Cold-Moist Stratification Improves Germination in a Temperate Terrestrial Orchid

Authors: Poff, Kirsten E., Sharma, Jyotsna, and Richards, Matt

Source: Castanea, 81(4) : 292-301

Published By: Southern Appalachian Botanical Society

URL: https://doi.org/10.2179/16-098

CASTANEA 81(4): 292–301. DECEMBER

Cold-Moist Stratification Improves Germination in a Temperate Terrestrial Orchid

Kirsten E. Poff,[1] Jyotsna Sharma,[1]* and Matt Richards[2]
[1]Department of Plant and Soil Science, Texas Tech University, Lubbock, Texas 79409
[2]Atlanta Botanical Garden, Atlanta, Georgia 30309

ABSTRACT Seed dormancy is a common evolutionary adaptation in temperate plant taxa. Dormancy mechanisms can prevent seeds from germinating at inopportune times, such as a cold period. We report the influence of pregermination stratification treatments on in vitro seed germination and seedling development in *Platanthera chapmanii*, a rare temperate terrestrial orchid native to the southeastern USA. Seeds were subjected to 0, 8, or 12 wk of cold-moist stratification at 5°C; mean seed viability was 89%. At 9 mo after plating, seeds exposed to 8 and 12 wk of stratification resulted in higher germination (Stage 1; 32% and 35%, respectively) in comparison to 25% germination in nonstratified seeds. Once a protocorm developed a leaf primordium (i.e., reached Stage 2), development to Stage 3 (root development) was independent of the pregermination treatments. Exposure to artificial lights for 3, 4, and 5 mo resulted in 32%, 44%, and 63% of the Stage 2 seedlings, respectively, developing into Stage 3 photosynthetic root-bearing seedlings. Our results indicate that in vitro seed germination in this temperate terrestrial orchid can be improved by using cold stratification. Furthermore, leaf- and root-bearing seedlings can be obtained through the methods reported herein.

Key words: Asymbiotic germination, cold-moist stratification, Orchidaceae, plant conservation, seed dormancy, sterile culture.

INTRODUCTION Seed dormancy is a consequence of evolutionary adaptation and an important survival mechanism in many plant species (McMahon et al. 2011). Dormancy prevents seed germination until adverse conditions abate, because seedlings that develop in unfavorable environmental conditions may perish without reproducing. Types of dormancies exhibited across the plant kingdom vary by species, and are a result of the specific adaptations of a species to its local climatic conditions. Typically, temperate species tend to manifest seed dormancy during cold periods. Within the temperate biomes, seeds of species native to colder latitudes may be adapted to longer dormancy periods compared to those that occur in the warmer temperate regions. Furthermore, the mechanism of dormancy may be physical or physiological, and sometimes a complex combination of dormancies may occur in seeds in response to evolutionary pressures (Baskin and Baskin 1998). For example, physical dormancy is a type of exogenous dormancy that requires scarification for water to pass through the impermeable layers of a seed coat (Baskin and Baskin 1998). Seeds with other dormancies, such as physiological or chemical dormancy, may be permeable to water, but require a metabolic change to occur before germination (Baskin and Baskin 1998). Understanding the dormancy mechanisms in seeds has implications for plant reproductive ecology, biology, and propagation.

The microscopic dust-like seeds in the family Orchidaceae, the largest angiosperm family, with an estimated 25,000 to 30,000 species, represent a variety of complex seed dormancy mechanisms (Johansen and Rasmussen 1992, Rasmussen 1995, Lauzer et al. 2007). When used for in vitro propagation, orchid seeds can require pregermination treatments to overcome physical and/or physiological dormancy (Johansen and Rasmussen 1992, Rasmussen 1995, Zettler et al. 2001, Sharma et al. 2003, Lauzer et al. 2007). In

*email address: jyotsna.sharma@ttu.edu
Received May 12, 2016; Accepted September 27, 2016.
DOI: 10.2179/16-098

