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Freeze–thaw events differently affect survival of seeds of two native and two invasive woody species

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Abstract

Current and projected changes in climatic conditions (e.g., increased freeze–thaw events) may have detrimental effects on the survival of seeds that are essential for plant regeneration in forest ecosystems. We conducted a factorial experiment manipulating thermal regime (daily freeze–thaw event vs. a constant-freeze treatment) and fungicide application to examine how freeze–thaw events affect seed survival of two native tree species (*Pinus strobus* and *Acer saccharum*) and two invasive shrub species (*Lonicera maackii* and *Rhamnus cathartica*). *Pinus strobus* seeds exhibited increased survival after freeze–thaw treatment, while *A. saccharum* survival was uniformly low and unaffected by freeze–thaw treatment. Freeze–thaw treatment significantly reduced survival of seeds of *R. cathartica* and *L. maackii*. Fungicide led to significantly higher survival only for *L. maackii*, and only when exposed to the freeze–thaw treatment. Our study highlights how recruitment of some invasive woody shrub species, but not two native tree species, may be significantly reduced by increased climatic variability.

Key words: climate change, freeze–thaw cycles, fungal pathogens, invasive shrubs, plant recruitment, seed survival

Introduction

Understanding factors that affect the survival of seeds is important because seed loss and germination failure are major causes of recruitment failure in forests (Clark et al. 1999; Ibáñez et al. 2007). Seed survival is affected by many factors, including granivory, seed pathogens, and stressful abiotic conditions (Baskin and Baskin 2014); seed dormancy plays an important role because seeds may fail to germinate (and thus spend more time susceptible to factors that kill seeds) or seeds may break dormancy at a suboptimal time, subsequently experiencing stress following germination (Baskin and Baskin 2014). One potential cause of recruitment failure may be exposure to early season freeze–thaw events or “false springs” (e.g., Connolly and Orrock 2015; Guiden et al. 2018). Freeze–thaw events are characterized as periods of unusual warming in winter or early spring, followed by a return to extreme cold. Given the significant effect of freeze–thaw events on seed germination and viability of herbaceous plants (Connolly and Orrock 2015) and projections of increased frequency and severity of freeze–thaw events (Allstadt et al. 2015; Hatami and Nazemi 2022), understanding how freeze–thaw events affect the germination and viability of seeds of woody plant species is important for understanding potential changes in plant recruitment in forest ecosystems.

Freeze–thaw events may affect seeds via at least two mechanisms: by directly damaging seed tissue and by increasing the susceptibility of seeds to pathogens, as damaged seeds may be more readily infected by pathogens (Connolly and Orrock 2015). Deciphering the role of these mechanisms is important because it can inform mitigation strategies (e.g., use of fungicide seed coatings to reduce pathogen attack, seasonal timing planting) as well as help guide predictions of which species will be more susceptible to the deleterious effects of freeze–thaw events. Although fungal pathogens may affect the survival of seeds of forest plant species (Blaney and Kotanen 2001, 2002), it is not known whether freeze–thaw events modify the susceptibility of seeds of woody plants to fungal seed pathogens.

We conducted a factorial experiment to evaluate how freeze–thaw cycles and fungal pathogens affect the survival of seeds of four woody plant species. Our experiment explicitly included a fungicide treatment to help explore the effect of fungal pathogens in affecting seed survival in the presence of freeze–thaw cycles (Connolly and Orrock 2015); the use of fungicide for exploring the role of fungal pathogens in affecting seed fate has been informative in other studies evaluating many other species (e.g., Blaney and Kotanen 2001, 2002; Orrock et al. 2012; Connolly and Orrock 2015). We focused on two widespread invasive shrub species (*Rhamnus cathartica* and *Lonicera maackii*) that have deleterious effects in Midwestern forests (Gorchov and Trisel 2003; Knight et al. 2007) and two native tree species (*Acer saccharum* and *Pinus strobus*) that are important components of the forest plant community (Rogers et al. 2022). The goals of our experiment were to determine whether freeze–thaw events affect the survival of seeds of native tree species and seeds of invasive shrubs and to determine whether fungal pathogens were a significant component of the effect of freeze–thaw events on seed survival.

