darkness varied among the medicinal woodland herbs. *Actaea racemosa* radicles emerged at similar fractions independent of whether seeds were stratified or incubated in light and darkness ($F_{3, 12} = 2.48$, $P = 0.11$). However, cotyledons only emerged when seeds were incubated in light ($F_{3, 12} = 127.32$, $P < 0.0001$; Table 1.2). For *H. canadensis*, a majority of seeds rotted in the dark stratification/dark incubation treatment. Removing this treatment from the analysis resulted in an unbalanced data structure; thus, the following *F*–values are derived from a Type III analysis (Littell et al. 2002). *H. canadensis* radicles ($F_{2, 9} = 1.27$, $P = 0.33$) and cotyledons ($F_{2, 9} = 0.24$, $P = 0.79$) emerged independently of light and darkness, although cotyledons that emerged in darkness were etiolated and chlorotic. Radicles ($F_{3, 12} = 6.81$,

$P < 0.006$) and cotyledons ($F_{3, 12} = 4.55$, $P < 0.02$) emerged at significantly lower fractions when *S. canadensis* seeds were stratified in darkness compared to light.

*Effects of gibberellic acid ($GA_3$)*

There was a significant temperature × $GA_3$ interaction on *A. racemosa* ($F_{6, 36} = 3.64$, $P < 0.006$) and *S. canadensis* ($F_{6, 36} = 7.54$, $P < 0.0001$) seed germination. Whereas no seeds germinated in the control or 100 mg/L $GA_3$ treatment, approximately 54% and 48% of the *S. canadensis* seeds subjected to 1000 mg/L $GA_3$ germinated in the 20/10 ºC and 15/6 ºC thermoperiods, respectively (Table 1.3). In the 15/6 ºC thermoperiod, *A. racemosa* seeds subjected to 1000 mg/L $GA_3$ germinated to three times the rates of the control (Table 1.3). All *H. canadensis* seeds in $GA_3$ treatments rotted.

*Effects of simulated winter and spring temperatures on seedling emergence*

Seedling emergence fractions were enhanced significantly when *A. racemosa* ($F_{3, 36} = 73.0$, $P < 0.0001$) and *H. canadensis* ($F_{3, 36} = 124.2$, $P < 0.0001$) seeds experienced longer periods at simulated winter temperatures (Figure 1.3). However, the effects of stratification time depended on incubation temperature for *A. racemosa* seedlings ($F_{6, 36} = 7.09$, $P < 0.0001$) but not *H. canadensis* seedlings ($F_{6, 36} = 4.77$, $P = 0.58$). *A. racemosa* seedlings emerged only at 30/15 °C themoperiod in the control (Figure 1.3). *H. canadensis* seedlings emerged at significantly lower fractions in the 30/15 °C thermoperiod relative to the 15/6 °C thermoperiod across all stratification treatments ($F_{2, 36} = 4.53$, $P = 0.02$).

*Seed burial*

Germination phenologies of buried seed populations were consistent with the laboratory simulations (Figure 1.4). Laboratory germination of *A. racemosa* and *H. canadensis* seeds were restricted to the appropriate seasons, autumn and spring, respectively. In contrast, at the last summer retrieval date, *S. canadensis* seed populations (both +/− elaiosomes) germinated in the laboratory although no germinates were found in seed packets at this retrieval (Figure 1.4).

For *A. racemosa*, the fraction of dormant seeds remaining after two annual cycles in the laboratory simulation were slightly lower than those in the burial study (Figure 1.5), although this was only marginally significant ($F_{1, 7} = 3.82$, $P = 0.09$). Both the laboratory simulation and burial study indicated that the *H. canadensis* seed population was depleted after two annual cycles (Figure 1.5). The effects of elaiosome removal on dormant seed fractions depended on whether *S. canadensis* seeds were buried in the field or placed in the laboratory simulation (elaiosome × treatment: $F_{1, 12} = 6.21$, $P = 0.03$; Figure 1.1.5). Elaiosome removal had no effect on dormant seed fractions in the laboratory simulation, (lsmeans test: $P = 0.27$), but significantly enhanced dormant fractions in the burial study (lsmeans test: $P < 0.0001$).

## Discussion

*Dormancy and the seasonal temperature cycle*

Laboratory experiments and the field burial study confirmed the strong seasonal germination patterns described previously for mesic forest herbs (Baskin and Baskin 2001). In southern Ohio, seeds of *H. canadensis* and *S. canadensis* are dispersed in early-

July and early-June, respectively, and those seeds that germinate the next spring experience a summer → autumn → winter → spring temperature sequence. Seeds of *A. racemosa*, on the other hand, are dispersed in mid- to late-September and experience only a brief period (~ 2 wks) of warm (25-30 °C) temperatures prior to an autumn → winter → spring temperature sequence. Despite contrasting dispersal phenologies, these woodland herbs exhibit similar dormancy-break phenologies in that MPD is broken slowly during the autumn and winter seasons. Seedling emergence is then cued by cool, spring temperatures, enabling seedlings to emerge rapidly in a window when they are at a low risk of mortality due to late winter frosts but prior to complete closure of the forest canopy.

Despite strong similarities in seasonal dormancy-break patterns, primary dormancy levels (measured by germination potential) varied widely among the three species. When maintained at the control thermoperiods, newly dispersed seeds of *A. racemosa* began germinating 16 weeks sooner than the summer dispersed species. These results are consistent with Washtani and Masuda's (1990) prediction for spring germinators in seasonal temperate climates: summer dispersed species should have deeper dormancies than autumn dispersed species since the time period between dispersal and the appropriate germination season is much longer. Summer dispersed seeds with low levels of primary dormancy would be at greater risk of germinating during an ecologically inappropriate season.

The order in which seeds must experience seasonal temperature changes for dormancy-break is not interchangeable. Seeds of all species started in the winter treatment sequence did not completely germinate (radicle and cotyledons) until the

second spring following dispersal, after seeds experienced a summer → autumn → winter sequence. This general warm → cold temperature sequence for dormancy-break is consistent with the expected response of seeds with deep physiological dormancy (Baskin and Baskin 2001). The fact that final germination fractions for all species were similar among the summer and winter treatment sequences also suggests that dormancy-levels in seeds remain constant while they "await" the appropriate order of seasonal temperature cues.

However, summer temperatures play a differential role in the dormancy-break process. In *A. racemosa* and *H. canadensis*, germination fractions of newly dispersed seeds were similar in the autumn and summer treatment sequence, indicating that summer temperatures are not required for dormancy-break. Instead, summer temperatures maintain strong primary dormancy until seeds experience autumn temperatures which cue the underdeveloped embryos to grow. In contrast, *S. canadensis* seeds require summer temperatures to fully break dormancy in autumn. Other perennial woodland herbs that disperse seed in early summer, such as *Jeffersonia diphylla* (Baskin and Baskin 1989) similarly require summer temperatures for full dormancy-break.

Differences in dormancy levels among species were also reflected in their winter stratification requirements. Germination trajectories of *H. canadensis* seeds corresponded to the approximate three-month cold period seeds experience in the field. On the other hand, *A. racemosa* seeds lost dormancy long before the winter duration period would have ended, as seeds began germinating within four weeks in the short winter stratification treatment. This apparent incongruence in germination timing has also been observed in other mesic woodland herbs (Baskin and Baskin 1985b). Vegis (1964)

described this as a Type 2 germination pattern: as seeds pass out of dormancy they first gain the ability to emerge at higher temperatures but as seeds become less dormant they emerge at lower temperatures. However, this germination pattern is probably ecologically irrelevant because the warm temperatures (>25 °C) that seeds are responsive to in the laboratory fall above the maximum temperature conditions seeds would experience in their natural habitat during spring.

Some of the germination patterns reported in previous studies with these species can be explained by the seasonal temperature specificities described here. Davis (2000) reported that when fresh seeds were (1) stored in moist sand at 21 ºC for 30 d, (2) transferred to 5 ºC, (3) and then outplanted in spring, they did not germinate until the second spring season after dispersal (Davis 2000). In this instance, germination was delayed until the second spring season because, during the first year, seeds never experienced the warm/cool cycle (autumn) temperatures followed by winter temperatures that would have probably broken dormancy in a large fraction of the seed crop. Deno (1993) reported a similar pattern with *S. canadensis* and *H. canadensis* seeds germinated to low percentages (< 6%) when transferred through several cycles of a temperature sequence (90 d at 21 ºC → 90 d at 5 ºC) that did not include fluctuating autumn temperatures. Henkel and Klugh (1908) sowed *H. canadensis* seed populations outdoors immediately following dispersal (July) and then the following spring, presumably after storing seeds dry at ambient conditions. Seeds stored indoors and then planted outdoors the following spring did not germinate, because seeds never experienced the warm → cold temperature sequence, whereas 30% of the seeds planted in July germinated the following spring (Henkel and Klugh 1908). Interestingly, Reeleder (2003) reported that

*H. canadensis* seeds collected from a northern population germinated after fresh seeds were: (1) ripened for 5 d at ambient laboratory conditions, (2) stratified at 4 ºC for 100 d, (3) and then germinated at 20 ºC. In Ohio populations, *H. canadensis* seeds that were stratified first in cold (5 ºC) temperatures without a warm pre-treatment did not germinate at the first spring temperature sequence (Figure 1.2).

Data derived from my laboratory study provide practical solutions for germinating seeds of medicinal woodland herbs for conservation purposes (Table 1.4).
These species can germinate on a wide variety of substrates varying in pH, particle size, and water holding capacity, with the exception of *S. canadensis* seeds that germinated poorly on sand. It is not clear why sand inhibits dormancy-break in *S. canadensis*, although one explanation is that the sand has a greater particle size than the other substrates, which could reduce seed surface contact and impede water uptake.
Gibberellic acid can accelerate dormancy-break in *A. racemosa* seeds at the 15/6 °C thermoperiod, ultimately reducing germination time from ~30 weeks in the field to ~18 weeks in controlled conditions. Given that summer temperatures are not required for dormancy-break in *A. racemosa* and *H. canadensis*, seeds can be placed directly in autumn temperatures to cue embryo growth. For *S. canadensis*, the dormancy-break process is long and slow, even though gibberellic acid can substitute for the warm stratification treatment; strong primary dormancy in some seeds can only be broken with multiple annual temperature cycles.

*Double dormancy*

In laboratory and field burial studies, there was no evidence of double dormancy in *S. canadensis* seeds, as radicle emergence only occurred in autumn. In woodland herbs with double dormancy (e.g., *Arisaema triphyllum* and *Trillium grandifolium*), radicles emerge the first spring following dispersal but the cotyledons do not emerge until the second spring, thus two years (or two winters) are needed for seedlings to fully develop (Baskin and Baskin 2001). Further, all *A. racemosa* and *S. canadensis* seeds with radicles emerged in autumn were committed to emergence (or death) the following spring, even when seeds experienced complete darkness in seed packets buried shallowly in the forest soil. This contrasts with the "blind" germination phenology observed in the woodland herb *Arisaema dracontium*, where seeds with emerged radicles that did not experience spring light cues failed to produce an aboveground shoot; instead, seedlings remained belowground as a root system awaiting the next spring season (Yang et al. 1999).

*Delayed germination*

Germination of a seed crop in these medicinal woodland herbs is spread across multiple germination seasons, indicating formation of a short-term persistence seed bank (sensu Walck et al. 2005). Similar results were observed for an *A. racemosa* seed population sown in an unheated glasshouse where germination was spread over three germination seasons (Baskin and Baskin 1985a). Short-term persistent (<5 germination seasons) seed banks have been reported in other co-occurring perennial woodland herbs (e.g., Table 5.6 in Baskin and Baskin 2001) and woody species (Marquis 1975; Hille Ris Lambers et al. 2005) throughout the eastern deciduous forest. One advantage of delayed

germination is that it could buffer local populations from year to year variability in seed production and environmental conditions unfavorable (e.g. drought) for recruitment (Hille Ris Lambers et al. 2005).

Why do some seeds in the population germinate the first spring following dispersal whereas other delay germination? By periodically retrieving buried seed populations over a two year period and subjecting nongerminated seeds to optimum temperatures in the laboratory, I was able to distinguish whether seeds were in primary innate dormancy or were conditionally dormant, in that they could still germinate over a limited range of environmental conditions (Baskin and Baskin 2001). In the burial study, germination of *A. racemosa* and *H. canadensis* seeds in the field and laboratory tests were restricted to the appropriate seasons. Even under ideal conditions (i.e., constant imbibition, light, and optimum temperatures) in laboratory simulations, some seeds failed to germinate after the first seasonal temperature cycle, indicating that carry-over in the burial study cannot be attributed to lack of appropriate dormancy-breaking cues. Thus, strong primary dormancy ensures that a fraction of the newly dispersed seed population is devoted to carry-over, since seeds do not lose dormancy and re-acquire it during the annual cycle. In the case of *S. canadensis*, seed carry-over is mediated by both primary and secondary dormancy mechanisms. Some *S. canadensis* seeds were clearly in secondary dormancy because, in the last summer retrieval, no seeds (both +/− elaiosomes) had germinated *in situ* although a fraction of the seed population germinated in the 30 d laboratory test. Apparently some seeds lost dormancy after experiencing two winter and spring cycles but then re-acquired (secondary) dormancy during the summer.

This fraction of *S. canadensis* seeds that carried-over in field and laboratory simulations is comparatively greater than other perennial woodland herbs studied, wherein a majority of the seed population is committed to germinate at the first germination season (e.g., Thompson and Grime 1979; Baskin and Baskin 1988; Baskin and Baskin 2001). *Sanguinaria canadensis* seeds collected from a Kentucky population and subjected to the annual temperature cycles in an unheated glasshouse spread germination over eight years (Baskin and Baskin 2001), indicating that the results observed in this study are not exceptional. Collectively, these data indicate that in optimum conditions, *S. canadensis* is capable of forming a long-term persistent seed bank since germination of a single seed crop can extend across six seasons (Walck et al. 2005).

Such seed longevity seems unusual for a long-lived woodland herb in a stable forest system (see Pickett and McDonnell 1989), because long-term persistent seed banks are usually associated with species that have "fast" life-histories (e.g., high adult mortality, short life-cycles, and higher fecundity) and occupy habitats with frequent, catastrophic disturbances (Thompson et al. 1998). Further, 'bet-hedging' germination strategies evolve where climatic cues (e.g., precipitation) are unpredictably distributed, such as warm, low latitudinal deserts (Pake and Venable 1996; Clauss and Venable 2000). In temperate forests, however, perennial woodland herbs use the predictable onset of spring temperatures to cue germination (Baskin and Baskin 1988). Long-term seed persistence should be selected against in closed canopy woodlands, as forest plants evolved perenniality and larger seeds to cope with environmental heterogeneity (Venable and Brown 1988). Having larger seeds decreases the likelihood that seeds are

incorporated into the forest soil, which, in turn, make seeds more susceptible to depredation (Fenner and Thompson 2005).

Seed dispersal by ants could play an important demographic role in regulating dormancy-break in *S. canadensis* seeds. In the burial study, the removal of elaiosomes increased dormant seeds fractions. This contrasts with other studies that reported elaiosome removal either enhanced (Lobstein and Rockwood 1993; Ohkawara 2005) or had neutral effects on germination fractions (Lobstein and Rockwood 1993; Christian and Stanton 2004). The overall greater fraction of dormant *S. canadensis* seeds in the burial study compared to the laboratory simulation could be because darkness enhanced dormancy levels in buried seed populations; in the laboratory, elaiosome removal was not tested in complete darkness. This may explain why elaiosome removal had no effect on germination fractions in the laboratory simulation since seeds experienced a daily 12L:12D photoperiod.

Results from the burial study seem at odds with the hypothesis that elaiosome removal signals that ants have deposited seeds in sites favorable for immediate germination (Lobstein and Rockwood 1993), because one would expect smaller fractions of dormant seeds in the elaiosome removal treatment. After dispersing seeds to nest sites and removing the elaiosome, ants bury seeds in refuse piles. Perhaps an alternative explanation is that elaiosome removal enhances dormancy levels because it signals that seed's are buried, a prerequisite for seed bank formation (Fenner and Thompson 2005). This increases the seeds probability of long-term survival because (1) rodents are less likely to discover buried seeds when the elaiosome is removed (Heithaus 1981), and (2) seeds without the elaiosome are less prone to fungal attack compared to seeds with the

elaiosome attached (Christian and Stanton 2004). Low mortality and germination rates of seeds in the elaiosome removal treatment probably explain why exponential decay curves provided a poor fit to dormant seed fractions, explaining only 25% of the variance (Figure 1.4).

One caveat is that seeds can experience pathogen attack or depredation immediately following dispersal, implying that seed populations protected in packets (or germinated in controlled laboratory conditions) could overestimate the realized fraction of dormant seeds that carry-over across seasons (Hille Ris Lambers et al. 2005). Rodents can consume anywhere from 13-94% of newly dispersed *S. canadensis* seeds, and the probability of consumption increases when ants fail to effectively remove the elaiosome and bury seeds in safe sites (Heithaus 1981). Invasive exotic ants can also disrupt the ant-seed mutualism in *S. canadensis* populations, by dispersing seeds to unfavorable microsites, or by damaging seeds during transport and processing (Zettler et al. 2001). Given this spatial variability in post-dispersal seed loss and apparent year-to-year variation in seed production (Pudlo et al. 1980), dormant seed pools within *S. canadensis* populations are probably in disequilibrium.

*Regeneration ecology*

The consequences of delayed germination can be demographically important for woodland herbs whose perennating structures are harvested from the population, as dormant seed pools may be the only link for local persistence. For example, following harvest of most or all of the roots in a population, the woodland herb *Panax quinquefolius* (American ginseng) can recover numerically from dormant seed pools that occur at low

densities in the soil (Lewis 1988; Van der Voort et al. 2003). Although *A. racemosa* and *S. canadensis* can form dormant seed pools that persist for > 2 germination seasons, whether or not this could play a role in the post-harvest recovery of populations remains unclear. The apparent dispersal syndromes of these species (*A. racemosa*: gravity and *S. canadensis*: ants) imply that most seeds are distributed locally, indicating the potential for seeds to buffer populations from localized disturbances such as human harvesting. In other clonal woodland herbs with weak dispersal mechanisms, recruitment from seed is under strong selection pressure and becomes increasingly important in instances where genets experience high turnover rates (Eriksson 1989; Cain and Damman 1997; Damman and Cain 1998).