addition to overcoming dormancy, orchid seeds have specific nutrient requirements to induce asymbiotic germination. This is partially to make up for the lack of mycorrhizal associations that are considered necessary for germination and development of most orchid species in the wild (Rasmussen 1995). For some species, seed dormancy can be overcome by exposing seeds to cold, moist conditions through a treatment known as stratification (Rost et al. 2006). For example, seeds of *Platanthera praeclara* Sheviak and M.L. Bowles, which is native to the midwestern USA, germinated in vitro only after they were exposed to 4- or 6-mo cold-moist stratification periods (Sharma et al. 2003). Seeds of *P. leucophaea* (Nutt.) Lindl., a sister species, have also been documented to require at least 2 mo of cold stratification to germinate (Stoutamire 1996). Another study on in vitro seed germination of *P. leucophaea* documented that nonstratified seeds showed $\leq$5% germination, whereas 8-wk and 16-wk stratification increased germination up to 20% and >30%, respectively (Bowles et al. 2002). Conversely, seeds of some species require scarification, or the weakening or opening of the seed coat, to induce germination. Seeds of *P. integra* (Nutt.) A. Gray, a species native to the southeastern USA, responded to surface sterilization and scarification with a 1:1:1 (v:v:v) solution composed of ethanol, 5.25% sodium hypochlorite, and deionized water. Germination percentage and protocorm development increased to 14.5% and 27.2% in seeds scarified for 1 or 2 hr, respectively, in comparison to 1.6% and 6% with shorter scarification treatments (1 min or 30 min, respectively; Zettler et al. 2000). Given that *P. integra* is native to acidic bog habitats in warmer temperate zones, its seeds may have developed a physical dormancy mechanism instead.

Considering that the family Orchidaceae exemplifies evolutionary advances in angiosperms, and that a majority of orchid taxa are rare, and routinely require specialized in vitro propagation techniques (Dressler 1981, Rasmussen 1995), knowledge of species-specific propagation protocols is necessary to produce propagules for both research and conservation. However, empirically developed propagation protocols exist for relatively few species. This lack of knowledge is especially evident in the temperate terrestrial orchid taxa native to North America, perhaps because of a perceived lack of their commercial value and specificity of propagation protocols at the species level. Simultaneously, conservation threats (i.e., changes in land use) to rare plants are increasing globally (Swarts and Dixon 2009). In fact, little is known about the biology and ecology of most orchid species. *Platanthera chapmanii* (Small) Luer, a rare species native to the southeastern USA, faces similar knowledge gaps. This severely understudied taxon has a geographic range limited to northern Florida, southeast Georgia, and southeast Texas (Poole et al. 2007). Because of conversion from native longleaf pine (*Pinus palustris* Mill.) to industrial pine forest and urban development, the quality of habitat in which *P. chapmanii* naturally occurs continues to decline (Gilliam and Platt 2006). Populations of *P. chapmanii* are often small, with $\leq$10 individuals, and the only large population with $\geq$100 flowering individuals occurs in southeast Texas (Richards and Sharma 2014). Peak flowering time for *P. chapmanii* is between late July and early August, when individual plants produce single inflorescences with $\geq$60 orange flowers (Liggio and Liggio 1999). *Platanthera chapmanii* is assumed to be an obligate outcrossing or facultative outcrossing species, as is the case with most species of *Platanthera* (Argue 2012). According to a multispecies study conducted on the coastal plain of Florida and Alabama, *P. chapmanii* has been documented to be pollinated by several species of long-tongued butterflies, including *Phoebis sennae* L., *Papilio troilus* L., *Papilio palamedes* Drury, and *Papilio marcellus* Cramer (Argue 2012). In southeast Texas, *Papilio palamedes* has been documented as carrying *P. chapmanii* pollinia (J. Sharma, pers. obs.). If pollination and fertilization are successful, each flower can potentially produce a capsule containing thousands of dust-like seeds. Capsule dehiscence typically occurs in October, and, subsequently, seeds are presumed to experience cold and moist conditions during the winter before environmental conditions change to facilitate germination and seedling development in early March. Information on reproductive biology and natural recruitment is not available for *P. chapmanii*. However, a preliminary study reported in vitro propagation, culture, and outplanting of laboratory-raised plants into the wild for population augmentation (Richards and Sharma 2014). Although Richards and Sharma (2014) provided preliminary infor-

mation on *P. chapmanii* germination and development, empirical data were not included.

The objective of this study was to quantify the influence of cold stratification on in vitro seed germination and plant development in a North American terrestrial temperate orchid, *P. chapmanii*. Considering that the seeds of *P. chapmanii* are exposed to average minima as low as –9°C at 32°N and –7°C at 29°N (USDA, NRCS 2016), we hypothesized that non–cold-moist–stratified seeds of *P. chapmanii* will exhibit a lower germination percentage in comparison to cold-moist stratified seeds. Furthermore, we expected that seeds stratified for 8 wk at 5°C will yield similar germination and plant development as those stratified for 12 wk at 5°C. This expectation was based on the relatively short (~10–40 d below 0°C) cold period that the species experiences across its natural distribution (Richards and Sharma 2014, NOAA, NCEI 2016).

