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A SIMPLE GERMINATION PROTOCOL FOR *EX SITU* PROPAGATION OF THE ENDANGERED *CAREX LUPULIFORMIS*

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ABSTRACT. Plant reintroductions have become an important component of species recovery strategies. To favor establishment and survival rates of reintroduced specimens, the use of mature individuals is often recommended. Producing individuals from seed can be challenging, because little is known about the germination requirements of many endangered species. Here, we investigated whether *Carex lupuliformis* achenes can be germinated at high rates under semi-controlled *ex situ* conditions. More specifically, we aimed to determine which simple stratification technique allows higher/faster germination rates, whether scarification speeds up the germination process, and which light intensity allows higher/faster germination rates. We found that a brief cold-wet stratification (one month in wet sand) increases the likelihood that *C. lupuliformis* achenes will germinate, but that a similar germination rate can be obtained by storing achenes at 4°C for six months in a plastic bag. Although scarification did not affect final germination rates, scarified achenes germinated significantly faster than unscarified ones. Finally, we found that a light intensity of 25% resulted in significantly higher final germination rates than lower light intensities. In conclusion, our experiments showed that *C. lupuliformis* is easy to propagate *ex situ*, as a variety of treatments resulted in relatively high germination rates.

Key Words: False hop sedge, germination, endangered species, light, scarification, stratification, *Carex lupuliformis*, propagation

With a growing number of species at risk of extinction, there is an urgent need for effective preservation strategies. In recent years, reintroductions have become an important component of species recovery schemes (Godefroid and Vanderborght 2011; Liu et al. 2015). When properly planned, reintroductions can allow recovery of population size and genetic diversity (e.g., Daws and Koch 2015; Fant et al. 2013; Noël et al. 2011). However, there is usually a high level of risk involved, with biological success often low or unpredictable (Dalrymple et al. 2012; Drayton and Primack 2012; Godefroid et al. 2011; Liu et al. 2015).

To favor establishment and increase survival rates of reintroduced plant specimens, the use of mature individuals is often recommended

(Albrecht and Maschinski 2012; Arguraiuja 2011). Producing plants from seed can be challenging, because little is known about the germination requirements of many endangered species. Furthermore, ensuring these plants reach maturity is often more expensive and time- and infrastructure-demanding than direct seeding. Consequently, developing flexible, practical and labor-saving, yet still reasonably effective germination protocols presents a number of challenges. Since few conservation agencies possess enough growth chambers to propagate hundreds of individuals for reintroduction trials, seeds need to be grown in greenhouses or outdoors, where germination conditions cannot be controlled precisely. Also, for seedlings to mature, seeds must be sown on soil, which presents more heterogeneous conditions than the agar generally used for germination tests. Additionally, controlled environment germination tests usually focus only on those conditions that can produce optimal germination rates. Yet, speed of germination is also of great importance when resources are limited, and the factors that promote it may differ from those that promote high germination rates. For instance, stratification and scarification of seeds might not be necessary to obtain high germination rates, but might allow faster germination (Schütz and Rave 1999; Schütz 2000).

In our study, we investigated the possibility of germinating *Carex lupuliformis* Sartwell ex Dewey achenes (dry fruits that contain the seed) at high rates in semi-controlled *ex situ* conditions (greenhouse environment). More specifically, we aimed to determine which simple stratification technique allows higher and/or faster germination rates, whether scarification speeds up the germination process, and which light intensity allows higher and/or faster germination rates.

MATERIALS AND METHODS

Study species. *Carex lupuliformis* is a wetland perennial that grows mostly under canopy openings in seasonally flooded forested wetlands (COSEWIC 2011; Hill 2006; Thompson and Paris 2004). Distributed sporadically across eastern North America, it is rare and endangered throughout most of its range (COSEWIC 2011; Thompson and Paris 2004). *Carex lupuliformis* is a cespitose or long-rhizomatous species that can reproduce both vegetatively and sexually. It is characterized by a high production of viable achenes. Germination tests in a controlled environment have shown that 100% of *C. lupuliformis* achenes (gathered from 19 plants in two Québec populations) can germinate when sown on 1% agar and kept at a temperature of 25°C during the day (8 h) and 10°C at night (16 h; Royal Botanic Gardens 2015). Although the species' other

specific germination requirements are unknown, the probability that wetlands *Carex* seeds will germinate is usually higher in light, after cold-wet stratification (due to primary dormancy), and when perigynia are removed (Jones et al. 2004; Schütz 2000). Scarification is not a common requirement for *Carex* germination (Bond 1999), but it can induce faster and greater results in some species (Hoag et al. 2001).