Dormant seed pools contribute little to the post-harvest recovery in *H. canadensis* because populations regenerate from residual vegetative propagules left in the soil (Van der Voort et al. 2003; Sanders and McGraw 2005a; Albrecht and McCarthy 2006). In established *H. canadensis* populations, recruitment from seed is rarely observed (Sinclair and Catling 2000; Sanders and McGraw 2002; Sanders and McGraw 2005b), and large ramets that clonally propagate via rhizome fragmentation and adventitious roots maintain local population growth (Sinclair et al. 2005). Seed set is not constrained by outcrossing or pollinator activity (Sinclair et al. 2000; Sanders 2004). The results presented here indicate that the low rates of seed recruitment are not attributed to specialized germination requirements, suggesting that selection for seed recruitment within established populations is low. Alternatively, recruitment limitation at local scales may be attributed to seed predators or density-dependent mortality at seed and seedling stages. Indeed, birds are probably important long-distance dispersers of *H. canadensis* seeds

(Sinclair et al. 2000), affording escape from established populations where fungal pathogens appear to accumulate over time (Rock 2000). A combination of relatively large seeds and adaptation for long-distance dispersal is consistent with Eriksson's (1989; 1992) description of a clonal plant that is less likely to repeatedly recruit from seed within established populations.

*Conclusions*

Determining the seed dormancy and germination characteristics is especially important for slow-growing medicinal plants that face persistent pressures in the wild. Medicinal woodland herbs disperse seed with varying levels of primary dormancy, although seedling emergence in the wild is timed to a brief period in spring. Seeds must experience the proper order of temperature sequences for dormancy-break and germination. Thus, seeds cannot be induced into germinating in artificial conditions, although the dormancy-break period can be shortened in *A. racemosa* and *H. canadensis* seeds. Delayed germination occurs in all species but to varying degrees. Seed bank densities in *S. canadensis* are contingent on whether ants remove the elaiosome and whether seeds are buried. Seed banks may be demographically important for the recovery of natural populations, particularly in *S. canadensis*.

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Table 1.1. Effects of substrate on germination fractions of three medicinal woodland herbs. Different letters within a row are significantly different according to least-squares mean tests ($P < 0.05$). Separate tests were conducted for each species.

| Species | Field soil | Peat moss | Sand | Mix |
|---|---|---|---|---|
| *Actaea racemosa* | 0.84 ± 0.02a | 0.81 ± 0.02a | 0.91 ± 0.02a | 0.80 ± 0.05a |
| *Hydrastis canadensis* | 0.73 ± 0.02a | 0.80 ± 0.04a | 0.77 ± 0.05a | 0.80 ± 0.04a |
| *Sanguinaria canadensis* | 0.34 ± 0.04a | 0.34 ± 0.02a | 0.06 ± 0.04b | 0.28 ± 0.03a |

Table 1.2. Effects of light and darkness on germination fractions (mean ± 1 se) of three medicinal woodland herbs. *H. canadensis* seeds stratified and germinated in the dark treatment rotted. Different letters within a column are significantly different according to least-squares mean tests ($P < 0.05$). Separate tests were conducted for each species.

| Species | Light regime | | Radicle emergence (mean ± SE) | Cotyledon emergence (mean ± SE) |
|---|---|---|---|---|
| | Stratified in: | Germinated in: | | |
| (A) *Actaea racemosa* | | | | |
| | Light | Light | 0.95 ± 0.03a | 0.77 ± 0.06a |
| | Light | Dark | 0.97 ± 0.05a | 0 ± 0b |
| | Dark | Light | 0.99 ± 0.01a | 0.81 ± 0.06a |
| | Dark | Dark | 0.97 ± 0.09a | 0 ± 0b |
| (B) *Hydrastis canadensis* | | | | |
| | Light | Light | 0.75 ± 0.04a | 0.57 ± 0.07a |
| | Light | Dark | 0.81 ± 0.04a | 0.67 ± 0.10a |
| | Dark | Light | 0.74 ± 0.07a | 0.57 ± 0.08a |
| | Dark | Dark | – | – |
| (C) *Sanguinaria canadensis* | | | | |
| | Light | Light | 0.15 ± 0.04a | 0.13 ± 0.03a |
| | Light | Dark | 0.19 ± 0.04a | 0.17 ± 0.05a |
| | Dark | Light | 0.06 ± 0.02b | 0.05 ± 0.02b |
| | Dark | Dark | 0.04 ± 0.01b | 0.04 ± 0.01b |

Table 1.3. Effects of gibberellic acid ($GA_3$) on seed germination fractions (mean ± 1 se) of *Actaea racemosa* and *Sanguinaria canadensis*. Means with different letters are significantly different according to least-squares mean tests ($P < 0.05$). Separate tests were conducted for each species.

| Species | $GA_3$ concentration (mg/L) | Thermoperiod (° C) | | | |
|---|---|---|---|---|---|
| | | 5 | 15/6 | 20/10 | 30/15 |
| *Actaea racemosa* | | | | | |
| | 0 | 0 ± 0a | 0.23 ± 0.06b | 0 ± 0a | 0 ± 0a |
| | 100 | 0 ± 0a | 0.58 ± 0.08c | 0.06 ± 0.02f | 0 ± 0a |
| | 1000 | 0 ± 0a | 0.80 ± 0.07d | 0.14 ± 0.04b | 0 ± 0a |
| *Sanguinaria canadensis* | | | | | |
| | 0 | 0 ± 0a | 0 ± 0a | 0 ± 0a | 0 ± 0a |
| | 100 | 0 ± 0a | 0.05 ±0.02b | 0.02 ± 0.02b | 0.01 ± 0.01a |
| | 1000 | 0 ± 0a | 0.48 ± 0.08c | 0.54 ± 0.06c | 0.18 ± 0.05d |

Table 1.4. Optimum temperature sequences that maximize germination rates in seeds of medicinal woodland herbs.

| Species | Optimum temperature sequence | Time to seedling emergence (weeks) |
|---|---|---|
| *Actaea racemosa* | 15/6 °C + 1000 mg/L $GA_3$ (8 wk) → 5°C (8 wk) → 30/15 °C (2 wk) | 18 |
| *Hydrastis Canadensis* | 20/10 ºC (4 wk) → 15/6 ºC (4 wk) → 5 ºC (12 wk) → 20/10 ºC (6 wk) | 26 |
| *Sanguinaria canadensis* | 30/15 ºC (12 wk) → 20/10 ºC+ 1000 mg/L $GA_3$ (4 wk) →15/6 ºC (4 wk) → 5 ºC (12 wk) → 15/6 ºC (6 wk) | 38 |

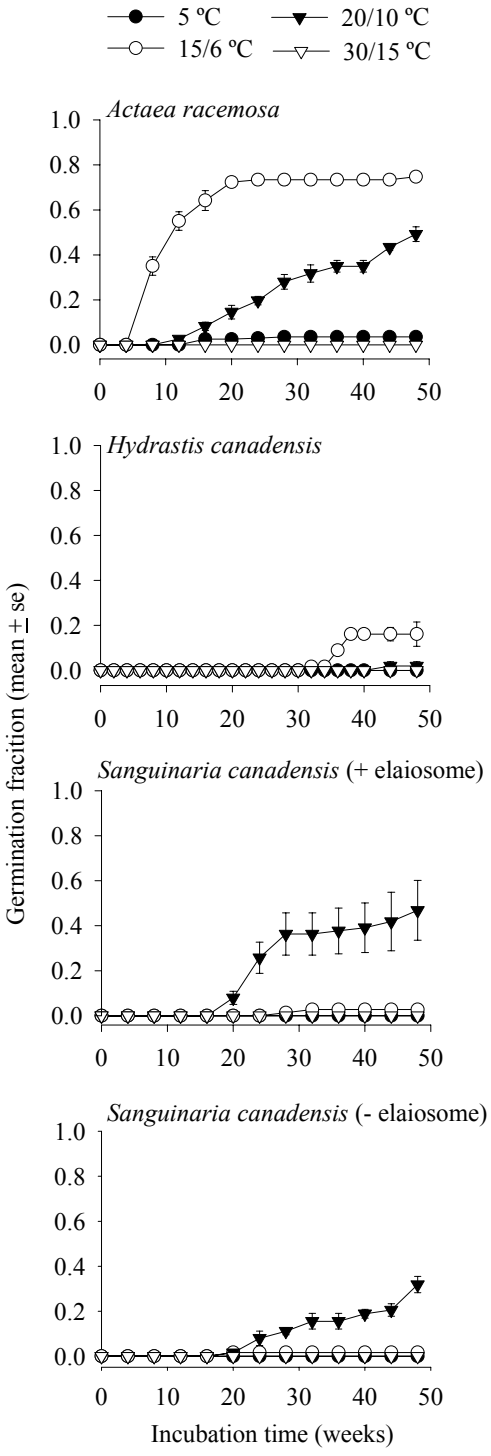


Figure 1.1. Cumulative germination fractions (mean ± se) for seeds of medicinal woodland herbs maintained continuously for 48 wk at four thermoperiods.

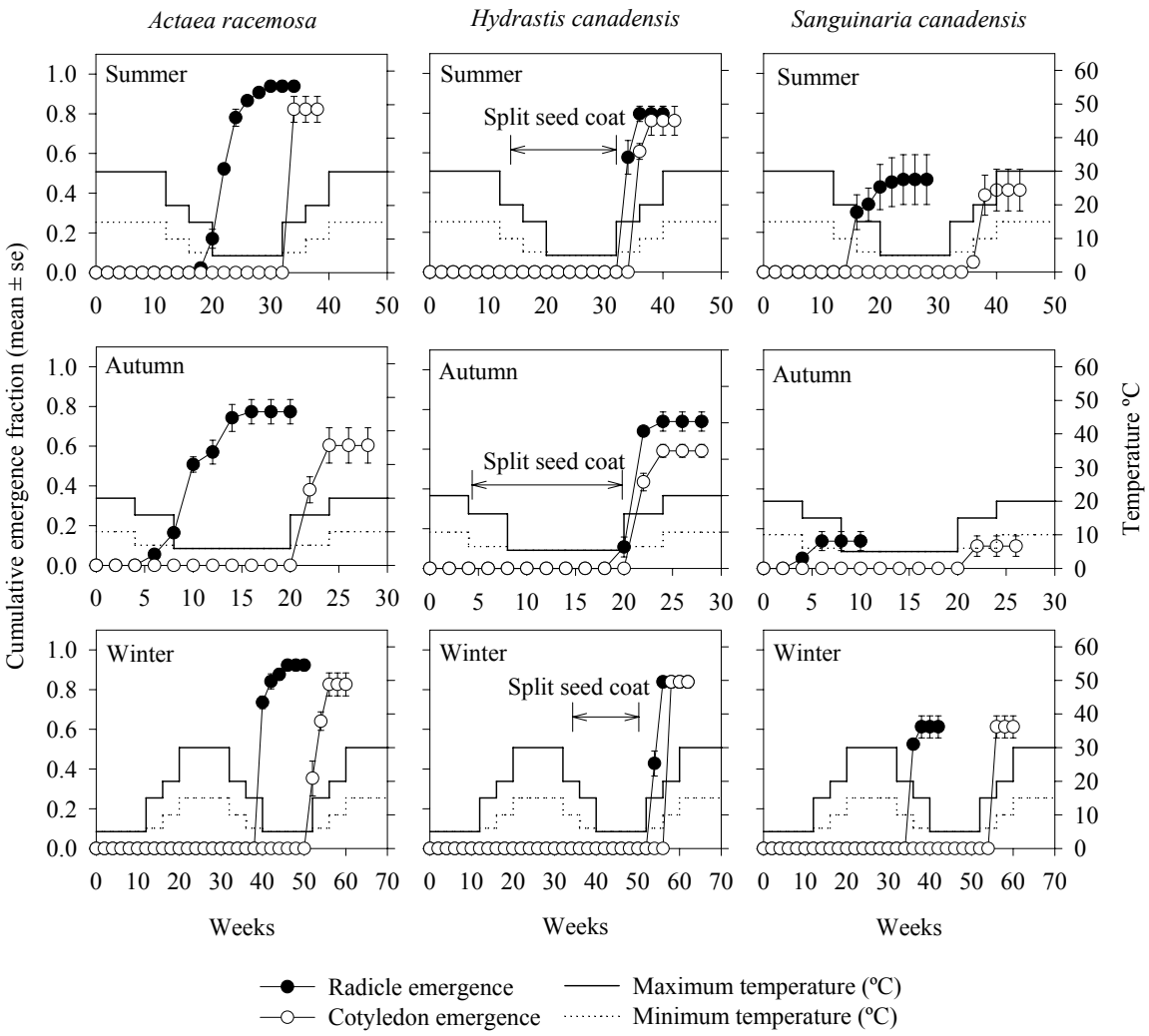


Figure 1.2. Cumulative germination fractions (mean ± se) for seeds of medicinal woodland herbs transferred through simulated temperature sequences started at summer (30/15 °C), autumn (20/10 °C), and winter (5 °C).

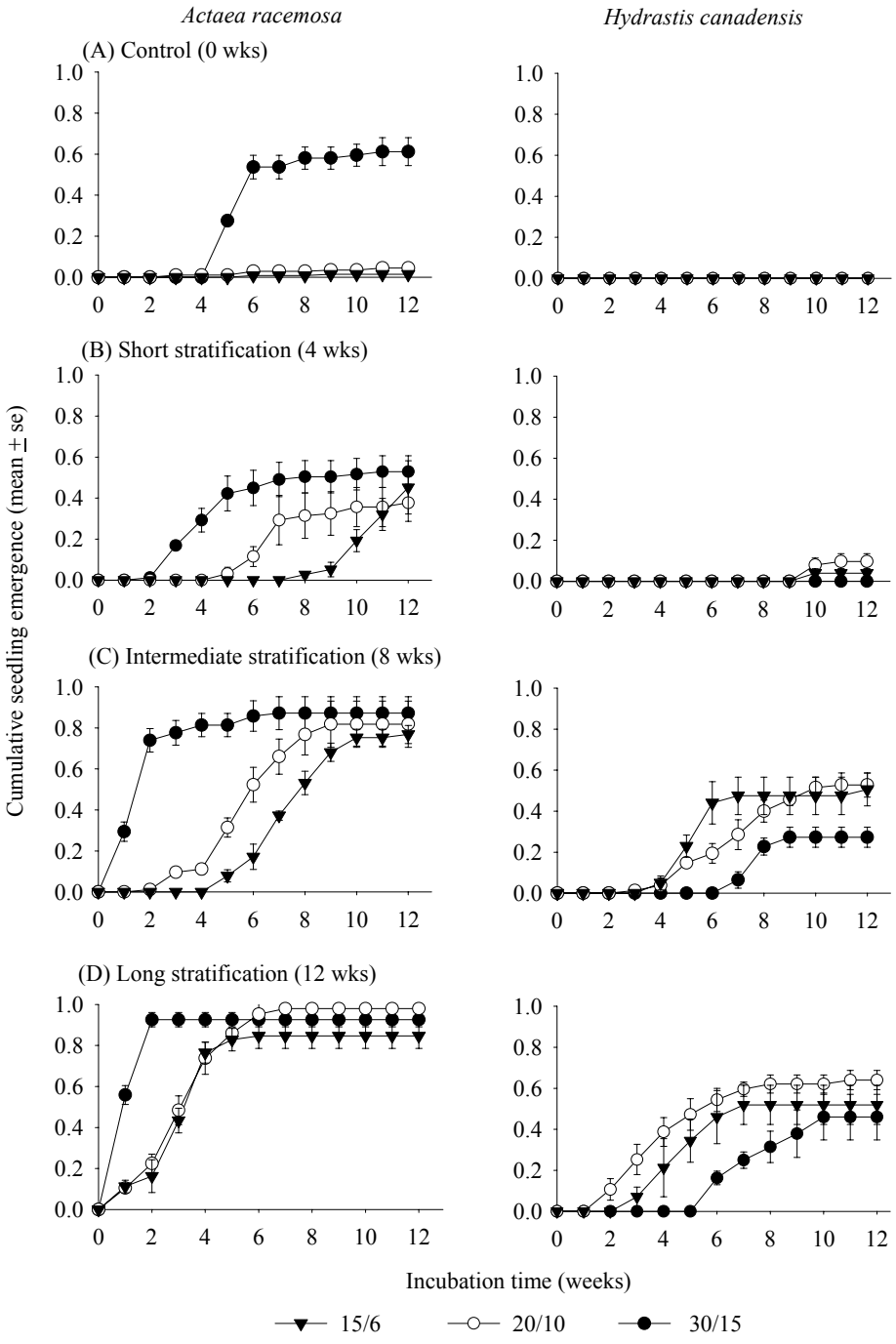


Figure 1.3. Seedling emergence trajectories (mean ± se) of *Actaea racemosa* and *Hydrastis canadensis* across three thermoperiods in the (A) control (no winter temperatures), (B) short (4 wks), (C) intermediate (8 wks) and (D) long winter (12 wks) stratification (5 °C) treatments.