MATERIALS AND METHODS

Seed Stratification

Seeds were collected from multiple capsules in October 2014 from haphazardly selected individuals of *P. chapmanii* from approximately 20 individuals in a population in southeast Texas. A maximum of one seed capsule was collected from each selected plant. Capsules were placed on a filter paper at room temperature at approximately 40% relative humidity to allow them to desiccate further and dehisce. Seeds were then collected and placed in a 1.5-ml glass vial. The vial containing the seeds was stored over silica gel desiccant at –20°C until further use. Time of storage at –20°C varied from 1 mo (seeds in the 12-wk treatment), to 2 mo (seeds in the 8-wk treatment) or 3 mo (seeds in the 0-wk treatment).

Seeds were prepared for the 8- and 12-wk cold-moist stratification treatments by first surface sterilizing them with a 0.6% sodium hypochlorite solution for 3 min. Because the seeds in the 0-wk treatment were not cold-moist stratified, surface sterilization was not performed until after the cold-moist stratification period was completed for the other two treatments. Seeds were then rinsed in sterile, ultrapure water and approximately equal portions were placed in each of two 2-ml safe-lock microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing approximately 1.0–1.5 ml sterile ultrapure water. The vials were inverted several times, wrapped in aluminum foil, and stored at 5°C for their respective stratification periods (Sharma et al. 2003). We staggered the timing of initiating the stratification treatments (8 and 12) to allow for the seeds in all three treatments to be plated at one time.

Once the stratification treatments had been applied, the seeds from both 8- and 12-wk treatments were again surface sterilized by submerging in a 0.6% sodium hypochlorite solution for an additional 6 min prior to plating on sterile nutrient medium to ensure surface sterility after cold-moist stratification. At this time, seeds from the 0-wk treatment were removed from storage and surface sterilized by submerging in a 0.6% solution of sodium hypochlorite for 12 min. The difference in total sodium hypochlorite exposure time from 9 min (8- and 12-wk cold-moist stratification) to 12 min (0-wk cold-moist stratification) prior to plating of seeds was to ensure that the embryos softened by cold-moist stratification were not damaged during the second surface sterilization procedure.

Seed Plating and Germination Assessment

After the seeds were subjected to their respective pregermination treatments, they were plated onto sterile P723 medium (Phytotechnology Laboratories, Overland Park, Kansas) contained in sterile, single-use Stericon-4 237-ml polystyrene containers (Phytotechnology Laboratories) in February 2015. Approximately 80 ml of medium was used per vessel and between 100 and 500 seeds were spread onto each vessel. Each of the three cold-moist stratification treatments was replicated 30 times with an experimental unit defined as one culture vessel. After the seeds were plated, a dissecting microscope was used to count and record the total number of seeds within each of the 90 vessels. At the same time, a count of viable seeds was performed. Within the 30 containers representing the 0-wk stratification treatment, each seed was observed for presence of a healthy embryo (defined as a clear, hyaline, rounded embryo) to be categorized as a viable seed. However, water imbibition instead was used as a measure of viability for seeds in the remaining 60 plates containing seeds that were cold-moist stratified for 8 or 12 wk. A swollen embryo (indicating imbibition) was counted as a viable embryo (Figure 1).

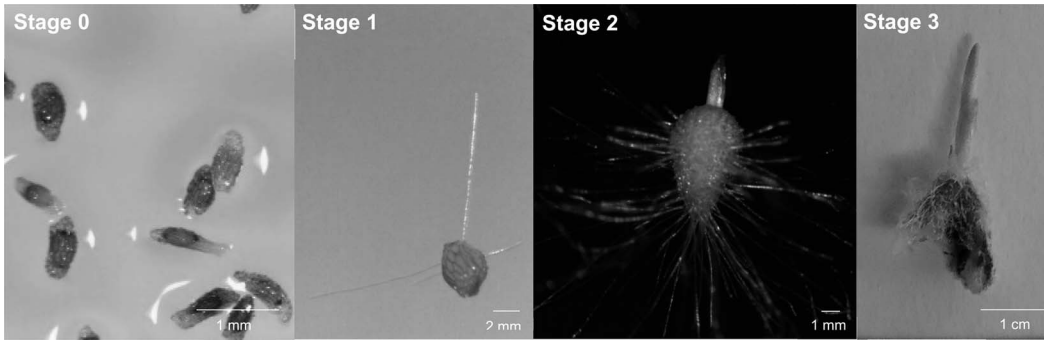

Figure 1. Seed germination and plant development in *Platanthera chapmanii* were recorded by using four categories: Stage 0 (no germination); Stage 1 (germination; rhizoid development); Stage 2 (leaf primordium development); Stage 3 (root development).