Stratification and scarification. In September 2008, 1200 mature achenes were collected from three individuals grown at the Montréal Botanical Garden from achenes gathered in 2005 from three wild individuals belonging to a single population of nine individuals. Although seeds collected from few individuals may have a lower genetic diversity and individuals grown in botanical gardens can have a reduced dormancy (Eßlin et al. 2011), we did not collect achenes from wild plants to avoid impacts on wild populations as much as possible (only 31 wild individuals were known in Québec at the beginning of the experiment).

Achenes were randomly divided into 120 sets of ten achenes. They were dried for a period of two days in a paper bag at room temperature prior to treatment, to avoid rotting (moisture was still present in the embryo, but there was no surface moisture). Achenes were then stored under 12 different conditions (10 sets per condition; Table 1) expected to be representative of methods used by not-for-profit conservation agencies. These treatments varied in regard to the duration of cold stratification (zero, one, five or six months at 4°C) and type of storage medium (paper or plastic bag, wet sand). In our study, cold stratification did not necessarily involve keeping achenes in a moist environment, which differs from the common definition of cold stratification (Baskin and Baskin 2001). Non-stratified achenes (i.e., zero months of cold stratification) were kept at room temperature for six months in paper bags. A group of fresh achenes sown in a greenhouse immediately after collection was used as a control. The other groups were sown in the same greenhouse in February 2009 after their respective treatments and complementary time at room temperature in paper bags. The sand used for stratification was sterilized for 30 minutes in an autoclave prior to experiments. Storage in wet sand without cold stratification was not tested. One day prior to sowing, all achenes were manually removed from their perigynia and five sets of achenes per stratification condition were manually scarified with 220 grit sand paper. The other five sets of achenes remained unscarified.

A homemade mix composed of bark compost, blond peat moss (one fifth), and sand (one sixth) was used as substrate. Sets of ten achenes were sown in trays containing 72 germination cells of 16 cm^2 (one set per cell). Achenes were sown on the surface, an equal distance apart, then sprinkled

Table 1. Code and description of the 12 treatments used to promote *Carex lupuliformis* germination. For each treatment, different sets of achenes were also scarified or non-scarified.

Code	Treatment
Fresh	Untreated achenes sown directly after collection
0plas	No stratification – achenes kept at room temperature for 6 months in a plastic bag
0pap	No stratification – achenes kept at room temperature for 6 months in a paper bag
1plas	One-month cold stratification (4°C) in a plastic bag
1sand	One-month cold stratification (4°C) in wet sand
1pap	One-month cold stratification (4°C) in a paper bag
5plas	Five-month cold stratification (4°C) in a plastic bag
5sand	Five-month cold stratification (4°C) in wet sand
5pap	Five-month cold stratification (4°C) in a paper bag
6plas	Six-month cold stratification (4°C) in a plastic bag
6sand	Six-month cold stratification (4°C) in wet sand
6pap	Six-month cold stratification (4°C) in a paper bag

with a thin layer of soil and watered to ensure optimal contact with the substrate. Transparent plastic covers were used to avoid rapid desiccation. Replicates were divided into five randomized blocks of 24 cells (12 storing conditions × two scarification treatments). Seeded trays were monitored daily, and the experiment was stopped when two consecutive weeks had passed without any further germination (total 95 days). Minimal day- and night-time temperatures in the greenhouse were respectively 10°/4°C in February–March, 15°/12°C in April–May, 23°/21°C in June–August, 18°/10°C in September, and 12°/4°C in November–December.