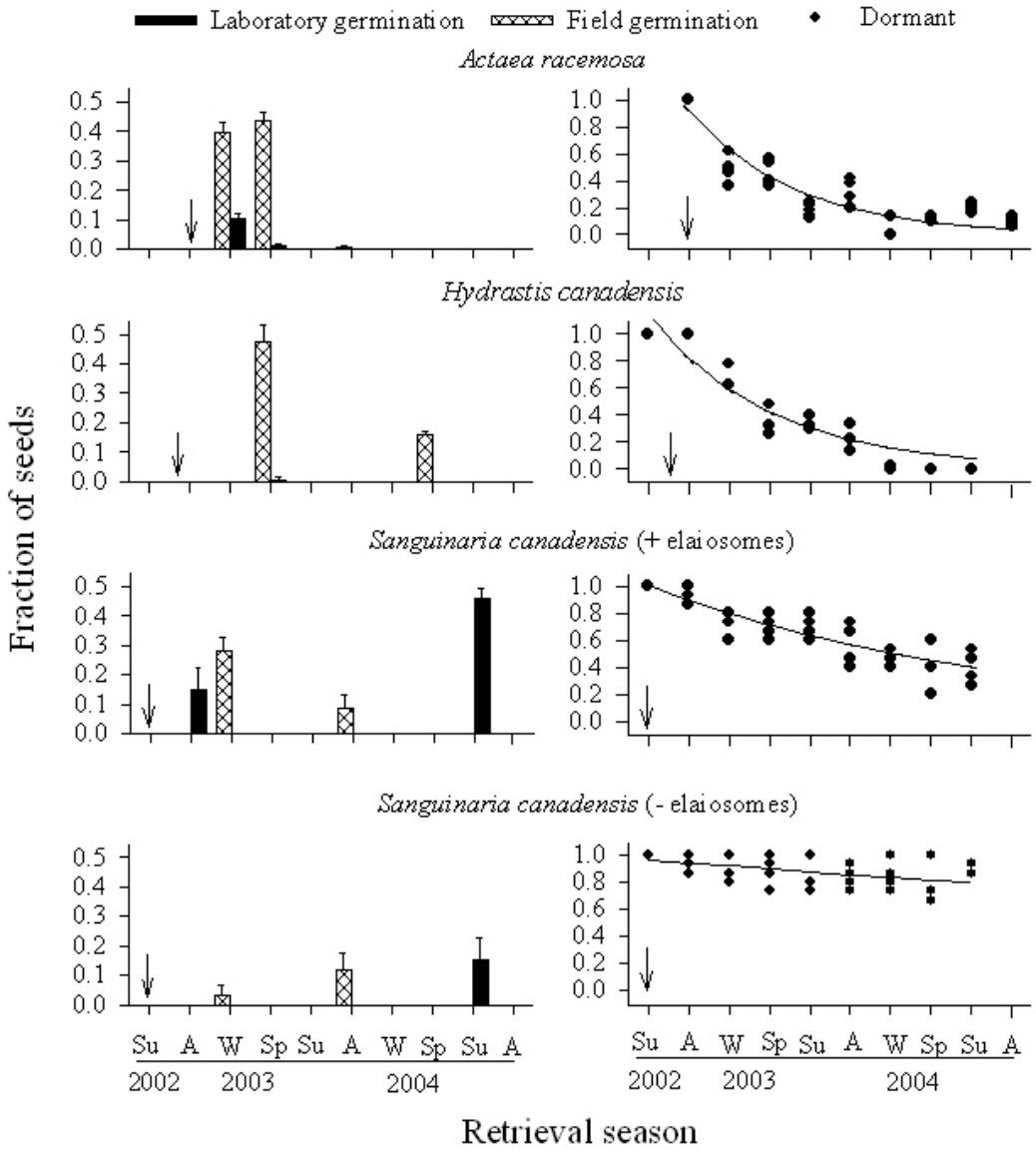


Figure 1.4. The fate of seed populations buried approximately 2 cm belowground in forest soil over a two year period. Germination refers to seeds with emerged radicles. Arrows indicate when seeds are naturally dispersed.

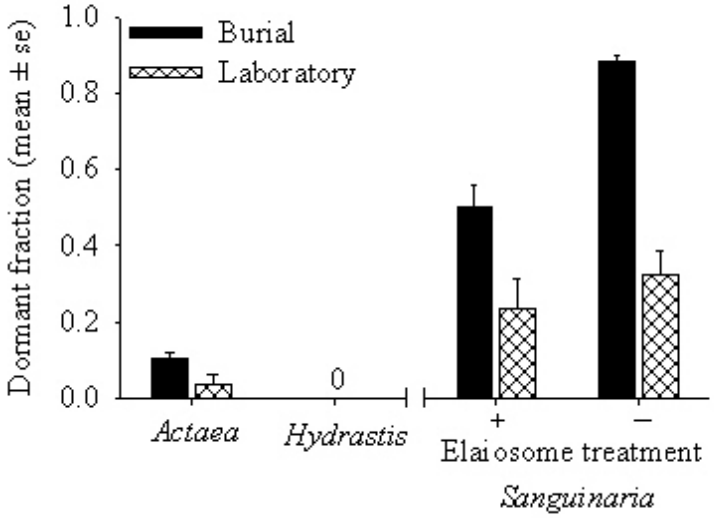


Figure 1.5. The fraction of dormant seeds (mean ± se) after two annual cycles in a laboratory simulation and two years of burial (approximately 2 cm belowground) in forest soil.

## Chapter 2: Seedling recruitment dynamics of medicinal woodland herbs: effects of environmental heterogeneity

### Introduction

In the eastern deciduous forest of the unglaciated Alleghany plateau, fine-scale environmental heterogeneity (e.g., leaf litter) is superimposed on topographically driven micro-climatic gradients, creating a mosaic of different "safe sites" (sensu Harper 1977) for germination and establishment. Strong microhabitat specificity exhibited by many woodland herbs suggests that seedling recruitment may be constrained by certain micro-environmental conditions (Bratton 1976a,b; Hicks 1980; Beatty 1984). Experimental studies have demonstrated that, in some woodland herbs, seedling recruitment is restricted to certain combinations of forest floor micro-habitats (Whigham et al. 1993; Vasseur and Gagnon 1994). By contrast, other studies have found that woodland herbs can germinate and establish across an array of different microsites suggesting that recruitment is limited primarily by seed availability rather than by specific environmental requirements for seedling establishment (Eriksson 1994,1995; Drayton and Primack 2000; Vellend et al. 2000; Fröborg 2001).

The 'litter limitation' hypothesis proposes that leaf litter is an important filter of postdispersal recruitment in temperate woodland herbs (Facelli and Pickett 1991; Eriksson 1995; Fröborg and Eriksson 1997). In small seeded species, leaf litter can intercept germination cues (e.g., light) and act as a physical barrier to seedling emergence (Fenner and Thompson 2005). Alternatively, leaf litter can facilitate establishment of larger seeded species by protecting seeds from vertebrate predation

(Fröborg and Eriksson 1997), and buffering seeds and seedlings from desiccation by moderating evaporative moisture loss at the soil-litter interface (Facelli and Pickett 1991; Facelli and Ladd 1996). The effects of litter, however, can vary widely and species-specific responses to litter often depend on a complex interaction between seed size, litter type, and litter depth (Facelli and Pickett 1991; Molofsky and Augspurger 1992; Xiong and Nilsson 1999; Fröborg 2001).

In this study, I examined seed and seedling recruitment probabilities of four medicinal woodland herbs — *Actaea racemosa* L. (black cohosh), *Hydrastis canadensis* L. (goldenseal), *Panax quinquefolius* (American ginseng), and *Sanguinaria canadensis* L. (bloodroot) — indigenous to eastern North America. These species are harvested from the wild and globally traded in the botanical medicines industry (Robbins 1999). Due to over-exploitation, habitat alteration, excessive deer pressure, and poor dispersal ability, recent evidence suggests that these species are experiencing population decline throughout portions of their natural ranges (Sinclair and Catling 2000; Sanders and McGraw 2002; Fankland and Nelson 2003; Ruhren and Handel 2003; McGraw et al. 2005; Mulligan and Gorchov 2005). Consequently, these species are often absent from successional forests that seemingly provide suitable habitat for colonization and establishment (van Manen et al. 2005). In order to formulate effective restoration and management plans, experimental studies are needed to evaluate what types of forest patches are suitable for seedling recruitment in these species.

Experimentally dispersing seeds into unoccupied sites is one option for restoring and managing forest plant populations (Primack and Miao 1992; Drayton and Primack

2000; Flinn and Vellend 2005). Seed addition studies explicitly test for local recruitment limitation by seed availability and determine how early life-history stages respond to micro-environmental heterogeneity in the field (Eriksson 1995; Ehrlen and Eriksson 2000). In a factorial arrangement, I added seeds of the target species to unoccupied forest microsites defined by varying levels of leaf litter. I superimposed fine-scale litter treatments on opposing landscape positions (north vs. south) and asked the following questions: (1) How does variation in litter levels affect emergence? (2) Can litter effects change on opposing landscape positions? (3) Do larger seeded woodland herbs exhibit greater recruitment probabilities?

## Materials and Methods

### *Study Species and System*

The four polycarpic woodland herbs selected for study represent a range of life-history traits including seed size and dispersal syndrome (Table 2.1). Each species exhibits morphophysiological dormancy (MPD), and seedling emergence occurs during a brief period in spring just prior to canopy closure (Baskin and Baskin 2001). Due to conservation concerns, both *H. canadensis* and *P. quinquefolius* are included in the Appendix II of the Convention on International Trade in Endangered species (CITES; Robbins 2000).

This study was conducted in a second-growth forest watershed at the Appalachian Forest Resource Center (AFRC) in Meigs County, Ohio (39º5´N, 82º9´W), located in the unglaciated Western Alleghany Plateau region of the Appalachian highlands. This highly

dissected region is characterized by repeated sequences of narrow ridgetops and stream valleys that bracket steep forested slopes (total relief < 100 m; King 1979). Strong physical gradients on these opposing north and south aspects interact with local geomorphological processes, resulting in greater soil pH, base-cation concentrations, A horizon thickness, and soil moisture availability on north facing slopes (Hutchins et al. 1976; Small and McCarthy 2002).

Secondary forests at this site are classified as mesophytic (sensu Dyer 2006) with *Liriodendron tulipifera* L. and *Acer saccharum* Marsh. dominating north-facing slopes and mixed-*Quercus* communities dominating southerly aspects and ridgetops. The woody plants *Asimina triloba* L. Dunal and *Lindera benzoin* L. Blume form dense patches that are interspersed in the understory. Natural and transplanted populations of the target species are distributed throughout the site, indicating that environmental conditions are suitable for the persistence of these species. Soils at this site are described as Alfisols and Ultisols in the Upshur-Gilphin series, which are overlain on sandstone and shale bedrock containing interbedded bands of limestone and coal (Gilmore and Bottrell 2000). Deep to moderately deep, well-drained silt loams to silty clay loams characterize soils on the forest slopes (Gilmore and Bottrell 2000). Climate is humid continental, with an average annual precipitation of 105 cm falling uniformly throughout the year; mean annual temperature ranges from -2 °C in January to 23 °C in July (NOAA 2004).

*Experimental design and sampling*

In order for my results to be relevant to ecological restoration and management activities, I used commonly implemented techniques in woodland herb restoration to optimize seedling recruitment (Cech 2002). In a completely randomized split-plot design, I established three 4 × 4.5 m plots each on opposing north- and south-facing aspects in the summer of 2003. To control for a moisture-fertility gradient that extends from the valley floor to the ridgetop, plots were placed at mid-slope positions (5-15% incline) in relatively uniform canopy and microtopgraphic conditions. All saplings (<3 cm dbh) and shrubs (multi-stemmed woody plants) were cleared within and in a 2 m buffer around each plot. Occasional ground-layer vegetation such as evergreen ferns and perennial herbs were carefully transplanted out of each plot and into adjacent empty patches. In June 2003, I standardized seedbed conditions across all plots by using a Mantis portable tiller/cultivator to lightly till the $A_1$ horizon down to a depth of approximately 5 cm.

Fresh seed of *Actaea racemosa*, *Hydrastis canadensis*, and *Sanguinaria canadensis* were collected from populations distributed throughout the site, most of which were transplanted from regional forests for conservation purposes. Because of limited seed availability at the study site, 12-month-stratified *Panax quinquefolius* seed was purchased from an Ohio propagator and sown in October 2003. Prior to sowing *S. canadensis* seeds, I removed the elaiosome from half of the seeds because a prior laboratory study showed that seeds without elaiosomes germinated to slightly greater percentages than seeds with the elaiosomes left intact (Lobstein and Rockwood 1993).

Seed viability, based on testing a random subset of seeds with a tetrazolium stain test, was > 90% for all species.

Main plots were divided into twelve 1 × 1.5 $m^2$ quadrats ($n$ = 72 quadrats), each separated by a 0.5 m buffer to minimize disturbance during census. For each species, 100 fresh seeds were sown evenly in a grid pattern in three randomly selected quadrats within every main plot (total of 7200 seeds sown). For *S. canadensis*, each quadrat was further divided so that one half received 50 seeds with an elaiosome and the other half received 50 seeds without an elaiosome. However, cumulative seedling emergence (over two years) among *S. canadensis* seeds with and without the elaiosome were only marginally different ($F$ = 3.60, $P$ = 0.07), thus I collapsed the two different seed samples together for all subsequent analyses. Seeds were hand planted at a depth of ca. 1.5 cm in the soil and litter was re-distributed uniformly over plots to reduce predation (Chambers and MacMahon 1994; Garcia et al. 2002). Two 4 × 1 m belt transects that ran parallel to both sides of all main plots ($n$ = 6) were established to monitor background emergence.

Maximum potential emergence was evaluated by sowing 100 seeds of each species into 4 plastic flats filled with standard potting soil mix. Seeds were scattered over the surface and then covered by approximately 2 cm of additional potting soil and a thin layer of leaves which were removed before spring emergence. Flats were placed outside beneath a grove of *Acer saccharum* trees so that seeds could experience the annual temperature and moisture cycle. A metal screen was placed over the flats to prevent predation; flats were watered during the growing season as needed. Over a 2 year period,

flats were checked for emergence every week in spring and periodically checked during the growing season. All emerged seedlings were counted and removed at each census.

Leaf litter depth was sampled in spring 2003 to determine realistic litter depths seeds may experience during spring emergence. Eight 30 m transects (2 per aspect) were placed randomly at mid-slope positions and every 2 m along each transect the depth of leaf litter (L+F layers) was recorded. Litter depths of 2 and 5 cm approximated the lower ($25^{th}$) and upper quartiles ($75^{th}$) of the distribution (median or $50^{th}$ quartile = 3.2 cm) of leaf litter levels across the forest floor, respectively. These two values were, therefore, chosen to represent "shallow" and "deep" litter depths in the experimental treatments.

In February 2004, each of three species quadrats in every main plot was randomly assigned into one of three seedling emergence conditions: litter removed, shallow litter (2 cm), and deep litter (5 cm). Because germination of a single seed crop of each study species may be spread across multiple years, the same litter treatments were reapplied to the quadrat in February of 2005. Seedling emergence was monitored at approximately 7 d intervals from March to May and all new seedlings were marked with uniquely labeled wooden stakes, so the fate of each emerged seedling could be monitored over time. Leaf litter levels within each quadrat were also checked and, if necessary, adjusted for uniformity within each microsite. Thereafter, seedling survivorship was monitored at 4-wk intervals until September, the period of aboveground senescence. In 2005, seedling survivorship was scored in April, June, and August. Ground-layer vegetation was clipped from all quadrats at each census to minimize competitive interference. During the June 2005 census, shoot height and leaf width for all surviving seedlings was measured to the

nearest 0.1 cm. For the compound leaves of *A. racemosa* and *P. quinquefolius*, I measured the width of the largest leaflet.

Light and soil moisture were monitored concurrently over the two-year study period. A quadrat representing each litter treatment was randomly selected within each plot and sampled ($n$ = 3) for volumetric water content (VWC) at monthly intervals during the growing season using a Hydrosense™ moisture monitor containing two 12 cm probes ($n$ = 54 measurements per census). To avoid pseudoreplication, replicate samples taken within each quadrat were later averaged for statistical analysis. All measurements were taken $\geq$ 3 d following rainfall. During cloudy days in July 2004 and 2005, understory light levels were quantified with hemispherical photographs taken at the center of each plot and 1 m in from each of the four corners ($n$ = 5 photographs per plot). Photos were taken with a 35-mm Nikon digital camera equipped with a Sigma 8-mm fish eye lens positioned approximately 1 m from the ground. Digital photographs were analyzed using the Gap Light Analyzer software program to determine percent open sky and the absolute amount of growing season radiation (direct and diffuse) transmitted through the canopy after accounting for local terrain and meteorological conditions (Frazer et al. 1999).

*Data Analysis*

All abiotic variables were analyzed using mixed-model analysis of variance (ANOVA) in PROC MIXED (SAS Institute 2001). I used a repeated measures mixed-ANOVA for soil moisture analysis, since VWC measurements were repeated at each census in quadrats over two growing seasons. In this analysis, litter and aspect were

considered the between-subject effects and time the within-subject effect. For total transmitted radiation and percent open sky, which was sampled once per year, I used a three-way mixed ANOVA considering aspect, litter, and year as fixed effects and plot as a random effect.

For all demographic analyses I considered aspect the whole-plot factor and litter and species the subplot factors. The six plots were considered random effects in all models and represent pseudoreplicates of each opposing aspect. Plot and the plot × aspect interactions were coded as the error terms according to the split-plot model design (Littell et al. 1996). For all subsequent parametric models I conducted two analyses. First, I included species in the model to test the alternative hypothesis that the effects of litter would vary according to species identity. I then omitted species from the model and ran a separate analysis for each of the four species to explore their individualistic responses to environmental variability.

The number of seedling that emerged in each treatment combination was compared with a generalized linear mixed model in (PROC GLIXMIX; SAS Institute 2001). Emergence counts were assumed to follow a Poisson distribution . Post-hoc multiple comparisons were conducted on least-square means with Bonferroni adjusted *P*-values.