After the initial seed count and viability assessment, seeds were stored at approximately 23°C in the dark. A visual assessment of germination and development was performed by inspecting all seeds in each experimental unit every 30 d. Germinating seeds and developing seedlings were categorized in one of the three categories: Stage 1, 2, or 3 (Figure 1). We defined Stage 1 as germination and the presence of one or more rhizoids; Stage 2 as the presence of a leaf primordium on the developing protocorm; and Stage 3 as the presence of at least one root.

Once a seed reached Stage 2, it was transferred to new containers with fresh P723 medium. We used sterile Magenta GA-7 vessels (Sigma-Aldrich, St. Louis, Missouri), with approximately 50 ml of P723 medium for the transfer. The newly transferred protocorms were then placed on a culture rack and exposed to 40-watt cool white fluorescent light bulbs to provide a photoperiod of 12 hr. The developing seedlings were subsequently examined every 30 d for further development. At 9 mo after the seed plating, young plants were individually examined for root development. After an assessment of root development, plants were placed in sterile PTcon 947-ml culture vessels (Phytotechnology Laboratories) containing approximately 150–200 ml of P723 medium. The experimental data collection for this study was considered complete at this time.

Data Analysis

A one-way ANOVA was performed with Stages 0–3 germination as the dependent variables and stratification treatment as the independent variable. We calculated the proportions in each stage as follows: (1) number of ungerminated seeds (Stage 0) was calculated by subtracting the number of seeds in each experimental unit that reached Stage 1 from the total number of seeds plated and then dividing that number by the total number of seeds plated; (2) number of Stage 1 protocorms in each experimental unit was calculated by recording the total number of seeds that reached Stage 1, as defined above, and dividing that number by the total number of seeds plated; (3) development to Stage 2 was calculated by recording the total number of seeds that developed to Stage 2 in each experimental unit and dividing that by the total number of seeds plated; and (4) development to Stage 3 was calculated by recording the total number of Stage 3 seedlings and dividing by the total number of Stage 2 seedlings that were obtained in each experimental treatment. The means were separated by using Fisher's least significant difference (LSD) test. To test whether each treatment received a similar number of seeds, we performed an ANOVA using the total number of seeds plated in each experimental unit. A similar procedure was also used to test the differences in viability among the plated seeds. Furthermore, a two-way ANOVA was performed with stratification period and duration of exposure to light as the two independent variables and Stage 3 proportions as the dependent variable. Means were separated by using Fisher's LSD test. All statistical analyses were performed using RStudio 0.99.842 (RStudio Team 2015) using the agricolae package with an α of 0.05.

Table 1. A one-way ANOVA was conducted to test the effect of cold-moist stratification (0, 8, or 12 wk) on seed germination and plant development in *Platanthera chapmanii*. Germination of seeds was categorized as Stage 0 (no further development), Stage 1 (germination; rhizoid development), or Stage 2 (leaf primordium development).

	Sum of Squares[y]	Mean Square	f Value	p Value[z]
strat_stage 0_t[x]	0.48	0.01	15.36	<0.001
strat_stage 0_v	0.59	0.01	17.82	<0.001
strat_stage 1_t	0.45	0.01	13.71	<0.001
strat_stage 1_v	0.56	0.01	16.21	<0.001
strat_stage 2_t	0.12	0.00	12.20	<0.001
strat_stage 2_v	0.15	0.00	11.46	<0.001
Total seeds	839,462	11,499	0.91	0.34
Viable seeds	658,411	9,019	0.95	0.33
Viability	0.14	<0.000	0.86	0.36

[x]t = proportions calculated by using total number of seeds that were plated; v = proportions calculated by using only the number of viable seeds.

[y]Factor level df = 1; residual df = 73.

[z]$\alpha = 0.05$.

RESULTS

Seed Germination

Among the calculated 22,348 individual seeds used across the three stratification treatments, mean viability was 89% (Tables 1 and 2). Results from an ANOVA and Fisher's LSD test showed that *P. chapmanii* seeds exposed to the 0-wk stratification treatment had lower Stage 1 germination percentage than the 8- and 12-wk cold-moist stratification treatments. When the total number of seeds sown was used as the denominator for calculating percentage of seeds that reached Stage 1 germination, the 0-wk stratification treatment had the lowest germination (mean = 22.8%; $p < 0.001$), whereas the means were statistically similar for the 8- and 12-wk treatments (28.7% and 30.7%, respectively) (Tables 1 and 2). Similar results were observed when the number of viable seeds was used as the denominator to calculate germination percentages. In this case, mean germination in the 0-wk cold-moist stratification treatment was 25.4% ($p < 0.001$), whereas we observed 32.4% and 35.1% germination among the 8- and 12-wk treatments, respectively. Finally, the highest percentage of ungerminated seeds was observed in the 0-wk stratification treatment (77.3% of all plated seeds and 74.6% of viable seeds), while both 8- and 12-wk stratification treatments had lower percentages of ungerminated seeds (Tables 1 and 2).