Light intensity. In September 2014, mature achenes were randomly collected from 20 individuals from five wild populations. Achenes were stored in plastic bags in darkness at 4°C until the beginning of the experiments in March. Achenes were then randomly divided into 90 sets of 40 achenes. Photographic 0.15, 0.3 and 0.6 Neutral Density filters (LEE Filters, Burbank, CA) were used to obtain seven different light intensities (approximately 70%, 50%, 25%, 12%, 6%, 3% and 1%). These filters reduce light intensity without changing wavelengths. Filters were shaped and glued to the tops and sides of 70 Petri dishes (10 sets per light level). Lower light intensities were obtained by combining filters, using up to three layers per Petri dish. Two other levels were achieved by forgoing filters (100%) or covering their tops and sides with two layers of aluminum foil (0%).

A layer of Berger® BM6 soil mix (containing 85% peat moss and 15% perlite; Berger Peat Moss, Saint-Modeste, Québec) considered

thick enough to block light (about 1 cm) was placed in each Petri dish. After manually removing all achenes from their perigynia, one set of 40 achenes was sown on the surface, all an equal distance apart, in each Petri dish. Ten replicates per treatment were divided into five randomized blocks (two paired replicates per block).

Light intensity in the greenhouse was monitored bi-weekly between 11am and 1pm using a portable HI97500 Lux meter (Hanna Instruments Ltd., Leighton Buzzard, U.K.). Light intensity ranged from about 50 $\mu mol/m^2/s$ to 200 $\mu mol/m^2/s$, with a mean of 150 $\mu mol/m^2/s$. Therefore, at noon on an average day, light intensities tested were approximately 150, 105, 75, 38, 19, 9, 5, 2 and 0 $\mu mol/m^2/s$. Achenes from all treatments were briefly exposed to full light intensity during sowing and monitoring. Because *Carex* species do not typically respond to light flashes (Schütz 2000), it is unlikely that germination rates were affected by these short periods of full light exposure. Petri dishes were monitored bi-weekly after initial germination and the experiment was stopped after four consecutive days without further germination (41 days), due to infrastructure constraints. Minimal day- and night-time temperatures were respectively 20° and 18°C, following a cycle of 14 h of light and 10 h of darkness. Mogul Base High Pressure Sodium lamps in the greenhouse shut off automatically if luminosity reaches >600 $\mu moles/m^2/s$. Water was provided daily to maintain substrate at saturation point.

Statistical analysis. For each experiment, the effects of different treatments on the number of germinated achenes were tested using repeated measures mixed model ANOVAs with blocks considered as a random effect. Time was included as a variable in the analysis to investigate potential differences in germination speed and pattern between treatments. Since one combination (wet sand without cold stratification) was not tested during our stratification experiment, our factorial plan (cold stratification duration $\times$ storage) was incomplete. Thus, the effect of cold stratification itself was tested using contrasts between stratified and non-stratified treatments. All 12 stratification treatments could nonetheless be examined to determine their overall effect on germination rates. Additionally, a sub-analysis excluding non-stratified treatments was conducted to compare the effect of cold stratification duration and storage medium type. Tukey's post-hoc tests were used to identify differences between levels of various factors when they were significant (at $p<0.05$). Because the scarification treatment included two levels only (scarified/unscarified), a Student's T test was used as a post-hoc test. All statistical analyses were conducted using the JMP-9 software package (version 9.0.1; SAS Institute Inc., Cary, NC).

RESULTS

Stratification and scarification. Final mean germination rates ranged from 0 to 88% across stratification treatments, and differed significantly among treatments ($F_{(11,44)}$=22.93, $p<0.0001$; Figure 1). Final germination rates were significantly higher for stratified (mean 38%) than for non-stratified achenes (mean 25%; contrast test, $p=0.0021$). These rates were also significantly influenced by the duration of cold stratification ($F_{(2,\ 8)}$=13.63, $p=0.0026$) and storage medium ($F_{(2,\ 8)}$=65.36, $p<0.0001$). A significant interaction between cold stratification duration and storage medium was detected ($F_{(4,16)}$ =18.78, $p<0.0001$), however, the direction of the interaction remains uncertain (Figure 2). No effect of scarification ($F_{(1,\ 4)}$=0.10, $p=0.77$) and no interaction between cold stratification and scarification treatments on final germination rates were found ($F_{(11,44)}$=0.85, $p=0.60$).