Seedling survival time was analyzed using parametric and nonparametric methods (Fox 2001). Nonparametric estimates of seedling survivorship were based on the Kaplan-Meier product limit estimator of the survival function, $S(t)$, which measures the probability that an individual seedling survives beyond time, $t$. Seedling survivorship

curves among litter treatments on each aspect were compared using a log-rank test on estimated survival functions. For formal testing of main effects and their interactions on seedling survivorship, an accelerated failure time model was fit to survivorship data (AFAT; PROC LIFEREG; SAS Institute, 2001). Because AFAT models in SAS are incapable of handling random effects, I was unable to incorporate the plot effect in this analysis (Allison 1995). I conducted two separate analyses with the 2004 and 2005 cohort. Models with five different error distributions were run for each species and the model with the highest log-likelihood ratio was selected (Allison 1995; Fox 2001). When main and/or interactive effects were significant, I used a post-hoc multiple comparison test to calculate Wald test statistics from the estimated variance and covariance matrix based on the method described in Allison (1995).

Interactions of leaf litter with broad-scale environmental gradients could result in seed-seedling conflicts whereby patches that negatively affect one life-history stage can facilitate recruitment in another life-history stage (Schupp 1995). Thus, one must consider the net effects of environmental variation across multiple life-history transitions to properly assess recruitment outcomes. I calculated the probability of a seed transitioning into a 2-year seedling in each of six microsites and tested for differences with the same generalized linear model described previously.

A linear mixed ANOVA (PROC MIXED) was fit to seedling height and leaf width data with the split-plot model. However, because of differential survival among microsites, the data structure was necessarily unbalanced. I accounted for this by using Sattherwaite's method to determine the approximate denominator degrees of freedom in

the model (Littell et al. 1996). A full model testing for species effects was precluded by the large differences in variance among species that could not be stabilized through transformation.

**Results**

There were clear differences in understory light levels among opposing aspects (Figure 2.1). Total transmitted solar radiation and percent open sky were significantly greater on south plots than north plots (both *P*-values < 0.05), although there were no differences among years (aspect × time: both *P*-values > 0.4).

There were no differences in VWC among the litter treatments during any of the census periods. Differences in VWC among opposing aspects depended on time (Table 2). VWC was consistently greater on north plots than south plots during mid- to late-summer in both years (Figure 1). Palmer drought severity indices (PDSI), a standardized measurement of moisture conditions, indicated moderately wet conditions during the 2004 growing season, whereas growing season values in 2005 ranged from normal to mild drought (NOAA 2004).

Seedling emergence rates varied widely among species in the field ($F_{3, 44} = 77.78$, $P < 0.0001$). Overall, total seedling emergence across all microsites and years was 6% for *A. racemosa*, 20% for *H. canadensis*, 26% for *P. quinquefolius* and 15% for *S. canadensis*. Germination was spread over two-years for all species except *H. canadensis*. For *P. quinquefolius*, a majority (95%) of the seeds that germinated did so the first spring after sowing. Of the 276 *S. canadensis* seeds that germinated, 70% emerged during the

first spring. Of the 110 *A. racemosa* seeds that germinated, 42% emerged during the first spring. Cumulative germination in flats over two germination seasons was greater than in the field for *P. quinquefolius* (43%), *S. canadensis* (37%), and *A. racemosa* (28%), although germination was lower in flats for *H. canadensis* (8%).

Seedlings emergence was not homogenous among treatments and species (aspect × litter × species interaction: $F_{6, 44} = 2.89$, $P = 0.02$), indicating that these woodland herbs vary in their selectivity for certain micro-environments. *Actaea racemosa* and *Hydrastis canadensis* seedlings emerged at greater rates on north-facing plots compared to south-facing plots, whereas *P. quinquefolius* and *S. canadensis* seedlings emerged at similar rates on the opposing aspects (Figure 2.2). With the exception of *A. racemosa*, seedlings emerged at different rates among litter treatments, although for *H. canadensis* and *S. canadensis* litter effects depended on slope aspect (Figure 2.2). Compared to shallow litter microsites, deep litter microsites inhibited seedling emergence for all species (except *A. racemosa*) on both aspects. In litter free microsites, *H. canadensis* and *S. canadensis* seedling emergence was lower on south plots compared to north plots (Figure 2.2).

Seedling survival time varied significantly among species over the 720 d study period ($\chi^2 = 98.4$, $P < 0.0001$). Species exhibited differential survival times among litter treatments (species × litter: $\chi^2 = 17.40$, $P = 0.008$) and aspects (species × aspect: $\chi^2 = 25.78$, $P < 0.0001$), although the 3-way interaction was nonsignficant (aspect × litter × species interaction: $\chi^2 = 2.75$, $P = 0.84$). Nonparametric log-rank tests on *H. canadensis* seedlings showed that survival time in litter-absent microsites was greater than in litter-

present microsites, although this occurred only on north plots (Figure 2.3). This finding was confirmed by a significant aspect × litter interaction in the parametric analysis (Table 2.3; multiple comparison tests: bare vs. deep, $P = 0.03$; ambient vs. bare, $P = 0.03$; ambient vs. deep, $P = 0.87$). By contrast, litter significantly affected *P. quinquefolius* seedling survivorship time independent of aspect (Table 2.3), with survival times ranked in the following order across litter treatments: shallow > deep > bare (all multiple comparisons, $P < 0.03$). *S. canadensis* seedling survival was significantly greater on north plots than south plots, although there were no obvious litter effects (Table 2.3). The reverse occurred for *A. racemosa* seedlings, as survival time was significantly greater on south plots compared to north plots (Table 2.3). However, I consider the latter result spurious since residual plots suggested that no parametric model provided a reasonable fit to the data due to the small sample size with this species. There were no significant effects observed in the 2005 cohort of *S. canadensis* and *A. racemosa* (all *P*-values > 0.27).

Microsites selectively influenced seedling height and leaf width. Except for *A. racemosa*, all species exhibited significantly greater heights in microsites where litter was present compared to those where litter was absent, although this effect was more pronounced for *P. quinquefolius* on south plots (Table 2.4; Figure 2.4). Only *A. racemosa* leaf size exhibited a significant litter effect (Table 2.4). Mean height and leaf widths were greater for *S. canadensis* on north slope plots compare to south slope plots.

Cumulative transition probabilities summed across microsites indicated that large seeded species exhibited greater recruitment rates than small seeded ones (Table 2.5;

Figure 2.5). Species also responded differentially to slope aspect (Table 2.5). For *S. canadensis*, transition probabilities were lower across all south-facing plots compared to north-facing ones independent of litter conditions (Figure 2.5), although these results were only marginally significant (aspect: $F = 8.63$, $P = 0.09$; aspect × litter: $F = 0.60$, $P = 0.57$). By contrast, the effects of litter on transition probabilities depended on aspect for *H. canadensis* (aspect × litter: $F = 6.40$, $P = 0.02$) and *P. quinquefolius* (aspect × litter: $F = 8.89$, $P = 0.009$). Seedling recruitment rates were lowest in litter-free sites on south-facing plots (Figure 2.5). No differences in transition probabilities among microsites were detected for *A. racemosa* (all *P*-values > 0.27).

**Discussion**

Mesic woodland herbs of eastern deciduous forests are often characterized by high microhabitat specificity, limited dispersal, and low seedling establishment rates. Despite the large numbers of established populations comprising sexually reproducing individuals at our study site, colonization of new sites by seed is uncommon to rare in the study species (personal observation). Seedling emergence and establishment were observed in nearly every patch type suggesting that numerous microsites may be available for local colonization and restoration of woodland herbs. However, differential seedling recruitment among patch types was also observed for three of the four woodland herbs, indicating that local recruitment can be limited by a combination of seed availability and micro-environmental variation (Eriksson and Ehrlen 1992).

*How does variation in litter levels affect emergence?*

Although leaf litter is an important recruitment filter in most ecosystems (Xiong and Nilsson 1999), none of the woodland herbs considered here emerged exclusively in litter-free microsites. Instead, variation in leaf litter levels determined the direction and magnitude of litter effects on seedling regeneration: emergence was significantly lower in deep litter microsites relative to shallow litter ones in three of the four species. This pattern was consistent on both landscape positions, despite differences in the physical structure of litter on the opposing aspects. For example, litter composed of mixed-*Quercus* species (south slope plots) tends to be loose, curly and contain many interstices for seedlings to emerge, whereas litter of mesophytic species (*Acer* and *Liriodendron*) forms compact mats held together by fungal hyphae, which could act as a stronger mechanical barrier. This supports conclusions drawn from Peterson and Facilli's (1992) work with early-successional woody plants, in that the amount of litter is often more important than the type of litter during early-life history stages.

Results presented here support the hypothesis that deep litter levels reduce emergence in woodland herbs by acting as a mechanical barrier (Sydes and Grime 1981b; Facelli and Pickett 1991; Eriksson 1995). It seems unlikely that an altered light environment in litter sites influenced germination, because in laboratory studies these species can break dormancy in complete darkness, and there is no evidence they gain a light requirement following shallow burial. Seeds that gain a photoblastic requirement following dispersal are generally < 0.1 mg (Grime et al. 1981), and dependency on light as a germination cue is inversely related to seed size (Milberg et al. 2000). Although leaf

litter could reduce emergence by moderating diurnal temperature fluctuations that trigger germination (Thompson et al. 1977), glasshouse and field studies have shown that many mesic woodland herbs germinate freely beneath litter (reviewed in Eriksson 1995; Baskin and Baskin 2001). Fröberg's (2001) generalizations in Scandinavian temperate forests, that woodland herbs with seeds > 3 mg emerge freely beneath forest litter, were also consistent with this study and reinforce the predictive relationship between species-specific seed mass and emergence probabilities beneath litter in forest communities.

Greater amounts of energy reserves in large seeds of late-successional forest species allow for greater stem elongation to penetrate physical barriers (Thompson 1987). In three of the four species, seedlings tended to grow taller in microsites where litter was present, and etiolated hypocotyls were noted in seedlings that germinated in the darkness in laboratory studies. This is consistent with Grime's (1979) conclusion that many perennial woodland herbs exhibit emergence strategies and seedling geometries, including robust shoot morphologies, to cope with physical barriers on the forest floor. However, litter depths deeper then the ones administered in this study undoubtedly become limiting, even for the most litter tolerant woodland herb (Sydes and Grime 1981b,a; Beatty and Sholes 1988).

*Can litter effects change on opposing landscape positions?*

Litter can facilitate emergence in sites where microclimatic conditions are more extreme by moderating temperature and moisture fluctuations (Callaway and Pugnaire 1999), which are two critical factors that determine demographic outcomes in the early

life-history stages of forest plants. Although I observed insignificant moisture differences between bare and litter microsites, our measurements represented moisture content at deeper soil depths and may not have captured micro-environmental differences in moisture at the soil-litter interface. McKinney (1929) demonstrated that moister spring seedbeds were maintained in litter microsites compared to bare-ground areas because forest litter lowers surface air temperatures, thereby preventing water vapor diffusion at the soil surface. In this study, the presence of leaf litter may have buffered germinating seeds from the greater moisture deficits and low temperatures in forest patches where moisture regimes were less stable. For example, emergence probabilities of *H. canadensis* and *S. canadensis* were lower on bare ground microsites than shallow litter microsites only on the drier, south slope plots. Similar "mulching" effects were reported in experiments with seeds of *Banksia* spp. (Enright and Lamont 1989) and *Eucalyptus* spp. (Facelli and Ladd 1996), and Becerra et al. (2004) demonstrated that litter facilitated recruitment in *Beilschmiedia miersii* only when soil moisture became limiting.

Previous studies with woodland herbs have shown that the probability of seed germination can depend on soil moisture availability (Vasseur and Gagnon 1994; Baskin and Baskin 2001). In general, many late-successional woodland herbs with relatively large seeds tend to be more sensitive to dessication, and warmer, drier conditions can accelerate seed death (Fenner and Thompson 2005). Thus, the facilitative role litter can play in maintaining optimum seedbed conditions on drier sites seems particularly relevant when restoring woodland plants from seed.

In most instances, survival times were similar among litter microsites on both aspects, indicating neutral effects of litter on seedling survival. Broad overlap in species-specific survival times among litter treatments suggests that factors other than litter could have contributed to seedling mortality. I observed little evidence of herbivory during the study; although I cannot completely rule this out since herbivore access/exclusion was not included as an independent variable. Unexplained residual variance in mortality could result from some underlying environmental gradient not measured, such as soil biota or small-scale differences in soil fertility (e.g., Lechowicz and Bell 1991). Experimental studies with temperate forests herbs, however, have observed similar seedling recruitment rates across contrasting soil fertility conditions (Eriksson 1995; Ehrlen and Eriksson 2000 ; Vellend et al. 2000; Graae et al. 2004). An alternative explanation is that the random mortality rates observed here are an outcome of predators, pathogens, and micro-disturbances continually removing individuals from the population (Cook 1979).

Litter-free microsites increased survival time in *H. canadensis* seedlings only on north slope sites. Although some studies with herbaceous species have shown that litter facilitates greater survival rates compared to litter-free sites due to the "mulching effect" (e.g., Fowler 1988), this can be offset by herbivore and pathogen communities that can accumulate in litter layers, particularly in mesic micro-environments (Facelli and Pickett 1991; Facelli and Ladd 1996). While it is only speculative that fungal pathogens were the primary cause of seedling mortality in this study, other studies have shown that *H. canadensis* is susceptible to the damping-off fungus, and disease is reportedly common in horticultural settings (Reeleder 2003). Further, Rock (2000) reported that experimental

litter disturbances, including low-intensity dormant season fires, were effective at reducing fungal pathogen (*Streptobotrys streptothrix*) incidence in an established *H. canadensis* population in a southern Appalachian forest.

Landscape position plays an import role in the seedling recruitment dynamics of mesic woodland herbs. Among treatments, transition probabilities were lowest in litter-free sites on the south slope in three of the four species. For *S. canadensis*, I observed reduced seed survival, growth, and transition probabilities on south slope plots independent of litter conditions, indicating that regeneration in this species is constrained by meso-scale environmental gradients. This reflects the natural distribution pattern of established populations in regional forests, which are restricted almost exclusively to moist, fertile north-facing slopes (Hutchinson et al. 1999). The lower emergence probabilities of *H. canadensis* on the south plots and greater transition probabilities on litter-free sites on the north-facing slope, also reflects this species fidelity to mesic sites in this region (Meyer and Parker 2003). By contrast, Sanders & McGraw (2005b) found that mature ramets of *H. canadensis* transplanted across a forest cove were unresponsive to micro-environmental gradients. This suggests that landscape position can operate on early-life history stages to constrain the local distribution pattern of some woodland herbs, and that mature ramets may be able to survive in a broader niche space than seedlings can successfully recruit.

*Do larger seeded species exhibit greater recruitment probabilities?*

Cumulative transition probabilities varied widely among species and corresponded to the predicted relationship with seed mass. In other seed addition studies, larger-seeded species consistently exhibit greater establishment rates than smaller-seeded ones (Moles and Westoby 2002). Indeed, larger seeds confer several advantages over smaller seeds in closed-canopied forests, including greater tolerance of low light levels, defoliation, nutrient shortages, and intermittent periods of drought (Grime and Jeffrey 1965; Leishman and Westoby 1994; Westoby et al. 2002). Because canopy gaps, a major axis of environmental variability in eastern forests, were not incorporated into this study design, light was probably a major factor limiting survival in these species. Although it is unknown how these species respond to canopy openings, differential responses to gaps could alter the establishment probabilities observed here (Collins et al. 1985).

*Implications for restoration*

Results from this study show how economically important woodland herbs germinate and establish in different patches in an eastern deciduous forest. The direction and magnitude of litter effects vary according to litter depth, species identity, and interactions with landscape position. Litter-free sites on south slopes are clearly unsuitable for seedling recruitment. Managers can use this information to optimize micro-habitat conditions for seedling recruitment in declining populations (e.g., Sinclair et al. 2005) and in the establishment of new populations in successional forests with a depauparate woodland herb flora.