Methods

Soil collection and preparation

Soil was collected from five locations within a mixed deciduous forest containing *A. saccharum*, *P. strobus*, *R. cathartica*, and *L. maackii* at the University of Wisconsin–Madison Arboretum, using methods similar to those that have been successfully used to examine freeze–thaw effects on an herbaceous plant species (Connolly and Orrock 2015). Each of the five locations was located at least 50 m apart to capture any potential variability in soil conditions and fungal pathogens. At each location, we removed leaf litter and other debris from the surface of a 1 m by 1 m plot. In late September, we collected the top 3 cm of soil in each plot (Connolly and Orrock 2015). We passed each soil sample through a 3.35 mm sieve to remove any debris and refrigerated the samples for 102 days at 8 °C. We homogenized 400 g of soil from each of the five soil locations to use in this study.

Seed collection and preparation

We obtained seeds of *P. strobus* and *A. saccharum* from the Wisconsin Department of Natural Resources. Both species were collected in Wisconsin as they ripened during the summer and fall of 2022 and kept in cold storage. We removed any dispersal structures from the seeds before placing them in the refrigerator to cold stratify. We collected *L. maackii* seeds between June 2022 and August 2022 from five urban forested areas in southern Wisconsin. At each collection site, we collected fruit from at least 10 individuals within a 30 m by 70 m area located at least 10 m from the forest edge. We collected 100 *R. cathartica* seeds from each of five individuals in the University of Wisconsin–Madison Arboretum on 28 September 2022. Following the collection of *R. cathartica* and *L. maackii* fruits, we placed fruits in a refrigerator at 8 °C until they could be cleaned. For each species and collection site, we passed the fruits through a 3.35 mm sieve and rinsed and dried the seeds. We removed any seeds that failed the apparent viability test, where seeds were squeezed with forceps to determine if they were full and structurally sound (Borza et al. 2007). We cold stratified all species for 60 days after they were collected or received. For *R. cathartica* and *L. maackii*, we randomly selected an equal number of seeds from each collection site.

Freeze–thaw treatment

After stratification, we conducted the freeze–thaw treatment. For each of the four species, we had 15 replicate samples of 7 seeds for each of the following four treatments: (1) constant freeze, fungicide addition; (2) constant freeze, no fungicide; (3) freeze–thaw treatment, no fungicide; and (4) freeze–thaw treatment, fungicide addition; this yielded a total of 60 samples per species (15 replicates × 4 treatment levels) and 240 total samples. To ensure that any effects of pathogens were due to pathogens encountered in soil and not due to pathogens already on the seed coat, we sterilized all seeds using successive washes of 70% ethanol solution for 1 min, a 7% hypochlorite solution for 1 min, and two rinses of deionized water for 1 min each. We filled sterile 15 mL plastic centrifuge tubes with 12 mL of the homogenized soil (average soil mass: 7.14 g). We added 1 mL of deionized water to the no fungicide control tubes and 1 mL of a 0.5% Captan (N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide) solution to the fungicide treatment tubes. We placed seven seeds of a single species in each tube, then vigorously inverted the tube five times to coat the seeds in soil. Before returning the prepared samples to the freezer, we randomized placement in trays of 30 tubes each, separating the freeze–thaw treatment and constant freeze groups. For six consecutive days, we placed samples in the freeze–thaw treatment group in an 8 °C refrigerator for 8 h per day, before returning them to the −10 °C freezer overnight (Fig. S1). Meanwhile, samples in the constant freeze group stayed in the freezer for the duration of the treatment period (6 days). We randomly rearranged all trays within the refrigerator and freezer each time the treatment samples were moved.