The one-month cold stratification in wet sand resulted in the highest mean germination rate (88%), but this was not significantly different from the mean germination rates associated with five-month cold stratification in wet sand (70%) and six-month cold stratification in a plastic bag (68%; Figure 1). For all stratification durations tested, there was no statistical difference between the mean germination rates obtained by achenes stratified in a plastic or paper bag and non-stratified achenes, with the exception of achenes stratified for six months in a plastic bag. The mean germination rate of fresh achenes (46%) was significantly lower than that associated with one-month cold stratification in wet sand, but significantly higher than those associated with no stratification in a plastic bag and with five-month cold stratification in a plastic or paper bag.

The effects of stratification duration and of storage medium differed significantly over time ($F_{(6,\ 231)}$=11.28, $p<0.0001$; $F_{(6,\ 231)}$=21.68, $p<0.0001$; Figure 3). However, an interaction between stratification duration and storage medium in germination rates over time was found ($F_{(13,\ 231)}$=8.08, $p<0.0001$). Although, germination onset and final germination rates were similar between scarified and unscarified

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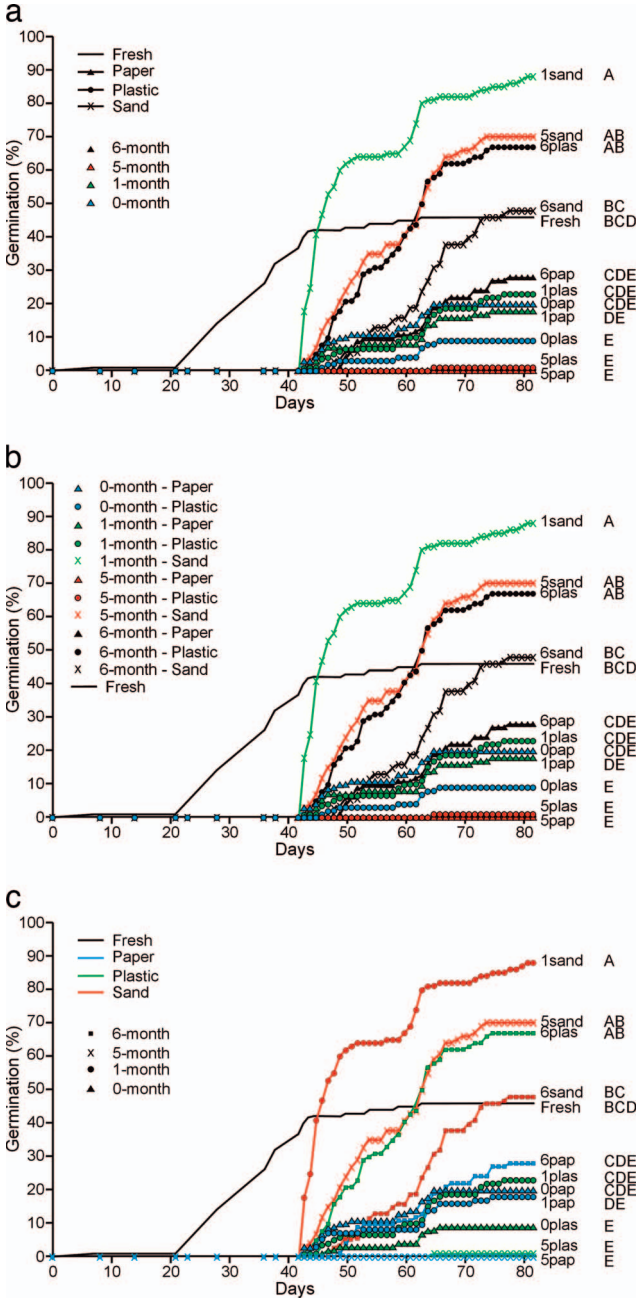
Figure 1. Mean germination rates (%) of *Carex lupuliformis* achenes over time under 12 different cold stratification treatments. Scarified and non-scarified achenes of each stratification treatment are combined. Means with different letters differ significantly at final outcome (Tukey test, $p<0.05$). See Table 1 for treatment codes. The graph is limited to 84 days.