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Table 2.1. Characteristics of woodland herbs used in an experimental seed addition study.

| | Diaspore type | Seed mass (mg)[a] | Dispersal | |
|---|---|---|---|---|
| | | | Mode | Season |
| *Actaea racemosa* | Seeds | 2.73 ± 0.06 | Gravity | August-October |
| *Hydrastis canadensis* | Aggregate of berries | 24.8 ± 1.0 | Ingestion | June-July |
| *Panax quinquefolius* | Drupes | 61.7 ± 0.6 | Ingestion | August-October |
| *Sanguinaria canadensis* | Seeds | 10.62 ± 0.2 | Ants | May-June |

[a] Based on random sampling of four batches of 50 fresh seeds each (mean ± 1se)

Table 2.2. Repeated measures ANOVA results on volumetric water content (VWC) in experimental study plots during 2003 and 2004 growing season.

| | Df | *F* | *P* |
|---|---|---|---|
| Between-subjects | | | |
| Aspect | 1, 12 | 0.65 | 0.44 |
| Litter | 2, 12 | 0.26 | 0.78 |
| Aspect × Litter | 2, 12 | 0.08 | 0.93 |
| Within-subjects | | | |
| Time | 12, 144 | 146.78 | <0.001 |
| Time × Aspect | 12, 144 | 4.25 | <0.001 |
| Time × Litter | 24, 144 | 0.94 | 0.55 |
| Time × Aspect × Litter | 24, 144 | 0.89 | 0.61 |

Table 2.3. Effects of aspect and litter on seedling survival time in an accelerated failure time analysis.

| | | *Actaea racemosa*[1] | | *Hydrastis canadensis*[1] | | *Panax quinquefolius*[2] | | *Sanguinaria canadensis*[2] | |
|---|---|---|---|---|---|---|---|---|---|
| | df | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Aspect | 1 | 5.85 | 0.02 | 0.03 | 0.87 | 0.35 | 0.56 | 24.65 | <0.0001 |
| Litter | 2 | 5.04 | 0.08 | 2.70 | 0.26 | 17.21 | 0.0002 | 1.56 | 0.46 |
| Aspect × Litter | 2 | 5.59 | 0.06 | 7.66 | 0.02 | 3.99 | 0.14 | 2.99 | 0.22 |

Error distribution: [1]Weibull, [2]Gamma

Table 2.4. *F*-ratios and *P*-values from a mixed-model ANOVA testing for treatment effects on seedling a) shoot height and b) leaf width. *Acra = Actaea racemosa, Hyca = Hydrastis canadensis*, *Paqu = Panax quinquefolius*, and *Saca = Sanguinaria canadensis*.

| | | *Acra* | | *Hyca* | | *Paqu* | | *Saca* | |
|---|---|---|---|---|---|---|---|---|---|
| | df | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| a) Shoot height | | | | | | | | | |
| Aspect | 1 | 0.28 | 0.61 | 0.47 | 0.53 | 4.18 | 0.17 | 47.08 | <0.0001 |
| Litter | 2 | 0.84 | 0.46 | 3.35 | 0.04 | 22.25 | <0.0001 | 7.32 | 0.001 |
| Aspect × Litter | 1 | 0.91 | 0.36 | 1.61 | 0.20 | 4.05 | 0.02 | 1.75 | 0.18 |
| b) Leaf width | | | | | | | | | |
| Aspect | 1 | 0.27 | 0.62 | 2.75 | 0.16 | 3.92 | 0.09 | 27.17 | <0.0001 |
| Litter | 2 | 6.65 | 0.02 | 0.05 | 0.95 | 1.25 | 0.29 | 2.24 | 0.11 |
| Aspect × Litter | 1 | 17.98 | 0.002 | 0.19 | 0.82 | 0.24 | 0.79 | 0.55 | 0.58 |

Table 2.5. Generalized linear mixed-model results on the probability of transitioning from a seed to 2-year seedling for four woodland herbs.

| | df | *F* | *P* |
|---|---|---|---|
| Species | 3,44 | 81.85 | < 0.0001 |
| Litter | 2,44 | 3.55 | 0.04 |
| Litter × Species | 6,44 | 0.43 | 0.86 |
| Aspect | 1,2 | 7.97 | 0.10 |
| Aspect × Species | 3,44 | 7.78 | 0.0003 |
| Aspect × Litter | 2,44 | 7.67 | 0.001 |
| Aspect × Litter × Species | 6,44 | 2.00 | 0.09 |

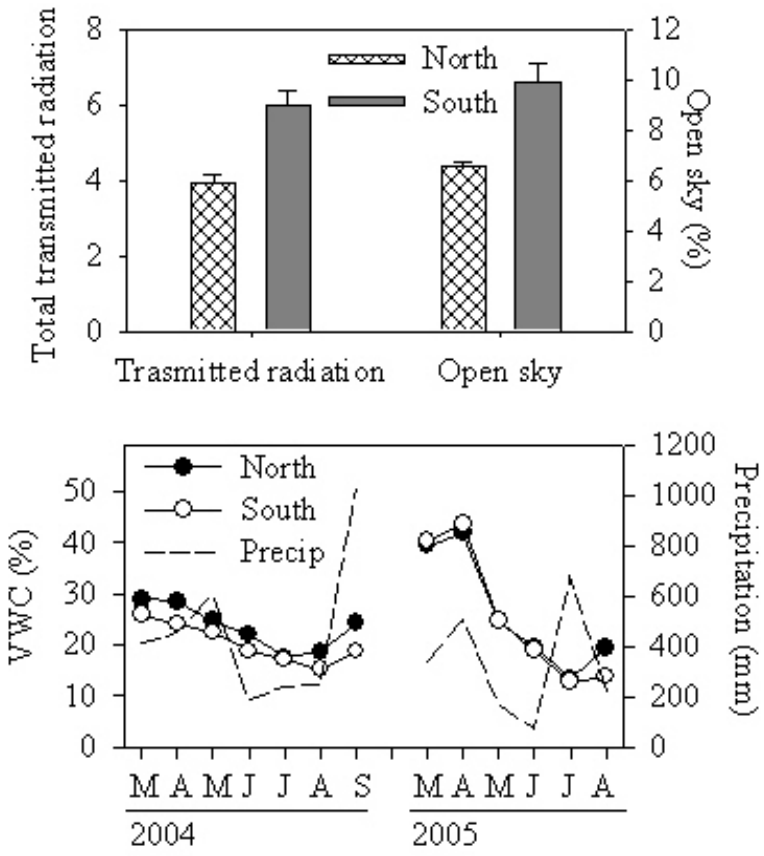


Figure 2.1. Understory light availability and soil moisture levels on opposing aspects in a second-growth deciduous forest.

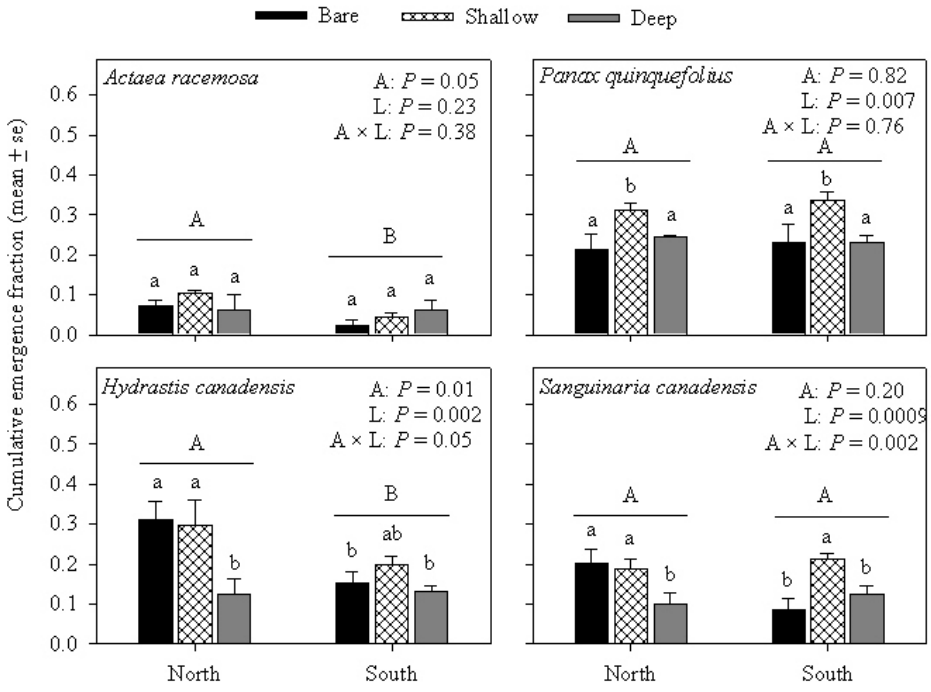


Figure 2.2. Cumulative seedling emergence (mean ± se) of four woodland herbs over two years in a seed addition study. *P*-values are from a generalized linear mixed model analyzing treatment effects on seedling emergence fractions (proportion of emerged seedlings/proportion of seeds sown). Different lower case letters indicate significant differences ($P < 0.05$) among litter treatments. Different underlined upper case letters indicate significant differences among aspects ($P < 0.05$). Aspect = A (df =1); Litter = L (df = 2). Denominator df = 8.

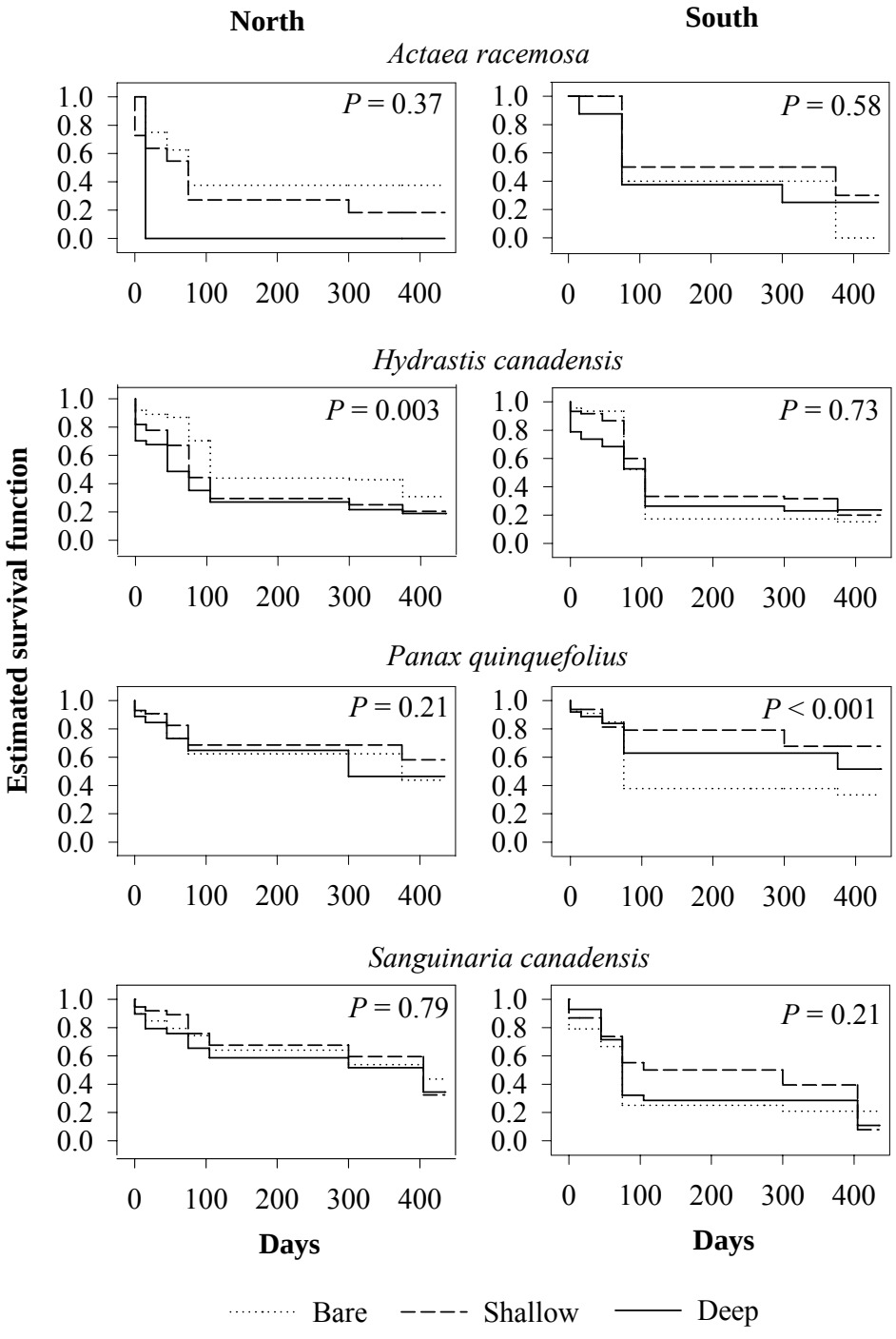


Figure 2.3. Kaplan-Meier survival estimates of seedlings in three litter treatments superimposed on north and south aspects. Differences in survival curves among litter treatments were examined separately on each aspect with a log-rank test. Treatment interactions were examined using parametric methods (see text).

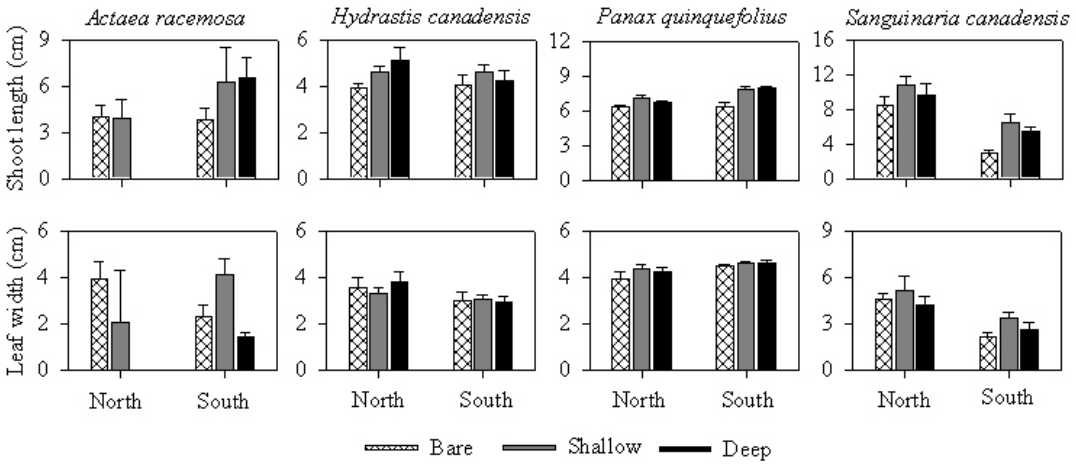


Figure 2.4. Shoot height and leaf width of seedlings growing in three litter treatments replicated on north and south aspects. See Table 4 for statistical analysis.

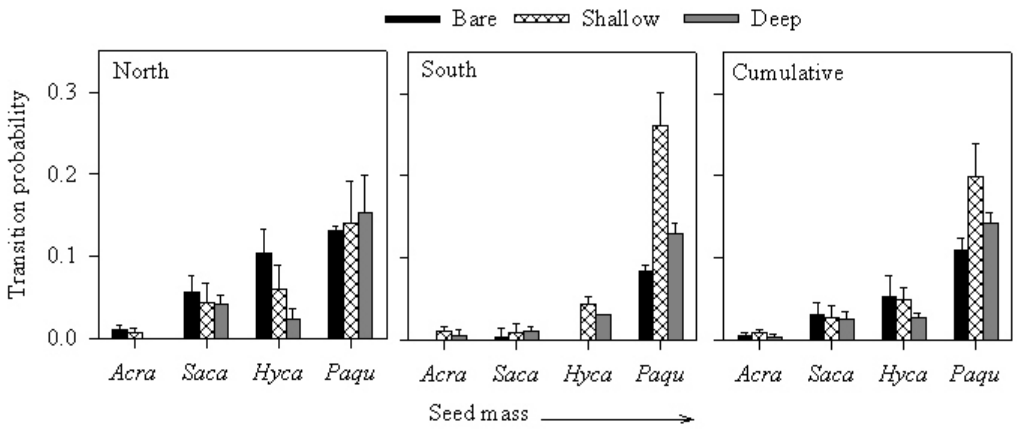


Figure 2.5. Seed → seedling transition probabilities of four woodland herbs. Species are listed in increasing order of seed size. *Acra* = *Actaea racemosa*, *Hyca* = *Hydrastis canadensis, Paqu* = *Panax quinquefolius*, *Saca* = *Sanguinaria canadensis.* See Table 2.4.

## Chapter 3: Seed dormancy and germination in the medicinal woodland herbs, *Collinsonia canadensis* L. (Lamiaceae) and *Dioscorea villosa* L. (Dioscoreaceae)

### Introduction

*Collinsonia canadensis* L. (Lamiaceae) and *Dioscorea villosa* L. (Dioscoreaceae), commonly known as stoneroot and wild yam, respectively, are medicinal woodland herbs native to the temperate forests of eastern North America. The rhizomes and roots of *D. villosa* contain diosgenin, a biochemical precursor in the synthetic production of progesterone and other corticosteroids, while the dried rootstocks of *C. canadensis* are used as a diuretic, and the leaves may improve capillary function which aids in the healing of skin wounds (Foster and Duke 1991). Because of their popularity in Europe and North America as botanical dietary supplements, these species are wild-harvested from public forestlands (Robbins 1999), and/or cultivated at small-scales under a forest canopy by private landowners to supply herb companies which manufacture value-added medicinal products from rootstock material (Krochmal 1968).

With the ever-escalating global demand for botanical forest products (Vance 1995; Foster 1997; Freese 1998), the cultivation of shade-requiring native medicinal herbs under forest canopies is an important conservation management and development strategy in temperate woodlands (Teel and Buck 2002). Medicinal plant management programs encourage the planting of seed and/or root material to either augment preexisting forest populations or establish new populations in forests where they are locally absent. Yet reliable information on the population ecology of many botanical forest products is recurrently cited as an impediment to conservation management efforts

(Robbins 2000; Duchesne and Wetzel 2002; Vance 2002). Thus, the purpose of this study was to investigate seed germination and dormancy in the increasingly important medicinal forest herbs *D. villosa* and *C. canadensis*.

Although the dormancy-breaking and germination requirements for many polycarpic woodland herbs are well documented (Baskin and Baskin 2001), little is known concerning the seed germination biology of *Collinsonia canadensis* and *Dioscorea villosa*. Neither species are cited in reviews on the seed germination ecology of temperate forest herbs (Baskin and Baskin 1988; Baskin and Baskin 2001). Martin (1946) observed that *C. canadensis* seeds have large, functionally developed embryos, whereas seeds of *D. villosa* had small, functionally underdeveloped, capitate-shaped embryos. When embryos are underdeveloped at maturity, seeds are considered morphologically dormant because they require some pretreatment for embryos to grow to some critical threshold for full germination (Baskin and Baskin 2001). Terui and Okagami (1993) reported that *D. villosa* seed air-dried for 50 d germinated at rates < 50% at a constant temperature of 25 ºC, whereas seeds that were cold stratified for 120 d germinated to > 95% over a range of warm constant temperatures (20-25 ºC). However, previous studies with *D. villosa* seeds investigated germination behavior under constant laboratory temperatures. Dormancy-breaking and seed germination conditions in nature involve daily temperature oscillations and the natural progression of seasonal temperature changes (Thompson and Grime 1983; Probert 2000; Baskin and Baskin 2001). Consequently, it is essential to evaluate seed germination behavior over a range of thermoperiods and alternating temperature regimes in controlled conditions, and

concurrently conduct field experiments to fully characterize dormancy-break (Thompson and Grime 1983; Baskin and Baskin 2001).

I experimentally investigated dormancy-break in *Dioscorea villosa* and *Collinsonia canadensis* by employing a double germination phenology study or "move-along" experiment (Baskin and Baskin 2003). Based on prior information, I was able to eliminate morphological dormancy (i.e., underdeveloped embryos) as a potential dormancy type in *C. canadensis*. Although fresh seed could be nondormant (i.e., seed would germinate within 30 d), I hypothesized that *C. canadensis* seeds, which are dispersed in mid-autumn, would have a cold stratification requirement (i.e., physiological dormancy) that would delay germination until favorable spring conditions. Because *D. villosa* embryos are underdeveloped at maturity and appear to require cold stratification for full germination, I hypothesized that seeds would have some form of morphophysiological dormancy (i.e., seeds would require some critical temperature for embryo growth and seeds would require some dormancy-breaking pretreatment to germinate). By transferring imbibed seeds through a sequence of thermoperiods that approximate seasonal temperatures in eastern temperate forests, the move-along experimental template would test my hypotheses while also determining the optimum temperatures required for dormancy-break.