Quantifying seed survival

We quantified seed survival by conducting germination assays followed by viability tests for seeds that did not germinate. Following 6 days of the freeze–thaw treatment, we removed the seeds from the tube and rinsed them with deionized water to eliminate excess soil. For each tube, we placed a blotter paper in a 60 × 15 mm Petri dish, saturated the blotter paper with deionized water, arranged the seven seeds inside the Petri dish, and replaced the lid. We then randomly placed each of the 240 Petri dishes on one of eight trays. We randomly arranged the trays within a Percival Model E41L2C8 growth chamber with a 12 h photoperiod, and incrementally increased the temperature weekly to mimic an average spring (WISCONET, accessed: 21 November 2022) and meet the ideal germination temperatures for the four species in our study (Baskin and Baskin 2014). We set the initial temperature to 5 °C, increasing the temperature by 5 °C weekly to a maximum of 25 °C by week 5 (see Fig. S2). We conducted daily checks on all samples, rewetting the blotter paper as needed, and monitoring seeds for germination, which we defined as initial radicle emergence, as well as visual signs of fungal growth. Each day, we rotated the eight trays to different positions in the germination chamber, to ensure that all dishes experienced similar conditions. After 34 days in the growth chamber, daily seed checks were terminated and seeds that had not germinated were examined for apparent viability (Borza et al. 2007). As a final check to ensure that germination had ceased, we placed seeds back in the growth chamber at 25 °C and performed two additional checks, during which no germination occurred.

Data analysis

We conducted all analyses in R, version 4.4.1 (R Core team 2024). To examine how freeze–thaw cycles affect seed survival, we fit a generalized linear model with a binomial error distribution. The dependent variable in each model was the proportion of seeds that survived (composed of seeds

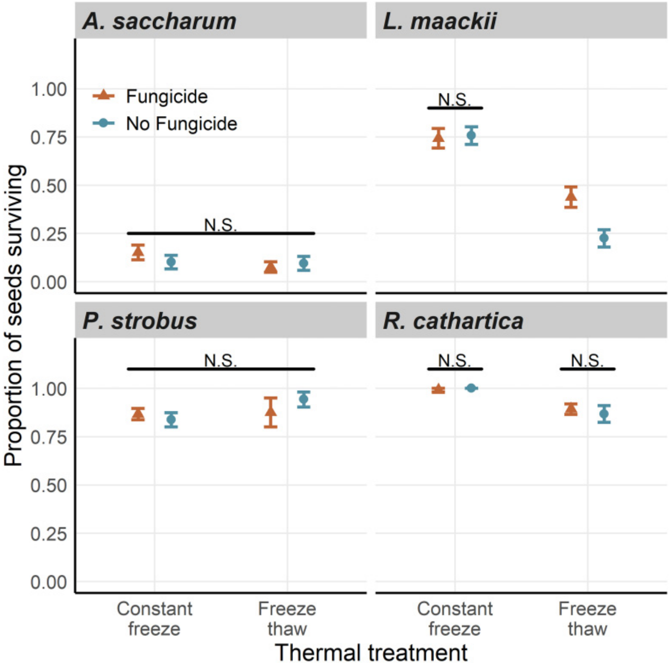
Table 1. Summary of generalized linear mixed effects model exploring the effect of freeze–thaw treatment (freeze–thaw or constant freeze), fungicide treatment (fungicide or no-fungicide control), plant species (two native tree species, *Acer saccharum* and *Pinus strobus*, and two woody introduced shrub species, *Lonicera maackii* and *Rhamnus cathartica*) and their interactions on the proportion of surviving seeds (i.e., seeds that either germinated or exhibited apparent viability at the end of the experiment).

	χ^2	df	*p* value
Freeze–thaw	9.001	1	**0.0027**
Fungicide	0.010	1	0.9205
Species	112.285	3	**<0.001**
Freeze–thaw:fungicide	0.004	1	0.9474
Freeze–thaw:species	41.375	3	**<0.001**
Fungicide:species	0.333	3	0.9538
Freeze–thaw:fungicide:species	6.712	3	*0.0817*

Note: Values of the test statistic represent Type III Wald χ^2 tests. Model terms that are significant at $\alpha < 0.1$ are italicized and terms significant at $\alpha < 0.05$ are in bold. Planned contrasts for the interaction terms are presented in Tables S1 and S2.

that either germinated or were viable). We included a random effect of tray to account for nonindependence of samples. Our model included three categorical variables (species, freeze–thaw treatment, and fungicide treatment) and their interactions. Because no *R. cathartica* seeds died in the control treatments, our data exhibit complete separation. To account for this, we used a Bayesian generalized linear mixed effects model implemented with the *blme* package (Chung et al. 2013). We used a normal (0, 3) prior and conducted prior predictive simulations to ensure that pre-data predictions spanned a range of biologically plausible outcomes. We used the *emmeans* package (Lenth et al. 2022) to generate means for the interpretation of treatment effects, as well as to conduct linear contrasts when significant interactions between freeze–thaw and fungicide were found in the main model for a species.