a
Germination (%)
Days
Fresh
Paper
Plastic
Sand
6-month
5-month
1-month
0-month
1sand A
5sand AB
6plas AB
6sand BC
Fresh BCD
6pap CDE
1plas CDE
0pap CDE
1pap DE
0plas E
5plas E
5pap E
b
Germination (%)
Days
0-month - Paper
0-month - Plastic
1-month - Paper
1-month - Plastic
1-month - Sand
5-month - Paper
5-month - Plastic
5-month - Sand
6-month - Paper
6-month - Plastic
6-month - Sand
Fresh
1sand A
5sand AB
6plas AB
6sand BC
Fresh BCD
6pap CDE
1plas CDE
0pap CDE
1pap DE
0plas E
5plas E
5pap E
c
Germination (%)
Days
Fresh
Paper
Plastic
Sand
6-month
5-month
1-month
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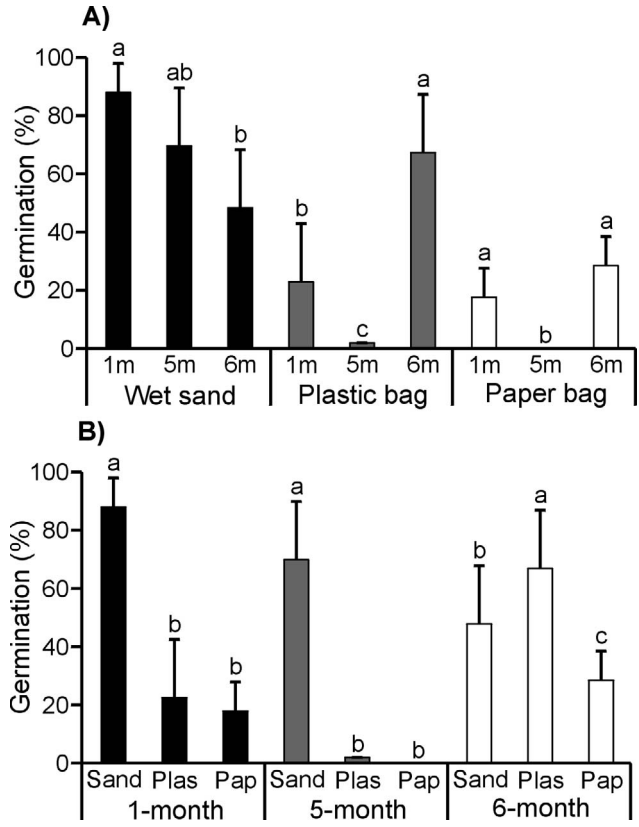

Figure 2. Mean germination rates (%) of *Carex lupuliformis* achenes at final time (A) by duration (one, five or six months) of cold stratification, in different storage media, (B) by storage medium, after different durations of cold stratification. Means with different letters (within a same comparison group; i.e., A: by storage media, B: by duration of cold stratification) differ significantly (Tukey tests, $p<0.05$)

achenes, the effect of scarification on germination rates changed significantly over time ($F_{(3, 231)}=7.13$, $p<0.0001$). Scarified samples had higher germination rates between 43–61 days after the beginning of experiments.

Light intensity. Final mean germination rates varied significantly among light treatments ($F_{(8, 32)}=34.93$, $p<0.0001$). Light intensities of 25% (i.e., an average of 38 μmol/m^2/s) resulted in significantly higher