## Materials and Methods

### *Species*

*Collinsonia* is a genus of 4 species restricted to eastern North America (Peirson et al. 2006). *Collinsonia canadensis* has the broadest distribution within the genus, occurring in early- to late-succesional woodlands from northern New England and southern Ontario to eastern Missouri, south to Louisiana and northern Florida, and east to New Hampshire (Peirson et al. 2006). In early spring, an aerial stem (sometimes 2 or more) bearing opposite leaves arises from a hard, knotty perenating rhizome (hence the common name stoneroot). Aerial shoots tend to branch near the apex, and flowering ramets terminate in branched panicles that bear hermaphroditic flowers from August into October. Flowers are yellow, lemon-scented and obligately xenogamous (Skinner 1976). Early observations show that bumble bees (*Bombus* spp.) were the primary pollinators in a naturally occurring population in an Ohio mixed-hardwood forest (Skinner 1976). The fruit is a set of one to three one-seeded nutlet that matures during leaf drop in mid-autumn. Seeds are gravity dispersed (Beattie and Culver 1981).

*Dioscorea* is largely a pantropical genus with six species occurring in eastern North America, the majority of which are restricted to the southeastern United States. The fleshy tubers and rhizomes of subtropical members of the genus *Dioscorea* are an economically important food crop. *Dioscorea villosa* is a dioecious, herbaceous vine that inhabits thickets, hammocks, and moist and dry woodlands from Connecticut to Wisconsin, west to Oklahoma, and south to the Florida panhandle (Al-Shebaz and Schubert 1989). Unlike *Dioscorea polystachya,* a non-indigenous sympatric congener, *D.*

*villosa* never produces vegetative propagules (i.e., bulbils) in its leaf axils (Raz 2003). In spring, aerial shoots arise from a perenating rhizome, and reproductive ramets develop inflorescences in the leaf axils from June thru July. Within a population, sex ratios tend to favor staminate plants, which typically outnumber carpellate plants by three to five times (Al-Shehbaz and Schubert, 1989). Staminate plants produce branched panicles that contain several small, sweetly scented flowers. In members of the genus *Dioscorea*, the stamens produce glutinous pollen (Al-Shebaz and Schubert 1989) which Coursey (1967) hypothesized was transported to carpelate plants by nocturnal insects. Carpelate plants produce small, solitary flowers at each node on a short spike (Al-Shehbaz and Schubert 1989). The fruit matures in mid-autumn and is a membranous, 3-valved loculicidal capsule with one or two winged seeds (diaspores) occupying each locule; Al-Shehbaz and Schubert (1989) hypothesized that diaspores in members of the genus *Dioscorea* are wind dispersed.

*Plant material*

I collected ripe seeds of *Collinsonia canadensis* from populations growing in mixed-oak second-growth forest in Vinton County, Ohio, on 23 Oct 2003, and at a roadside adjacent to a second-growth forest edge in Perry County, Ohio on 25 Oct 2003. Germination studies commenced on 29 Oct 2003. Since *D. villosa* populations in southern Ohio are patchily distributed and occur at low density, I was unable to collect enough seed from local populations for meaningful laboratory studies. Thus, in October 2002 I purchased seed from a reputable commercial supplier that propagates native plants

(Horizon Herbs Inc., Williams, Oregon). Purchased seed originated from plants that were originally sourced from wild populations in the central Appalachians and are currently growing in an agroforestry system (R. Czech, Horizon Herbs, *personal communication*). These plants have been under no artificial selection or breeding program.

*Germination experiments*

Initially, I checked for imbibition by comparing the mass of fresh seed to the mass of seed after incubation in moist conditions. Twenty seeds for each species were placed on moistened filter paper in a petri dish wrapped with plastic film for 24 h. Seeds were then removed from the dish, blotted dry with a paper towel, and weighed. Changes in seed mass were calculated by subtracting the mass of fresh seed after 24 hr exposure to moist conditions from the initial mass of fresh seed, and then dividing by the mass of initial seed.

Fresh seeds were placed on top of a 3:1 (v/v) mixture of potting soil and sand moistened with distilled water in 9 cm (diameter) plastic petri dishes. For each species, treatments consisted of four replicates of 30 seeds each ($N$ = 120 seeds/treatment). To determine if fresh seed of either species would germinate over a range of temperature conditions, a set of replicates was incubated in environmental chambers with a 14L:10D photoperiod (white fluorescent light) and 12 h/12 h thermoperiods of 30/15 ºC, 20/10 ºC, and 15/6 ºC, and a constant temperature of 5 ºC. All petri dishes were removed after a 4-wk incubation period and germination percentages were calculated. Seeds that had completely germinated (radicle and shoot emergence) were removed from the dish.

Dishes were then placed back in the germinators for an additional 40 wks and checked every 7 d for germination. Dishes were watered as needed so that seeds remained imbibed throughout the study.

A move-along experiment is particularly valuable when large sample sizes for extensive germination studies are difficult to obtain (Baskin and Baskin 2003). According to the move-along experimental template I simultaneously conducted the following two treatments, hereafter referred to as "warm treatment" and "cold treatment." In the warm treatment, a set of four replicate dishes for each species was incubated in the following sequence: 12 wk at 30/15 ºC → 4 wk at 20/10 ºC → 4 wk at 15/6 ºC → 12 wk at 5 ºC → 4 wk at 15/6 ºC → 4 wk at 20/10 ºC → 12 wk at 30/15 ºC. In the cold treatment, a set of replicates for each species was incubated in the following sequence: 12 wk at 5 ºC → 4 wk at 15/6 ºC → 4 wk at 20/10 ºC → 12 wk at 30/15 ºC → 4 wk at 20/10 ºC → 4 wk at 15/6 ºC → 12 wk at 5 ºC. The cold treatment would determine if seeds required cold stratification only or a cold followed by warm stratification, whereas the warm treatment would determine if seeds required a period of warm stratification or warm followed by cold stratification. The chosen thermoperiods represent average daily maximum and minimum air temperatures for our study region: 30/15 ºC for summer, 20/10 ºC for early autumn and late spring, 15/6 ºC for late autumn and early spring, and 5 ºC for winter (Baskin and Baskin 2003). To complement the move-along experiment, I conducted a germination phenology study only with *D. villosa* since I was unable to collect enough *C. canadensis* seeds for a statistical sample. I sowed 50 seeds in each of four flats filled with potting soil and covered with a thin layer of mixed-oak leaf litter to

prevent dessication. Flats were placed in a wood shade-house covered with lattice located at the Ohio University West State Street Research Gardens (Athens, Ohio). The shade-house was located within a fenced area where seeds were protected from predators but exposed to natural temperatures. Flats were watered as needed to maintain constant imbibition. Germination was checked on weekly basis for two years.

I also tested the response of seeds of both species to different periods of cold stratification (5 ºC). A set of replicates for each species was incubated for 4 wk at 5 ºC and 8 wk at 5 ºC, and then placed in warm/cool temperatures (20/10 ºC). Mean and standard errors for final germination percentages were calculated from each set of replicates (i.e., 4 petri dishes, 30 seeds/dish).

To determine whether an extended period of dry storage could alleviate dormancy for either species, 480 fresh seeds for each species were air-dried for two weeks and then stored in closed glass jars for six months at ambient laboratory conditions (ca. 21 ºC). At six months, seeds were removed from storage and placed on a moist germination mixture at each of the four thermoperiods ($N$ = 120 seeds for each species at each thermoperiod). Seeds were checked for germination after 28 d and results were compared with mean percentage of fresh seeds germinated after 4 wk at each thermoperiod with a one-way ANOVA.

**Results**

In the imbibition test, seed mass of both species increased by > 50% after a 24 h period in moist petri dishes, thus I concluded that seeds did not possess impermeable seed coats (i.e., physical dormancy).

Seeds of *Dioscorea villosa* exposed to natural temperatures in the shadehouse reached peak germination percentages the third week of April, when daily maximum and minimum temperatures ranged from 15 - 20 ºC to 3 - 10 ºC, respectively (Figure 3.1). No seeds germinated the following spring (2004).

In the move-along experiment, seeds initially started in the cold treatment (12 wk at 5ºC) germinated after transfer to warmer temperatures (Figure 3.2a). *Dioscorea villosa* germination peaked at 15/6 ºC, with 97.4% ± 2.6 (mean ± 1 SE) of seeds germinating before they were transferred to 20/10 ºC, where the remaining viable seeds germinated. In contrast, 41% ± 3.5 of *C. candensis* seeds germinated at the end of the 4 wk sequence at 15/6 ºC and 34% of seeds germinated after 3 weeks at 20/10 ºC (total germination: 97.5% ± 2.5; Figure 3.2a). For both species the germination mode was hypogeous, yet the radicle preceded shoot emergence by several days in *D. villosa*, whereas the radicle and shoot emerged concurrently in *C. canadensis*.

For *D. villosa*, seeds placed initially in the warm treatment did not germinate until transferred through the following sequence of temperatures: 12 wk at 5 ºC → 4 wk at 15/6 ºC → 4 wk at 20/10 ºC, seeds then germinated to 100% at 20/10 ºC (Figure 3.2b). In contrast, 27.0% ± 5.0 of *C. canadensis* seeds germinated during the initial 12 wk at 30/15 ºC but germination was subsequently suppressed when seeds were transferred through the

following temperature sequence: 4 wk at 20/10 ºC → 4 wk at 15/6 ºC → 12 wk at 5 ºC; seeds then germinated to 96.8% ± 1.9 after their second exposure to 15/6 ºC and 20/10 ºC (Figure 3.2b).

Neither species germinated within 4 wks in any of the control thermoperiods. However, both species germinated after a protracted time period in the controls but the thermoperiods at which dormancy break occurred differed between the two species. After 8 weeks at the 20/10 ºC thermoperiod, *D. villosa* seeds began germinating, although the speed of germination was slow and after 18 weeks germination stabilized at 61.9 ± 7.9% (Figure 3.3a). *Dioscorea villosa* seeds in the 15/6 ºC thermoperiod began germinating after 10 wk and germinated to high rates (80%) after a 24 wk period (Figure 3.3a). No *D. villosa* seeds germinated at 5 ºC or 30/15 ºC. In contrast, *C. canadensis* seeds began germinating after 4 weeks at 30/15 ºC and after 22 weeks at 5 ºC (Figure 3.3b). Seeds also began germinating after 22 weeks at 15/6 ºC but seed germination rates were < 20% (Figure 3.2b). Seed germination was negligible at 20/10 ºC (Figure 3.3b).

Each species responded differently to varying lengths of cold stratification. *Collinsonia canadensis* seeds maintained at 5 ºC for 8 weeks germinated to higher percentages at 20/10 ºC than seeds maintained at 5 ºC for 4 weeks, although germination was slow and did not begin until 8 weeks (Figure 3.4a). In contrast, *Dioscorea villosa* seeds germinated to similar percentages when held for 4 or 8 weeks at 5 ºC and then moved to 20/10 ºC (Figure 3.4b).

In the six-month dry storage treatment, no seedsof either species germinated after 28 d in of the thermoperiods. For *D. villosa*, the mean percentage of viable seeds was

significantly (one-way ANOVA, $F_{1,30} = 79.38$, $P < 0.0001$) lower at study termination with 6 month dry stored seed (39.3% ± 2.7) compared at our control thermoperiod experiment with fresh imbibed seed (70.8% ± 2.0). For *C. canadensis*, the mean percentage of viable seeds was significantly (one-way ANOVA, $F_{1,30} = 178.45$, $P < 0.0001$) lower at study termination with 6 month dry stored seed (24.1% ± 2.4) compared at our control thermoperiod experiment with fresh imbibed seed (75.5% ± 2.6).

## Discussion

### *Dormancy classification*

Over a range of simulated thermoperiods, fresh seed of *D. villosa* and *C. canadensis* failed to germinate after incubation for 30 d (or 4 wk), indicating that seeds were dormant at maturity (Baskin and Baskin 2001). Since effective dormancy-break in *D. villosa* and *C. canadensis* occurred following cold-stratification, results from the move-along experiment support the hypothesis that both species require cold stratification to alleviate physiological dormancy. Martin's (1946) and my own observations from longitudinal cross-sections of seeds indicate that *D. villosa* has small, underdeveloped embryos at maturity. If species with underdeveloped embryos require some time period for embryos to grow to some critical threshold before germination occurs, they are also morphologically dormant (MD; Baskin and Baskin 2001). Thus, the move-along experiment and shadehouse study support our initial hypothesis and confirm prior laboratory studies (Terui and Okagami 1993) that *Dioscorea villosa* seeds are morphophysiologically dormant (MPD).

Baskin and Baskin (2001) described 8 levels of MPD based on the response of seeds to three parameters: the effectiveness of gibberellic acid ($GA_3$) at breaking dormancy, temperatures required for dormancy-break, and temperatures required for embryo growth. Although I did not explicitly measure embryo growth in this study, Terui and Okagami (1993) found that when embryos were isolated from *D. villosa* seeds and placed in warm temperatures (14 -32 ºC) they germinated to high rates (> 80%), indicating that warm temperatures promote embryo growth and that after embryo growth occurs, cold stratification is not required for germination. In the three complex types of MPD (non-deep complex, intermediate complex, and deep complex), cold temperatures (5 ºC) are required for embryo growth, whereas embryo growth proceeds at warm temperatures in the five simple types of MPD (non-deep simple, intermediate simple, deep simple, deep simple epicotyl, and deep simple double; Baskin and Baskin, 2004). In four of the five simple levels of MPD, embryo growth occurs at warm temperatures but a sequence of warm followed by cold stratification is needed for full germination (radicle and shoot emergence) (Baskin and Baskin 2001; Baskin and Baskin 2004b). Since cold stratification breaks dormancy without a prior warm stratification treatment, and embryo growth appears to be delayed until warm temperatures occur in the spring, I conclude that *D. villosa* has non-deep simple MPD. This level of MPD has also been observed in other polycarpic perennials, such as *Chamaelirium luteum* (Baskin et al. 2001) and *Thalictrum mirabile* (Walck et al. 1999), that show similar phenological patterns in the field (i.e., seeds disperse in autumn and germinate in spring) and dormancy-break in the laboratory.

*Dormancy break: laboratory and field conditions*

In *D. villosa*, effective dormancy-break can occur over a prolonged time period when 12 hr of cold stratification (at 6 or 10 ºC) alternates with 12 hr of cool stratification (15 or 20 ºC). Conversely, these alternating temperatures were ineffective at breaking dormancy in *C. canadensis*, suggesting that temperatures higher than 5 ºC are ineffective at breaking physiological dormancy in this species. Although 5 ºC is often the optimum cold stratification temperature that alleviates dormancy in temperate woodland herbs (Baskin and Baskin 2001), temperatures ranging from 0 - 10 ºC may also be effective at overcoming dormancy (Bewley and Black 1982). For example, in temperate congeners, *D. japonica* and *D. septemloba*, effective dormancy-break occurred at 0 ºC whereas no or little germination was observed at 5 ºC (Okagami and Kawai 1982). Baskin et al. (2001) observed that with morphophysiologically dormant seeds of the woodland herb *Chamaelirium luteum*, dormancy-break occurred after 8 weeks at 15/6 ºC and 20/10 ºC thermoperiods. They hypothesized that cold temperatures (6 -10 ºC) alternating with cool temperatures (15 - 20 ºC) alleviate physiological dormancy while simultaneously promoting slow growth of the underdeveloped embryo. This type of dormancy break can be understood by examining the number of hours seeds were cold stratified. For example, with *D. villosa*, seeds would have been exposed to 840 h of cold stratification (or 12 h of cold stratification per day) when they began germinating after 70 d in the 15/6 ºC and 20/10 ºC thermoperiods. When seeds were given a constant cold stratification for a period of 4 weeks (or 672 h) and then moved into warmer temperatures (20/10 ºC), dormancy was broken and seeds germinated to high rates (> 80%) after 28 d. This suggests that

once the critical time period of cold stratification (5 - 10ºC) demanded by *D. villosa* seeds is achieved, seeds are capable of germinating in temperatures ≥ 15 ºC.

An ecological interpretation of our move-along experiment suggests that fresh seeds of both species overcome dormancy during winter and seeds rapidly germinate after exposure to warm days (15 - 20 ºC) and cold nights (6 - 10 ºC). This pattern of dormancy loss is typical of woodland herbs that naturally disperse seeds in mid-autumn, since delaying germination until spring ensures favorable conditions for seedling establishment (Probert 2000). Our phenology study with *D. villosa* in the shade-house confirmed the results of the move-along experiment since no dormancy-break occurred in *D. villosa* seeds during the autumn months. I also found no additional *D. villosa* germination after two years in the shadehouse (spring 2004), a germination behavior that corresponds with Thompson and Grime's (1979) Type II transient seed bank.

A common feature of species that display physiological dormancy is that germination occurs at temperatures uncharacteristic of those they would be experiencing in their natural habitat (Baskin and Baskin 2001). This is consistent with our results with fresh *C. canadensis* seed that began germinating at a slow velocity in warm (30/15 ºC) conditions after 4 weeks. However, warm stratification tends to be more effective at breaking dormancy in obligate winter annuals than polycarpic woodland herbs (Baskin and Baskin 2001). One possible reason for dormancy break at 30/15 ºC is this thermoperiod has a larger daily temperature amplitude than the 20/10 ºC and 15/6 ºC thermoperiods; large amplitudes have been found to trigger germination in other temperate herbs (Thompson and Grime 1983). However, in field conditions, the results

from our simulated mid- to late-autumn temperature (20/10 ºC and 15/6 ºC) treatments indicate that seeds would undergo conditional dormancy at the time of dispersal (mid-autumn), thus preventing epicotyl emergence in circumstances otherwise unfavorable for seedling establishment, i.e., late-autumn and winter freezing.