Table 2. Effect of cold-moist stratification (0, 8, or 12 wk) on seed germination was experimentally tested in *Platanthera chapmanii*. An ANOVA was conducted; germination of seeds was categorized as Stage 0 (no further development), Stage 1 (germination; rhizoid development), or Stage 2 (leaf primordium development). Mean number of seeds that were plated in an experimental unit, mean number of viable seeds, and mean percent viability are presented for each experimental treatment. Mean germination percentages were calculated by using total number of seeds and number of viable seeds separately. Means followed by the same letter in each column were statistically similar based on Fisher's least significant difference test.

Stratification (# of wk)	N[w]	Total Seeds (#)	Viable Seeds (#)	Viability (%)	Stage 0_t[z] (%)	Stage 0_v (%)	Stage 1_t (%)	Stage 1_v (%)	Stage 2_t (%)	Stage 2_v (%)
0	26	282	251	89.5[x] a[y]	77.3 b	74.6 b	22.8 b	25.4 b	11.6 b	13.0 b
8	26	361	321	89.2 a	71.3 a	67.7 a	28.7 a	32.4 a	14.1 a	15.7 a
12	23	223	198	88.3 a	68.4 a	64.0 a	30.7 a	35.1 a	15.5 a	17.4 a
p Value		0.34	0.33	0.36	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[w]A single vessel containing multiple orchid seeds served as an experimental unit.

[x]t = percentages calculated by using total number of seeds that were plated; v = percentages calculated by using only the number of viable seeds.

[y]Proportions were converted to percentages for presentation in the tables.

[z]$\alpha = 0.05$.

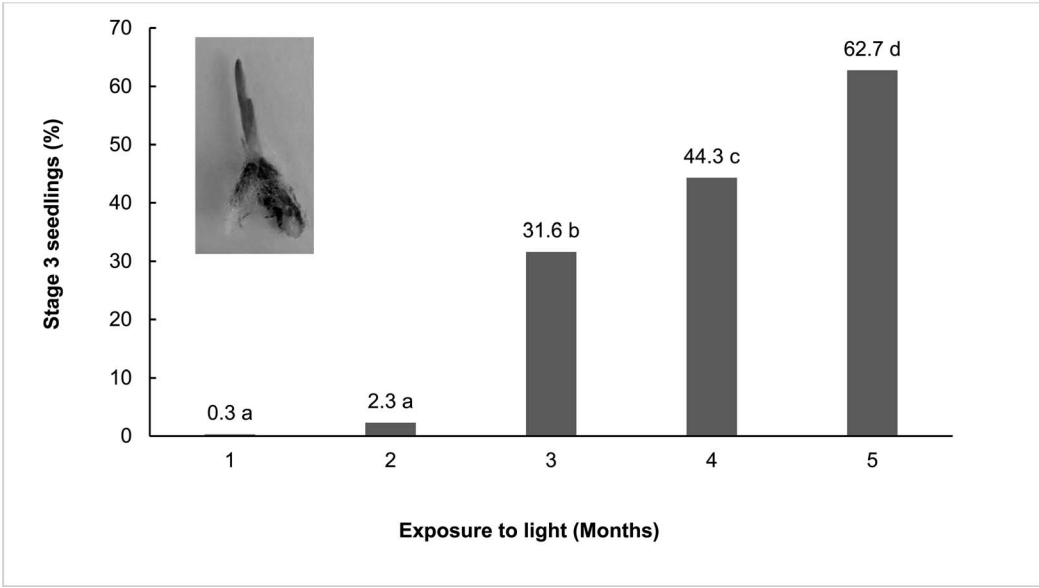

Figure 2. Proportion of Stage 2 seedlings of *Platanthera chapmanii* that reached the developmental Stage 3 after exposure to light. Duration of exposure to light (1–5 mo under 40-watt white florescent bulbs) influenced plant development to Stage 3. Pregermination stratification of seeds for 0, 8, or 12 wk did not influence development from Stage 2 to Stage 3; thus, the means were pooled across the three stratification treatments. Means followed by the same letter were statistically similar based on Fisher's least significant difference test.