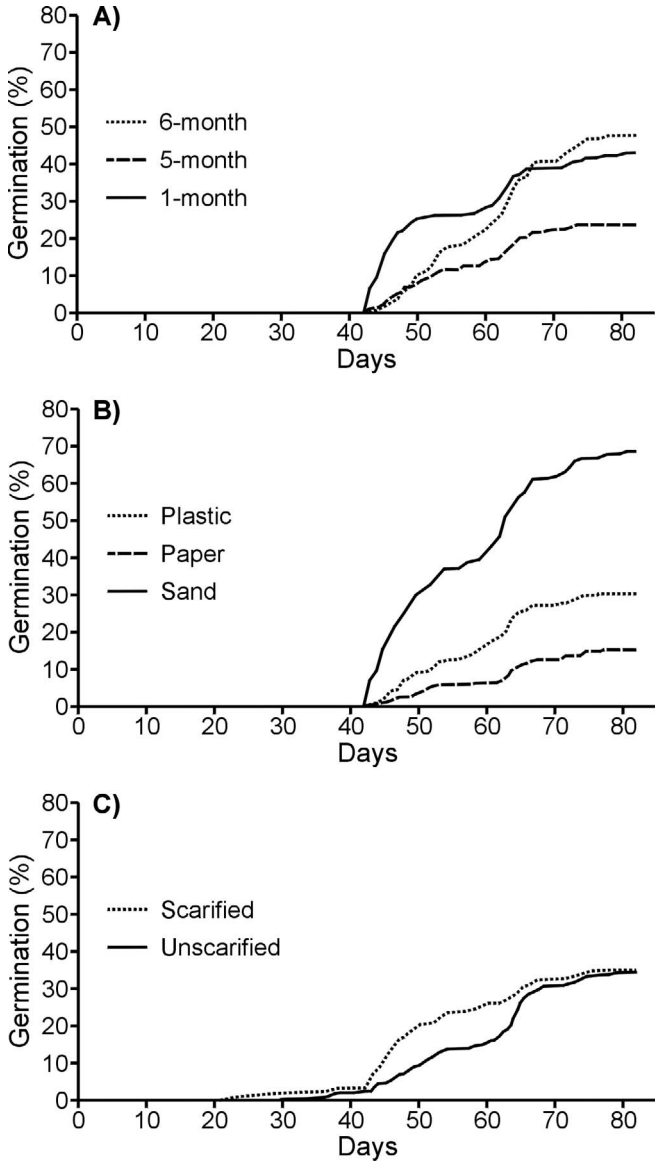

Figure 3. Mean germination rates (%) of *Carex lupuliformis* achenes over time (A) after different durations of cold stratification, (B) according to different storage media and (C) scarification treatment.

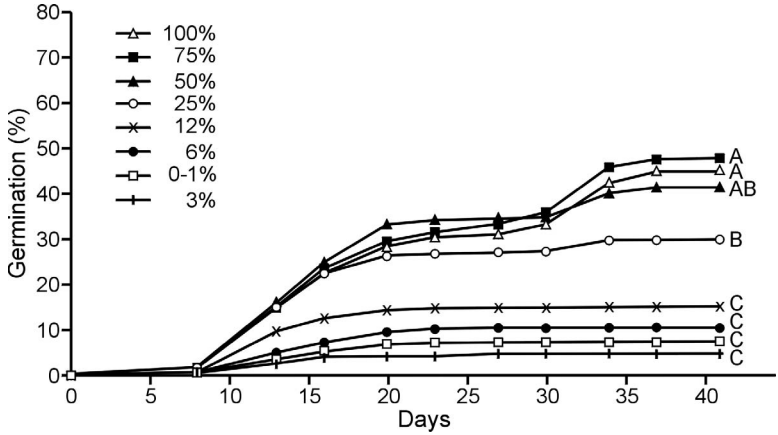

Figure 4. Mean germination rates (%) of *Carex lupuliformis* achenes over time under nine different light intensities. Means with different letters differ significantly at final outcome (Tukey test, $p<0.05$).

final germination rates than lower light intensities (Figure 4). The relationship of germination rates to light intensity differed over time ($F_{(22, 220)}=25.11$, $p<0.0001$), the threshold differentiation beginning 13 days after the experiments were initiated. Thirty days after sowing, a second germination wave was observed in samples under light intensities over 50% (75 μmol/m^2/s).

DISCUSSION

Given their limited resources, conservation agencies involved in reintroduction programs require simple and flexible, yet still reasonably effective, protocols to propagate rare plant species. Direct seeding can be an effective, cost- and time-efficient method. However, seedlings often emerge in very low numbers in natural wetlands (e.g., Kettenring and Galatowitsch 2011). For instance, several direct seeding attempts have been conducted with *Carex lupuliformis* in swamps in Québec, with no success (Langlois 2016). Plants established by direct seeding also tend to have lower survival rates than transplants (e.g., Guerrant and Kaye 2007; Jusaitis 2005).