*Cultivation recommendations*

Dry storage may overcome the cold stratification requirement for physiological dormancy loss in some herbaceous species (Baskin and Baskin 2001). Our results indicate that neither species afterripened (overcame dormancy) in the 6-month (~180 d of storage) dry storage treatment since no seeds germinated in 4 wks at any of the tested thermoperiods. While dry storage tends to alleviate primary dormancy in seeds that require warm stratification for overcoming PIM, as is often the case in winter annuals, it tends to be less effective at overcoming a cold stratification requirement in polycarpic perennials (Baskin and Baskin 2001). Alternatively, dry storage may induce seeds into protracted dormancies, although our investigation would not have detected this. A 6-month dry storage interval simulates a possible sequence for propagating plants from seed in forest cultivation systems: (1) fresh seed collected in October; (2) seed dry-stored in unchilled conditions during the winter; and (3) seed planted the following spring (April). If this sequence were followed in the field, based on our results from the move-along experiment it appears that *D. villosa* seed germination would not occur until the following spring (18 months after seed was collected) after seeds were exposed to a winter chilling period, an apparent requirement for full germination. However, *D. villosa*

seeds are capable of germinating following only 4 wks of cold stratification, suggesting that seeds sown in late winter or early spring may provide a long enough cold stratification period for full germination, although dry-storing seeds would result in a smaller viable seed population. While 4 and 8 wks of cold stratification did alleviate dormancy in *C. canadensis* seeds, seeds germinated at a slower speed and overall lower rates compared to the 12 wk cold stratification treatment in the move-along experiment, suggesting that longer periods of cold stratification may be more effective at breaking dormancy. From a forest cultivation perspective, I recommend that seeds of both species be sown shortly after dispersal. This would preclude viability loss incurred during dry-storage and ensure a long chilling period that is most favorable for full germination.

In conclusion, this study is the first description of physiological dormancy in *Collinsonia canadensis* and non-deep simple morphophysiological dormancy (MPD) in *Dioscorea villosa*. The temperatures requirements for dormancy-break identified in this study can guide direct seeding in the field and thereby facilitate conservation management strategies with often limited seed germplasm.

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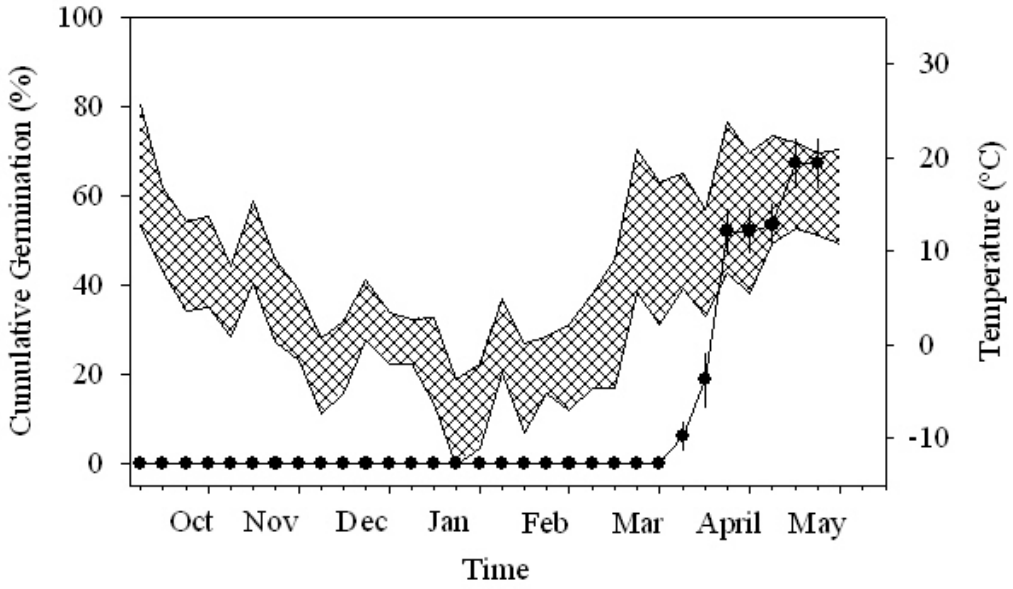


Figure 3.1. Cumulative germination percentages (mean ± 1 SE) for 200 seeds (4 replicates of 50 seeds) of *Dioscorea villosa* sown in fall 2002. Flats were placed in a wood shadehouse exposed to natural temperature conditions at the Ohio University West State Street Research Garden. Mean weekly maximum and minimum air temperatures (hatched areas) for 2002-03 were obtained from a National Climate Data Center weather observation station located in Athens, Ohio (39°21'N / 82°06'W) (NOAA 2004).

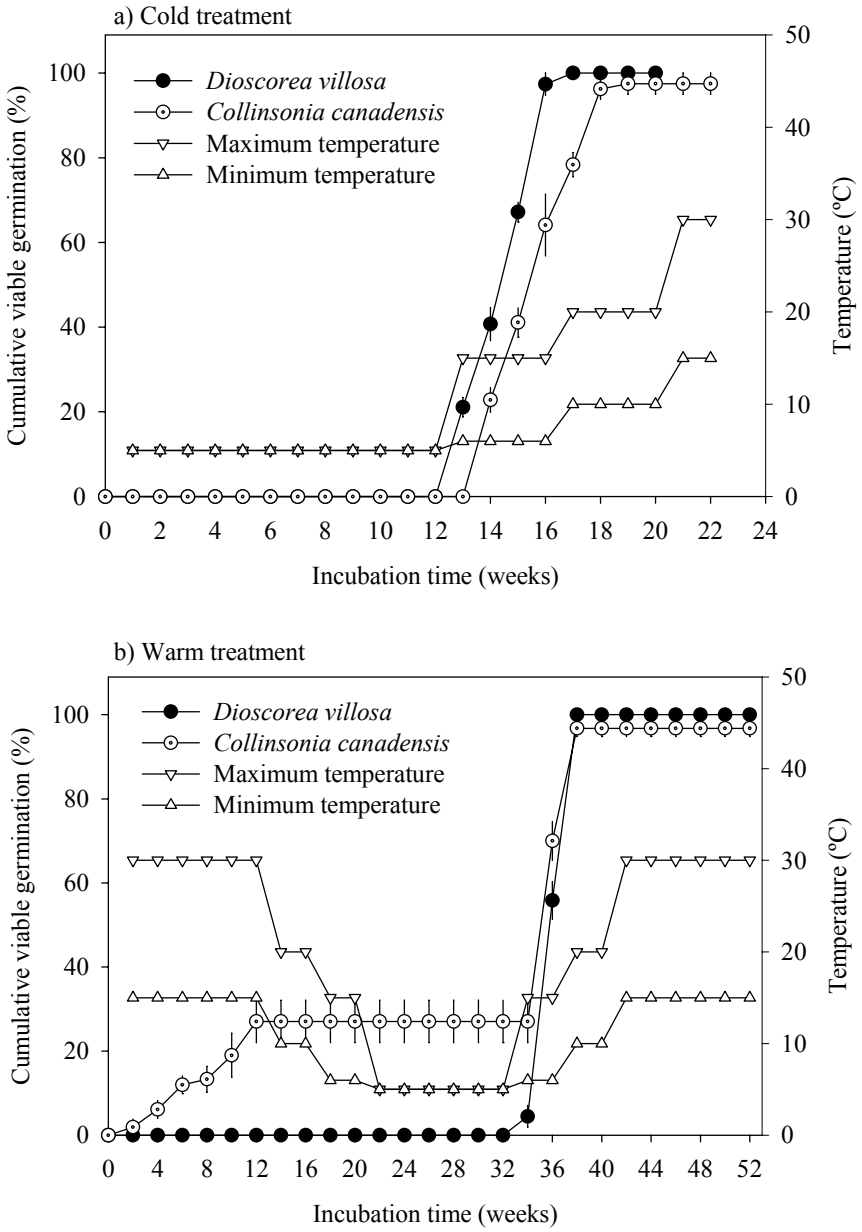


Figure 3.2. Cumulative viable germination percentages (mean ± 1 SE) for *Collinsonia canadensis* and *Dioscorea villosa* in the a) cold treatment sequence and b) warm treatment sequence of a move-along experiment.

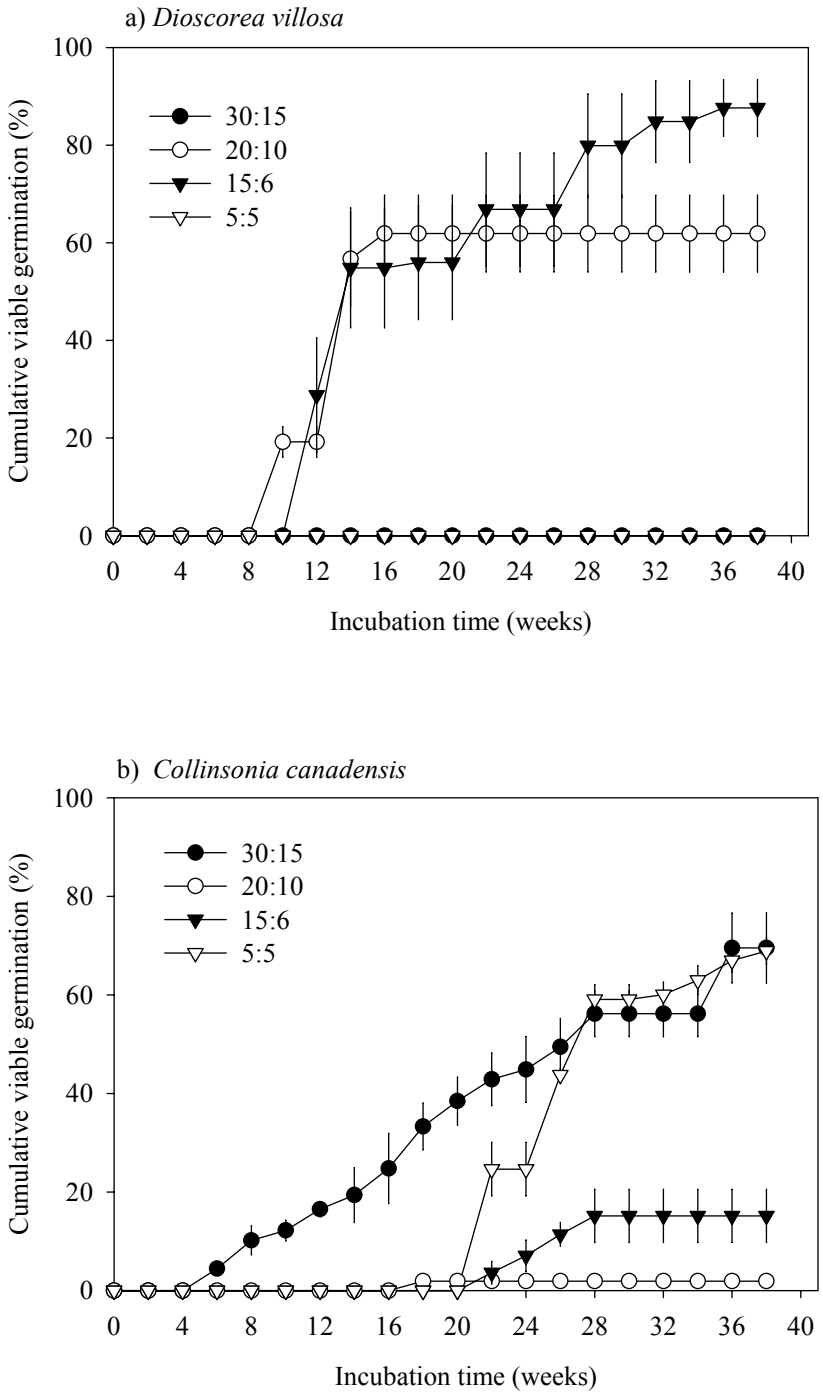


Figure 3.3. Cumulative germination percentages (mean ± 1 SE) for seeds of *Dioscorea villosa* and *Collinsonia canadensis* after incubation at four temperature regimes for 40 weeks.

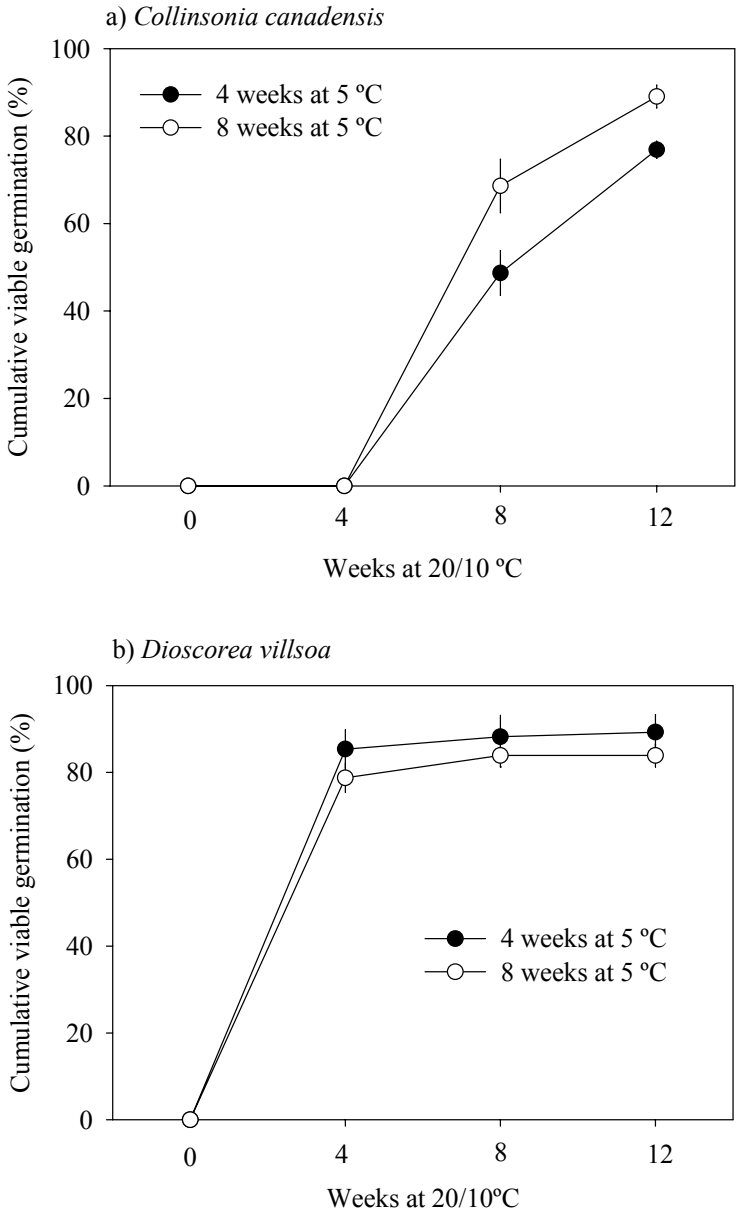


Figure 3.4. Cumulative viable germination percentages (mean ± 1 SE) for a) *Collinsonia canadensis* and b) *Dioscorea villosa* seeds given 4 and 8 weeks of cold stratification at 5 ºC and then transferred to warm temperatures (20/10 ºC), where cumulative germination was observed for 4, 8, and 12 weeks.

## Chapter 4: Effects of storage on seed dormancy and survivorship in black cohosh (*Actaea racemosa* L.) and goldenseal (*Hydrastis canadensis* L.)

### Introduction

Black cohosh (*Actaea racemosa* L.) and goldenseal (*Hydrastis canadensis* L.) are prized medicinal herbs indigenous to deciduous forests of eastern North America (Foster and Duke 1991). Rhizomes of these species are harvested increasingly from the wild for sale in a lucrative botanical medicines industry (Robbins 1999,2000). Although a culturally important practice, there is increasing concern that harvest of subterranean structures may be contributing to the depletion of these non-timber based resources (Robbins 2000). Consequently, proprietors of privately owned woodlands are now encouraged to propagate these species as an alternative to generating income from conventional timber-based activities, and/or harvest of these species from the wild (Teel and Buck 2002; Garrett 2003). Traditionally, medicinal woodland herbs are propagated vegetatively from material sourced from the wild, but there is an increasing push to use seed germplasm for propagation efforts in order to mitigate the long-term effects of harvesting on wild populations. However, conservation and management strategies are hampered by a poor understanding of their seed germination biology.

Like most members of the Ranunculaceae, newly dispersed seeds of *A. racemosa* and *H. canadensis* contain rudimentary embryos that are morphophysiologically dormant (MPD) (Baskin and Baskin 1985; Baskin and Baskin 2001). Newly dispersed seed of *A. racemosa* exhibits deep simple epicotyl MPD (Baskin and Baskin, 1985), whereas *H. canadensis* exhibits dormancy characteristics that appear intermediate between deep

simple and deep simple epicotyl MPD (Baskin and Baskin 2001). Thus, both species require a warm → cold temperature sequence to break primary dormancy and are characterized as strict spring germinators (Baskin and Baskin 2001).

A common practice among growers and propagators, to temporarily store freshly collected seed at room temperature or in cold refrigeration until outplanting in forest plots, frequently results in sporadic germination rates (Davis 1999; Cech 2002). Germination protocols for *H. canadensis* recommend that fresh seed be sowed immediately, as short-periods of dry-storing seed purportedly results in loss of seed viability and/or germinability (Hus 1907; Deno 1993; Davis 1999; Cech 2002). In contrast, Deno (1993) reported that dry storage at ambient laboratory temperatures for 6 months maximized germination of *A. racemosa* seed. To date, however, no studies have quantified the inter-relationship between storage conditions and seed survivorship.

Variation in germination rates may result from an interaction between length and temperature of storage conditions (Roberts 1973). Seeds can experience a wide variety of changes in dry storage including accelerated dormancy loss (Priestly, 1986), induction of a deeper dormancy (Edwards and El-Kassaby 1988), relief of germination requirements imposed on seeds at the time of dispersal (Probert *et al*., 1985; see also see review by Probert, 2000), and/or loss of viability (Murdoch and Ellis 2000).