Seedling Development

When data for seedling development from Stage 2 to Stage 3 were analyzed using a two-way ANOVA including stratification and duration of exposure to light, we did not detect any interactive effects or effect of stratification treatment on the means. However, we did detect an influence of time and duration of exposure to light on seedling development. In response to exposure to light, seedlings that reached Stage 2 required at least 1 mo of exposure to artificial lights to develop roots (i.e., to reach Stage 3; Figure 2). As the time after exposure to lights increased from 1 to 5 mo, the mean percentage of Stage 3 seedlings increased (Figure 2). While the means for 1- and 2-mo exposure were similar, 32%, 44%, and 63% of Stage 2 seedlings reached Stage 3 after 3, 4, and 5 mo, respectively (Figure 2). Even though not statistically different ($p = 0.27$), the absolute means of Stage 3 seedlings ranged from 16.5% (12-wk cold-moist stratification) to 22.1% (0-wk cold-moist stratification across the three stratification treatments) (Tables 3 and 4).

DISCUSSION

As in many plant species, seed dormancy in temperate terrestrial orchids is common (Johansen and Rasmussen 1992, Rasmussen 1995, Lauzer et al. 2007). The duration and type of seed dormancy, however, is often species and climate dependent. Temperate terrestrial species sometimes require a cold-moist stratification period of at least 8 wk to initiate germination (Rasmussen 1992, Sharma et al. 2003). Although Richards and Sharma (2014) reported propagation of *P. chapmanii* from seed after $\geq$12 wk of exposure to cold-moist stratification conditions, germination percentages were not quantified in their study; the resulting plants, however, were reported to survive for >3 yr. Furthermore, the authors documented reproduction and survival of the artificially propagated plants both in cultivation in a greenhouse environment and in the native habitat of the species (Richards and Sharma 2014).

In the present study, we report that cold-moist stratification improves asymbiotic in vitro germination among *P. chapmanii* seeds when compared to nonstratified seeds. An increase of approximately 10% was observed when seeds

Table 3. An ANOVA was conducted to test the effect of cold-moist stratification (0, 8, or 12 wk) on seedling development subsequent to leaf primordium development (Stage 2) in *Platanthera chapmanii*. The response variable used in the analysis was Stage 3 (root development).

	df	Sum of Squares[y]	Mean Square	f Value	p Value[x]
strat_stage3	125	8.29	0.07	1.24	0.27

[y]Factor level df = 1; residual df = 125.
[x]$\alpha = 0.05$.

were stratified for either 8 or 12 wk; however, we did not observe a differential influence of stratification on plant development past Stage 1 (germination and rhizoid development). While lower than the mean germination obtained after treating seeds with stratification, we did observe up to 25.4% germination in nonstratified seeds. Similarly, Zettler et al. (2000) reported a low (15%) germination in nonstratified *P. integra* seeds collected in North Carolina; however, seeds in their study were not plated on nutrient-rich asymbiotic medium. Cold-moist stratification treatments were not included in their study, hence, it is not known whether *P. integra*, which is native to similar habitats and climate as *P. chapmanii*, has similar pregermination stratification requirements. Another congeneric species native to the southeastern U.S., *P. clavellata* (Michx.) Luer, however, yielded much higher (47%) germination in nonstratified seeds collected from Tennessee and South Carolina (Zettler and Hofer 1998). Altogether, these two species have an overlapping distribution with *P. chapmanii* in southeast Texas and northern Florida, although *P. clavellata* extends also to northern latitudes in Quebec and Ontario (USDA, NRCS 2016). The germination percentage for nonstratified seeds reported in *P. clavellata* is higher than those observed both in *P. chapmanii* and *P. integra*. These data confirm that results from any individual species should not be broadly applied to even the congeners from similar habitats, and that species-specific studies are necessary to understand the nuances within each taxon (Stewart and Kane 2007, Swarts and Dixon 2007). It is also clear that additional or alternative pregermination treatments, such as scarification or different light/dark periods, should be examined for *P. chapmanii*. Simultaneously, it is possible that cold stratification may improve germination among *P. integra* and *P. clavellata* seeds. On the other hand, testing the efficacy of symbiotic fungi in improving germination in *P. chapmanii* might also be considered.

Table 4. Effect of cold-moist stratification (0, 8, and 12 wk) on seedling development subsequent to leaf primordium development (Stage 2) was experimentally tested in *Platanthera chapmanii*. An ANOVA was conducted; the response variable used in this analysis was Stage 3 (root development). Mean percentage of seedlings that were categorized as Stage 3 was calculated for each stratification treatment after a 5-mo exposure to 40-watt fluorescent bulbs to provide a photoperiod of 12 hr. Means followed by the same letter in a column were statistically similar based on Fisher's least significant difference test.

Stratification (# of wk)	n[z]	Stage 2 (#)	Stage 3 (%)
0	45	21	22.1 a[y]
8	46	34	16.5 a
12	36	28	16.5 a
p value			0.27

[z]A single vessel containing multiple orchid seeds served as an experimental unit.
[y]$\alpha = 0.05$.