Fig. 1. The effect of freeze–thaw treatment and fungicide application on the total proportion of seeds that survived (either by germinating or by remaining dormant but viable) for two native tree species (*Acer saccharum* and *Pinus strobus*) and two introduced shrub species (*Lonicera maackii* and *Rhamnus cathartica*). Within a panel, a solid bar indicates means that are not significantly different (i.e., $p > 0.05$). For example, there is a significant difference between freeze–thaw and constant-freeze treatments for *P. strobus* and *R. cathartica*, but no difference due to fungicide. For *L. maackii*, there was a significant interaction between freeze–thaw and fungicide treatment: fungicide nearly doubled the survival for *L. maackii* seeds that experienced the freeze–thaw treatment but had no effect on survival of *L. maackii* seeds that experienced the constant-freeze treatment. Error bars represent ±SE.

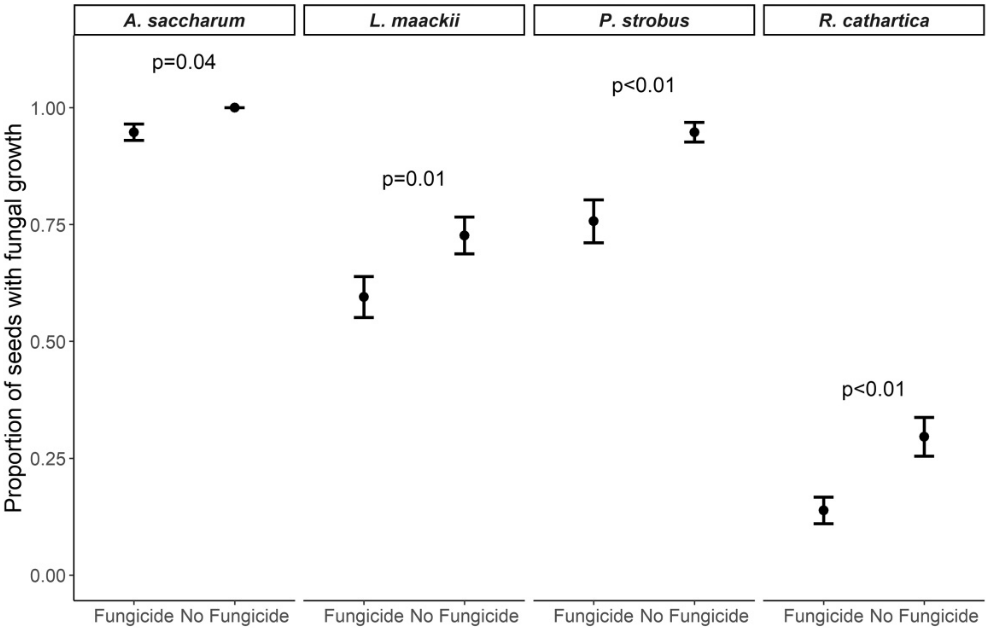


Results

We found strong evidence for species-specific effects of freeze–thaw treatment (species × freeze–thaw interaction, $p < 0.001$, Table 1), as well as evidence for species-specific effects of freeze–thaw and fungicide treatment (three-way interaction of species, freeze–thaw, and fungicide, $p = 0.081$; Table 1). In particular, the strong interaction between species and freeze–thaw treatment reflects significant differences in survival between freeze–thaw and constant-freeze treatments for *P. strobus* and *R. cathartica* (Fig. 1, Table S1). The three-way interaction was driven by *L. maackii* (Fig. 1): fungicide addition had no effect on survival of *L. maackii* seeds that experienced the constant-freeze treatment (Table S2), but fungicide nearly doubled survival for *L. maackii* that experienced the freeze–thaw treatment (Table S2). There was no interaction between fungicide treatment and species or fungicide treatment and freeze–thaw treatment (both $p > 0.94$; Table 1). There was no main effect of fungicide treatment, but there were significant main effects of species and freeze–thaw treatment (both $p < 0.003$; Table 1). Although the presence of significant higher order interactions requires consideration when interpreting main effects, the significant main effect of species reflects that, averaged across all of the other treatments and interactions, there were clear patterns of seed survival among species (Fig. 1). When averaged across all treatments, the proportion of seeds that survived was lowest for *A. saccharum* (0.10 ± 0.01 SE), followed by *L. maackii* (0.54 ± 0.03 SE). The proportion of seeds that survived was much higher for *P. strobus* (0.89 ± 0.02 SE) and *R. cathartica* (0.97 ± 0.01 SE); pairwise contrasts among species averaged across all other treatments were all significant ($p < 0.007$). Although fungicide treatment did not have significant effects on survival for three of the species we studied, fungicide treatment significantly reduced visible signs of fungal infection for all species studied (Fig. 2).