In order to clarify their response to artificial storage, we subjected seeds of *A. racemosa* and *H. canadensis* to a factorial combination of storage conditions in a controlled laboratory study to understand how storage temperature and duration affect seed survivorship and dormancy levels.

**Materials and Methods**

Ripe fruits were collected from cultivated populations of *A. racemosa* and *H. canadensis* growing on an experimental research farm in southeast Ohio during the autumn (September) and summer (July) of 2003, respectively. *Actaea racemosa* was growing beneath an artificial shade structure in an old-field whereas *H. canadensis* was growing in an adjacent second-growth (> 60 yr) mixed-mesophytic forest. All seed germplasm originated from plants that were previously growing wild in the Appalachian region but were transplanted for conservation purposes.

Post-harvest seed handling varied according to interspecific differences in fruit types and followed the general methods recommended for growers (Davis 1999; Cech 2002). *A. racemosa* follicles were allowed to dry at room temperature for 2 d after collection; seeds were then removed from follicles by gently tapping the infructescence against the sides of paper bags. Only brown, firm seeds were used for germination studies. *H. canadensis* seeds were depulped by soaking berries in distilled water for 24 h to loosen the fleshy pericarp. Fruits were then macerated by hand, and only seeds that sank in water were used for experimental study, since seeds that float are considered nonviable (Cech 2002). In a companion study, I found that seeds originating from these populations exhibited morphophysiological dormancy that was broken by a sequence of 12 wk at 15/6 °C → 12 wk at 5 °C (chapter 1), a result consistent with previous studies (Baskin and Baskin 1985; Baskin and Baskin 2001). Based on a random sampling consisting of four batches of 50 seeds each, 96.0 ± 1.4% (mean ± 1SE) and 91.5 ± 1.3% of the *A. racemosa* and *H. canadensis* seeds, respectively, were viable. Seed moisture

content, expressed as a difference between mass of 100 fresh seeds before and after being oven-dried at 105 ºC for 24 h, was 6.8% and 10.5% for *A. racemosa* and *H. canadensis*, respectively.

Seeds of each species were air-dried in the laboratory for 14 days and placed into glass jars sealed with a screw-cap lid. Equal numbers of jars were stored in a cold storage incubator (5ºC, relative humidity 50%) and in ambient laboratory storage (23ºC, relative humidity 15%) for 30, 60, 90, 180, 270, and 360 days. We chose these storage conditions in order to mimic how seed may be commonly stored prior to outplanting. At each storage interval, seeds were removed from storage treatments and placed onto a 1:1 mixture of moistened sand and potting soil, in glass Petri dishes (9-cm diameter). Germination tests were conducted in light-controlled germinators equipped with 20 W cool white fluorescent tubes. For dark germination treatments, Petri dishes were placed into steel canisters wrapped in aluminum foil. Each treatment condition consisted of 4 replicates of 50 seeds each for *A. racemosa*, and 4 replicates of 30 seeds each for *H. canadensis*. Primary root emergence was the criterion for germination.

In the first experiment, we quantified survivorship rates of laboratory stored seeds and checked for after-ripening that may have occurred during storage. After each time interval, seeds were removed from storage and germinated in light (12 h photoperiod) and complete darkness at each of the following thermoperiods: 5, 15/6, 20/10 and 30/15 °C. After 30 d, seeds were removed from the germinators and the number of germinates was counted. A control consisted of incubating fresh seed (14 d old) at each of the thermoperiods in light and darkness for 30 d. At the end of each experiment,

nongerminated seeds were scored as alive or dead based on a Tetrazolium stain test (Cottrell 1947).

In the second experiment, the effects of storage temperature and duration on seed germinability were evaluated. At each time interval, seeds were removed from storage and then transferred through the corresponding temperature sequence that alleviates dormancy in fresh seed of *A. racemosa* (12 wk at 15/6 °C → 12 wk at 5 °C ) and *H. canadensis* (12 wk at 15/6 °C → 12 wk at 5 °C → 6 wk at 15/6 °C; Baskin and Baskin 2001). Germination tests were conducted only in a simulated diurnal 12 h photoperiod, due to a limitation in the quantity of seed germplasm available for study. The experimental control consisted of transferring fresh seed (14 d old) of each species through its corresponding temperature sequence that breaks MPD.

Germination and survival data were analyzed using a generalized linear model (PROC GENMOD, SAS 2001) with a logit link function and binomial error structure, as the data were not normally distributed. Separate tests were conducted for each species. Germination fractions were calculated as follows: $G = V - D$, where $V$ represents the total number of viable seeds at the end of the incubation period and $D$ is the number of dormant or ungerminated seeds. Overdispersion was accounted for by dividing the likelihood ratio $\chi^2$ by the Pearson $\chi^2$ /df (pscale option in SAS), resulting in an analogous ANOVA $F$-test (Littell et al. 2002). Single degree of freedom contrasts were used test specific hypotheses when main effects were significant (Littell et al. 2002).

**Results**

Fresh seed of *A. racemosa* and *H. canadensis* failed to germinate over a range of thermoperiods in light or darkness after a 30 d incubation period, confirming that seeds were dormant at maturity (sensu Baskin and Baskin 2004). For both species, no germination was observed with seeds stored dry at ambient and cold temperatures and then tested over a range of thermoperiods in both light and darkness during the 30 – 360 d storage period (data not shown).

Seeds stored dry at ambient and cold temperature conditions exhibited differential survival rates within and among the two species (Figure 4.1). Overall, seeds of both species survived at significantly greater rates in ambient storage relative to cold storage conditions (Table 4.1). For *H. canadensis,* seed survival varied in the storage treatments over time (significant temperature × time interaction; Table 4.1). After 90 d, seed survival rates were similar, as 66% and 61% of *H. canadensis* seeds stored in ambient laboratory and cold temperature conditions, respectively, were viable. After 360 d, no *H. canadensis* seeds remained viable at ambient laboratory storage, and < 2% of seeds survived cold temperature storage.

Germination fractions of *A. racemosa* seeds stored in ambient laboratory and cold temperature conditions declined significantly over time (Table 4.1). Overall, germination fractions in *A. racemosa* seed were lower after storage compared to those of freshly dispersed seed (contrast fresh vs. stored, $P = 0.009$), whereas no differences were detected for *H. canadensis* (contrast fresh vs. stored, $P = 0.89$). In both species, freshly dispersed seed transferred through the dormancy-breaking temperature sequences

germinated to > 80%. *A. racemosa* seeds stored in ambient laboratory and cold temperature conditions for ≥ 270 d exhibited significantly lower germination percentages than seeds stored for ≤ 180 d (contrast 270 d vs. ≤ 180 d, $P = 0.02$; contrast 360 d vs. ≤ 180 d, $P = 0.0001$), suggesting that storage induced a 'deeper' dormancy (Figure 4.2). Germination fractions of *H. canadensis* seeds were relative constant over time (Figure 4.2), although seeds stored at cold temperatures germinated to slightly greater rates than seeds stored at ambient laboratory conditions (contrast ambient vs. cold, $P = 0.05$).

**Discussion**

*A. racemosa* and *H. canadensis* seed remained in primary dormancy throughout the 360 d storage period, as seeds failed to germinate when removed from each of the six storage intervals (spread over a 360 d period) and then subjected to the 30 d germination test over a range of constant thermoperiods in both light and darkness. Similarly, Hidayati *et al*. (2000, 20002) observed little after-ripening over a 12 and 24 month period, respectively, for MPD seeds of the temperate forest shrubs *Lonicera fragrantissima* and *Diervilla lonicera*. Dormant seed of the perennial woodland herbs, *Dioscorea villosa* and *Collinsonia canadensis* also did not after-ripen after six months in dry storage (Albrecht and McCarthy 2006). Evidently, dry-storage does not overcome the warm stratification requirement for MPD seeds because embryos must be imbibed at warm temperatures for growth and breaking of the morphological component of primary dormancy.

In both species, viable germination fractions of fresh seed moved through the dormancy-breaking temperature sequences were comparatively similar to those obtained with seed populations stored for short-periods (< 90 d) in ambient and cold temperatures. This indicates that short-term storage does not change dormancy-levels or the temperature requirements for dormancy-break in these species. This lack of physiological change in seeds may be related to their deep physiological dormancies, as only seeds with nondeep physiological dormancy should after-ripen in ambient dry storage conditions (Nikolaeva 1977).

Prolonged storage (> 180 d) in cold and ambient conditions reduced viable germination fractions in *A. racemosa* but not *H. canadensis* seed populations. This study was unable to determine if dormant *A. racemosa* seed subjected to prolonged storage (>180 d) gained an additional requirement to break dormancy, or whether seeds entered a 'delayed' dormancy, whereby seeds simply needed more than one exposure to a warm → cold temperature sequence for effective dormancy-break. Previous studies with newly dispersed seed (Baskin and Baskin, 1985) showed that germination in a small portion of a single seed crop is spread over multiple seasons, indicating that some seeds innately require at least two (sometimes three) warm → cold temperature sequences for dormancy-break. Plus, a previous study showed that dormancy-break occurs independently of light, even if seeds are subjected to complete darkness immediately following dispersal (M.A. Albrecht, unpublished data). Thus, it is unlikely that changes in photoperiod requirements were responsible for altering viable germination fractions. Dry-storage has been shown to induce and/or prolong dormancy in other species,

including *Eucalyptus pauciflora* (Beardsell and Mullet 1984) and *Pseudotsuga menziesii* (Edwards and El-Kassaby 1988).

Survivorship rates for *H. canadensis* seed populations that were dry-stored in ambient laboratory and cold temperature conditions were lower than those of *A. racemosa*. After 360 d of storage, > 97% of the *H. canadensis* seeds lost viability, indicating that *H. canadensis* seeds are relatively short-lived compared to orthodox seeds of other perennial herbs that reportedly can survive for several years at ambient laboratory temperatures (Priestly 1986; Walck et al. 1997). Seed propagation protocols recommend that *H. canadensis* seeds be sown immediately and never allowed to dry out, presumably because seeds lose viability when removed from moist environments (Davis 1999). Data presented here support this supposition, as approximately 30% of the *H. canadensis* seed population lost viability after only 30 d in both storage treatments. This apparent sensitivity of seeds to dry conditions may be one factor limiting *H. canadensis* distribution to moist microhabitats in closed-canopy deciduous forests (Sanders and McGraw 2005).

Because this study was designed to mimic current methods of short-term seed storage by growers, seed moisture levels were not manipulated, making it impossible to appropriately classify post-harvest seed storage behavior as intermediate, orthodox, or recalcitrant (Roberts 1973; Hong and Ellis 1996). According to predictions based on the association between seed storage behavior and seed moisture content at maturity, however, *A. racemosa* and *H. canadensis* seeds likely exhibit orthodox seed storage behavior (Hong and Ellis 1997).

From a propagation perspective, MPD represents a dual constraint, because morphological (MD) and physiological dormancy (PD) each impose a distinctive requirement that must be satisfied for seeds to completely germinate. This type of dormancy certainly imposes constraints on agronomic production of these species because seeds must first experience temperatures that break morphological dormancy, in this case fluctuating warm (15°C) and cold (5°C) temperatures (Baskin and Baskin 1985; Baskin and Baskin 2001, Albrecht and McCarthy, unpublished data), prior to the cold-stratification necessary for breaking PD of the epicotyls (*A. racemosa*: $\geq$ 4 wk at 5 °C, *H. canadensis*: $\geq$ 8 wk at 5 °C; Albrecht and McCarthy, unpublished data). Attempts to "trick" seeds into germinating in shorter time periods by reversing the order of temperature sequences are ineffective, because alleviation of epicotyl dormancy via cold stratification only works after warm temperatures break embryo and primary root dormancy (Baskin and Baskin 2001). Further, our results indicate that short or long periods of dry-storage do not overcome the specific temperature requirements for dormancy-break. Thus, germinating seeds of these medicinal woodland herbs is a slow process because of the specific temperature requirements that must be experienced for germination. Due to the loss of viability over time, storage methods employed in this study are unacceptable for long-term storage, particularly for *H. canadensis* seeds. Given that seed germplasm is often in limited supply for these species, sowing seeds immediately following dispersal in the field would maximize resources and seedling material for cultivation and restoration efforts.

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Table 4.1. Generalized linear model results for the effects of storage temperature and duration on a) survival and b) germination of *Actaea racemosa* and *Hydrastis canadensis* seeds after incubation in a temperature sequence that breaks primary root dormancy (see Methods).

| Effect | df | *Actaea racemosa* | | *Hydrastis canadensis* | |
|---|---|---|---|---|---|
| | | *F* | *P* | *F* | *P* |
| **a) Survival fraction** | | | | | |
| Temperature | 1,36 | 12.98 | 0.0009 | 11.10 | 0.002 |
| Time | 6,36 | 18.64 | 0.0001 | 75.13 | 0.0001 |
| Temperature × Time | 6,36 | 0.71 | 0.62 | 6.42 | 0.0002 |
| **b) Germination fraction** | | | | | |
| Temperature | 1,36 | 0.69 | 0.41 | 6.68 | 0.01 |
| Time | 6,36 | 16.65 | 0.0001 | 0.45 | 0.84 |
| Temperature × Time | 6,36 | 2.03 | 0.10 | 1.19 | 0.33 |

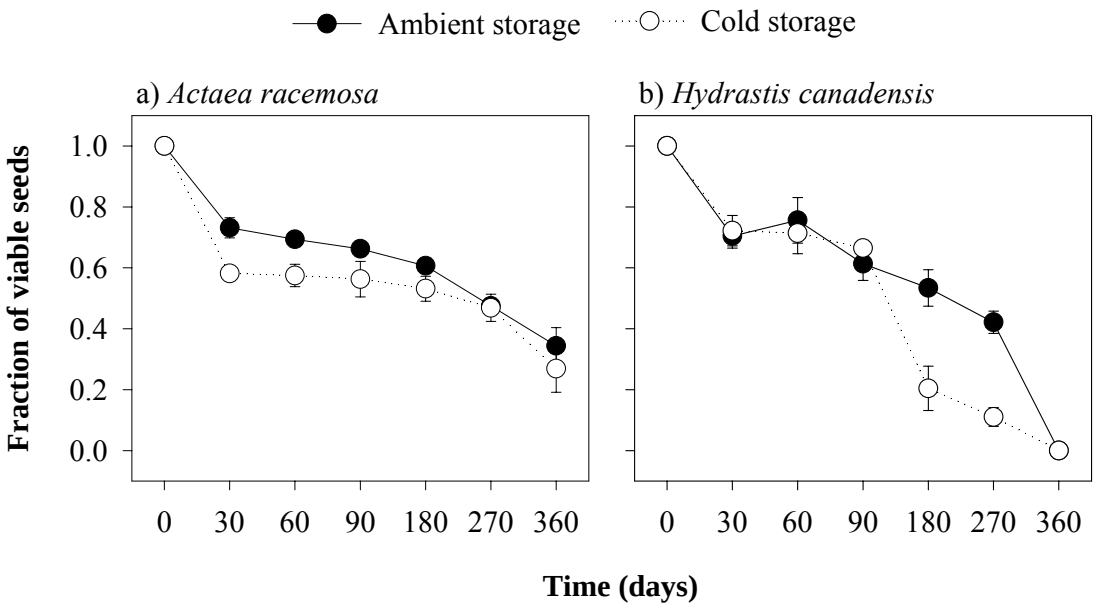


Figure 4.1. Seed survival of (a) *Actaea racemosa* and (b) *Hydrastis canadensis* in ambient laboratory (23ºC) and cold (5ºC) storage conditions.

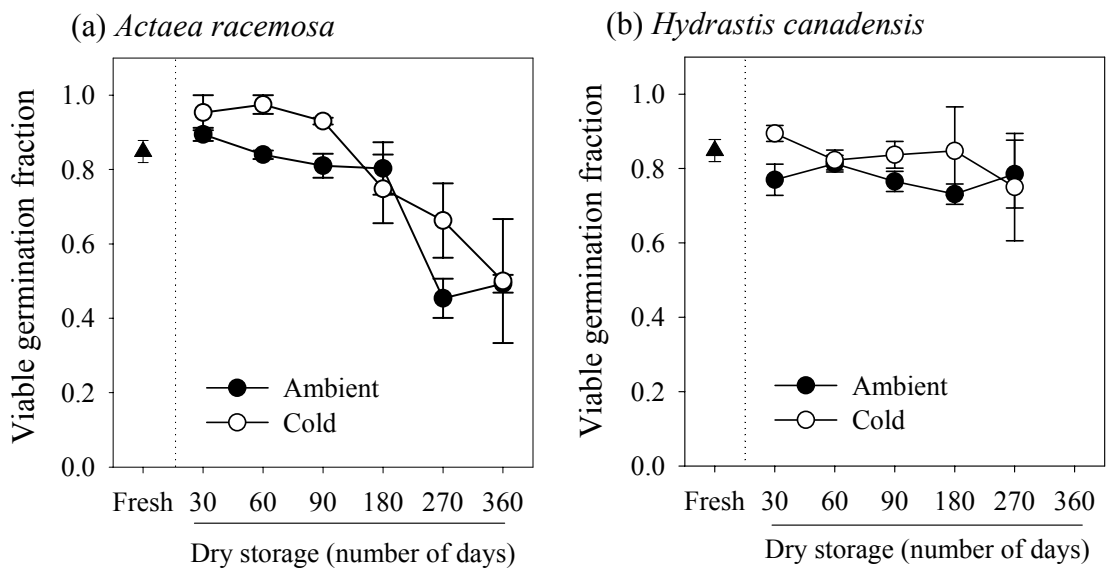


Figure 4.2. Fraction of dormant seeds of (a) *Actaea racemosa* and (b) *Hydrastis canadensis* following dry-storage at ambient laboratory (23ºC) and cold temperature (5ºC) conditions. Viable seeds that failed to germinate after dormancy-breaking temperature sequences were considered dormant.