The similarity between the germination percentages obtained from 8- and 12-wk stratification treatments in our study suggests that stratification periods longer than 8 wk may not be necessary to improve germination. In fact, it is possible that a stratification period between 0 and 8 wk could optimize germination in *P. chapmanii*. In southeast Texas, where the seeds for this experiment were collected, the average minima for the coldest month (January) ranged from –2°C to 4°C between 2012 and 2016, whereas the average maxima ranged from 15°C to 21°C between 2012 and 2016 (NOAA, NCEI 2016). Considering this, continuous cold-moist stratification at 5°C for 8 wk might have been excessive. Whether a shorter stratification duration, or other pregermination treatment combinations, would increase germination beyond 35.1% (maximum observed in this study) remains to be empirically tested, however. Additionally, because germination rates can vary among disjunct populations of the same

species, germination studies with seeds from additional populations of *P. chapmanii* could help clarify further the differences in germination in relation to provenance. Although the species has a wide range from east to west (81°W to 94°W), *P. chapmanii* populations are disjunct, small, and occur north to south within a relatively narrow latitudinal zone between 29°N and 32°N (NOAA, NCEI 2016). Seeds used in the current study were collected from a single population, although it is the largest documented population of the species and potentially the most genetically diverse. However, even this relatively large population may contain reduced genetic variation, considering the long interpopulation distances. In some plants, germination percentages correlate positively with genetic diversity and population size, as in the perennial prairie species, *Silene regia* Sims (Menges 1991). Similarly, the North American species, *Ipomopsis aggregata* (Pursh) V.E. Grant, exhibited reduced germination in seeds from populations with $\leq$100 individuals than seeds collected from larger populations (Heschel and Paige 1995). Conversely, a study on the perennial species, *Draba aizoides* Pall. Ex M. Bieb, showed that populations with lower genetic variation exhibited high germination rates when compared to populations with higher genetic variation (Vogler and Reisch 2013). Combined with population genetic diversity analyses, range-wide germination studies should be pursued to elucidate provenance differences and to assist with conservation of *P. chapmanii*.

In our study, a higher percentage of *P. chapmanii* seedlings developed roots as the duration of exposure to light increased. Exposure to light may facilitate development in plants; however, time and light were not taken into account as experimental treatments in this case. Consequently, *P. chapmanii* root development may be due to any number of factors. If given more time, more individuals may develop roots independent of light exposure. However, once *P. chapmanii* seedlings develop small roots, it is unlikely they would develop further in the absence of light (Hart 2012).

The effect of climate change is reported to be more severe on rare plants with fragmented populations. According to a review by Walther et al. (2002), the vegetative growth and flowering in multiple plant species in Germany is occurring progressively earlier in the year since the 1960s. The capacity of orchid seeds from temperate regions to germinate in the absence of stratification, as documented in this and other studies, could be an increasingly useful evolutionary adaptation as the climate changes (Canadell and Noble 2001). This strategy could allow natural recruitment and time for adaptation under milder climatic conditions.

Stratification treatments also may influence growth and development beyond germination in temperate orchid taxa. For example, seeds of *P. praeclara* stratified for 6 mo and cultured symbiotically developed roots after 60 d of culture. In comparison, the 4-mo stratification period did not yield root-bearing seedlings (Sharma et al. 2003). Considering that plants developed to Stage 3 (root-bearing, photosynthetic seedlings) in our study consistently across the three stratification treatments, it is evident that plant development up to 9 mo beyond germination (Stage 1) is independent of the pregermination stratification period in *P. chapmanii*. While additional reproductive biology and recruitment studies must be conducted for *P. chapmanii*, our results provide an effective and efficient protocol for generating plants of the species for experimental and conservation applications.

ACKNOWLEDGMENTS Funding from the Texas Parks and Wildlife Department is gratefully acknowledged for supporting portions of this study. We thank Joe Liggio for facilitating the collection of seeds in southeast Texas, and we also thank Jaspreet Kaur for her assistance with statistical analysis.

LITERATURE CITED

Argue, C.L. 2012. The pollination biology of North American orchids. Volume 1. Springer Publishing, New York, New York.

Baskin, C.C., and J.M. Baskin. 1998. Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press, New York, New York.

Bowles, M.L., K.A. Jacobs, L.W. Zettler, and T.W. Delaney. 2002. Crossing effects on seed viability and experimental germination of the federal threatened *Platanthera leucophaea*. Rhodora 104:14–30.

Canadell, J. and I. Noble. 2001. Challenges of a changing earth. Trends Ecol. Evol. 16:664–666.

Dressler, R.L. 1981. The orchids: natural history and classification. Harvard University Press, Cambridge, Massachusetts.