Stratification and scarification. The best mean germination rate obtained in our semi-controlled experiment was similar to rates found in a controlled environment (75–100%; Royal Botanic Gardens 2015), indicating that it is possible to obtain satisfactory germination rates

with a simple protocol involving only a few treatments for achenes. Overall, cold-wet stratification (i.e., stratification in wet sand) seemed to increase the chances of *Carex lupuliformis* achene germination, as achenes stratified in wet sand germinated at higher rates than achenes under most other treatments. A cold-wet stratification has frequently been demonstrated to increase the germination rate of *Carex* wetland species (e.g., Budelsky and Galatowitsch 1999; Esmaeili et al. 2009; Schütz and Rave 1999). For example, Schütz and Rave (1999) found higher germination rates in 28 of 32 temperate *Carex* species after cold-wet stratification. Interestingly, achenes stratified for six months in a plastic bag (after two days of drying in a paper bag) had germination rates statistically similar to those of achenes subjected to one or five month cold-wet stratification. Being stored in a plastic bag for six months likely allowed the achenes to retain more moisture than was the case for achenes stratified by the same method for shorter durations, as the former did not spend time at room temperature in a paper bag. Consequently, to attain high germination rates, we recommend stratifying *C. lupuliformis* achenes in wet sand or in a plastic bag preceded by a very brief period of drying to avoid achene rotting.

The duration of stratification generally influences germination rate, but the optimal length of time is species dependent (e.g., Koyuncu 2005; Schütz and Milberg 1997). In our experiment, the effect of cold-wet stratification was slightly lessened when treatment was prolonged. Therefore, a short cold-wet stratification is preferable to a longer one.

Stratification and scarification often accelerate germination (Schütz and Rave 1999). Yet, we observed the opposite effect for stratification alone as achenes sown directly after collection germinated much faster than achenes under other treatments. Different environmental conditions in the greenhouse may have been a causal factor, as fresh achenes were sown in September whereas achenes in other treatments were sown in February. The final mean germination rate of fresh achenes was, however, quite low (46%). On the other hand, we found that scarified achenes germinated significantly faster than unscarified ones in the second half of the experiment, although scarification did not affect the final germination rates. Therefore, the extra scarification step does not seem worth the effort for *Carex lupuliformis*.

Light intensity. The average germination rate of *Carex lupuliformis* was greater at higher light intensities (here, over 38 $\mu mol/m^2/s$), as is generally the case for wetland *Carex* (e.g., Baskin and Baskin 2001; Schütz 2000). This finding concurs with its habitat preferences since, at least in Québec, mature individuals of the species rarely grow farther than 15 m from the river shoreline, where the canopy is sparse and

flooding intensely disturbs the soil every spring (COSEWIC 2011). For germination in species of *Carex*s, a light requirement is usually coupled with a strong response to daily temperature fluctuations (Schütz and Rave 1999). These adaptations act as recognition mechanisms for surface proximity and canopy gaps. In our experiments, different light intensities between treatments could have influenced day-time temperature and, consequently, daily temperature fluctuations. Greater daily temperature fluctuations in treatments under higher light intensities could have facilitated germination triggering. Thus, *C. lupuliformis* response to daily temperature fluctuations should be tested in further experiments.

The best germination rate obtained in the light intensity experiment (48%), for which achenes were stratified for six months in a plastic bag, was lower than the mean germination rate obtained for the same treatment in the stratification experiment (68%). This difference may be due to intraspecific variations, as the achenes used for these experiments were collected years apart, from botanical garden-grown individuals (stratification experiment) and wild or reintroduced individuals (light intensity experiment). Different environmental conditions during seed maturation could also have induced intraspecific variation (Gutterman 2000). Such differences between the germination rates obtained could also be caused by different study conditions, such as the use of Petri dishes instead of trays in the light intensity experiment. The achenes also germinated much faster in the light experiment (5–7 days) than in the stratification experiment (21–42 days). Again, a difference in environmental conditions might be a causal factor. For instance, the addition of a thin layer of substrate on achenes prior to the stratification experiment might have reduced luminosity and consequently slowed germination.

In conclusion, our experiments showed that *Carex lupuliformis* is easy to propagate *ex situ* as a variety of treatments resulted in relatively high germination rates. Using scarified and/or untreated achenes sown directly after collection would result in faster germination, whereas using intact achenes stratified for one month in wet sand would result in higher germination rates. We were also able to determine the minimum amount of light necessary for optimal germination of *C. lupuliformis*. The combined effect of light intensity and stratification treatments would be interesting to investigate in future experiments.

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