Gilliam, F.S., and W.J. Platt. 2006. Conservation and restoration of the *Pinus palustris* ecosystem. Appl. Veg. Sci. 9:7–10.

Hart, J.W. 2012. Light and plant growth. Springer Science and Business Media, New York, New York.

Heschel, M.S. and K.N. Paige. 1995. Inbreeding depression, environmental stress, and population size variation in scarlet gilia (*Ipomopsis aggregata*). Conserv. Biol. 9:126–133.

Johansen, B. and H. Rasmussen. 1992. *Ex situ* conservations of orchids. Opera Bot. 113:43–48.

Lauzer, D., Renaut, S., St-Arnaud, M., and D. Barabe. 2007. In vitro asymbiotic germination, protocorm development and plantlet acclimation of *Aplectrum hyemale* (Muhl Ex Willd.) Torr. (Orchidaceae). J. Torrey Bot. Soc. 134: 344–348.

Liggio, J., and A.O. Liggio. 1999. Wild orchids of Texas. The University of Texas Press, Austin, Texas.

McMahon, M.J., A.M. Kofranek, and V.E. Rubatzky. 2011. Plant science. Prentice Hall, Upper Saddle River, New Jersey.

Menges, E.S. 1991. Seed germination percentage increases with population size in a fragmented prairie species. Conserv. Biol. 5:158–164.

NOAA, NCEI. 2016. Monthly summaries map (https://gis.ncdc.noaa.gov/maps, 29 March 2016). NCEI GIS Agile Team, Asheville, North Carolina 28801-5001 USA.

Poole, J.M., W.R. Carr, D.M. Price, and J.R. Singhurst. 2007. Rare plants of Texas. Texas A&M University Press, College Station, Texas.

Rasmussen, H.N. 1992. Seed dormancy pattern in *Epipactis palustris* (Orchidaceae): requirements for germination and establishment of mycorrhiza. Physiol. Plant., 86(1):161–167.

Rasmussen, H.N. 1995. Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, Cambridge, England.

Richards, M. and J. Sharma. 2014. Review of conservation efforts for *Platanthera chapmanii* in Texas and Georgia. Native Orchid Conf. J. 11:1–11.

Rost, T.L., M.G. Barbour, C.R. Stocking, and T.M. Murphy. 2006. Plant biology, 2nd ed. Thomson Corporation, Stamford, Connecticut.

RStudio Team. 2015. RStudio: integrated development for R. RStudio, Inc., Boston, MA (http://www.rstudio.com).

Sharma, J., L.W. Zettler, J.W. Van Sambeek, M.R. Ellersieck, and C.J. Starbuck. 2003. Symbiotic seed germination and mycorrhizae of federally threatened *Platanthera praeclara* (Orchidaceae). Am. Midl. Nat. 149:104–120.

Stewart, L.S. and M.E. Kane. 2007. Symbiotic seed germination and evidence for in vitro mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae) and its implications for species-level conservation. In Vitro Cell. Dev. Biol.-Plant 43:178–186.

Stoutamire, W.P. 1996. Seeds and seedlings of *Platanthera leucophaea* (Orchidaceae). p. 55–61. *In*: Allen, C. (ed.). Proceedings of the North American Native Terrestrial Orchid-Propagation and Production Conference. National Arboretum, Washington, D.C.

Swarts, N.D., and K.W. Dixon. 2007. Perspectives on orchid conservation in botanic gardens. Trends Plant Sci. 14:590–598.

Swarts, N.D. and K.W. Dixon. 2009. Terrestrial orchid conservation in the age of extinction. Ann. Bot. 104:543–556.

USDA, NRCS. 2016. The PLANTS Database (http://plants.usda.gov, 29 March 2016). National Plant Data Team, Greensboro, NC 27401-4901 USA.

Vogler, F. and C. Reisch. 2013. Vital survivors: low genetic variation but high germination in glacial relict populations of the typical rock plant *Draba aizoides*. Biodivers. Conserv. 22: 1301–1316.

Walther, G.R., E. Post, P. Convey, A. Menzel, C. Parmesan, T.J. Beebee, J.M. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change. Nature 416:389–395.

Zettler, L.W. and C.J. Hofer. 1998. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its

ecological implications. Environ. Exp. Bot. 39: 189–195.

Zettler L.W., S.L. Stewart, M.L. Bowles, and K.A. Jacobs. 2001. Mycorrhizal fungi and cold-assisted symbiotic germination of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley. Am. Midl. Nat. 145:168–175.

Zettler, L.W., J.A. Sunley, and T.W. Delaney. 2000. Symbiotic seed germination of an orchid in decline (*Platanthera integra*) from the Green Swamp, North Carolina. Castanea 65:207–212.