We assessed survival as the proportion of seeds that either germinated or did not germinate but were viable, and

Fig. 2. The proportion of seeds with fungal growth by fungicide treatment (averaging over the freeze–thaw treatment) for each of the four species in our study. Error bars represent ±SE. Differences in the proportion of seeds with fungal growth were significant for all four species (p value from linear contrasts following binomial GLMM): *Acer saccharum* ($p = 0.04$), *Lonicera maackii* ($p = 0.01$), *Pinus strobus* ($p < 0.01$), and *Rhamnus cathartica* ($p < 0.01$).



species also differed in the degree to which viable seeds germinated during germination trials or whether viable seeds remained dormant. The majority of the viable seeds of *A. saccharum* germinated (proportion germinating 0.59 ± 0.10), a smaller proportion of viable seeds of *R. cathartica* germinated (0.30 ± 0.03), and only a small proportion of the viable seeds of *L. maackii* (0.09 ± 0.02) and *P. strobus* (0.05 ± 0.02) germinated (i.e., over 90% of the viable *L. maackii* and *P. strobus* seeds were dormant).

Discussion

Because freeze–thaw events are predicted to become increasingly common in temperate ecosystems (Allstadt et al. 2015), understanding how these events affect seed survival is important for understanding recruitment in temperate forests. In finding that freeze–thaw events reduced survival of seeds of introduced shrub species and not seeds of native tree species (Table 1, Table S2, Fig. 1), and that the effect of fungal pathogens on an introduced shrub species (*L. maackii*) depends on freeze–thaw stress (Table 1, Table S2, Fig. 1), our results have several implications. First, our findings suggest that woody introduced and native species could experience differential recruitment limitation in future settings where freeze–thaw events are more common. Second, the effect of fungi on the survival of some species may depend on stress imposed by a freeze–thaw event. As such, our ability to predict the role of pathogens in affecting the woody plant species may depend on consideration of multiple stressors (such as freeze–thaw events) that may increase in frequency in the future.

Deleterious effects of freeze–thaw events on introduced shrubs but not native trees

Our results indicate that predicted increases in the prevalence of freeze–thaw events may have significant effects on recruitment of some, but not all, woody plant species, with potential implications for invasive shrubs in future forest communities. In particular, freeze–thaw treatments dramatically reduced survival of *L. maackii* (Fig. 1, Tables S1 and S2) and lowered survival of *R. cathartica*, even when protected from fungal attack (Fig. 1, Tables S1 and S2). The significant reduction in survival due to freeze–thaw treatments may have been due to physical damage caused by ice nucleation, physiological stress caused by shifting thermal regimes, or both. Our study also demonstrates that for some invasive species, understanding how fungal pathogens affect seed survival may depend upon the environment that seeds experience. We found evidence of an interaction between freeze–thaw treatment and fungicide for *L. maackii*; linear contrasts reveal that this interaction arises because there was no effect of fungicide on *L. maackii* in the constant-freeze treatments, but strong effects of fungicide addition on *L. maackii* survival in the freeze–thaw treatment (Fig. 1, Table S2). This finding suggests that physical seed damage caused by freeze–thaw treatment may increase the likelihood of death by fungal attack, and illustrates that our understanding of whether fungal pathogens have different effects on native versus introduced species could depend upon whether seeds experienced freeze–thaw events. Although several informative studies have explored the effect of fungal pathogens on native versus introduced seeds in some contexts (Blaney and Kotanen 2001, 2002; Orrock et al. 2012), understanding the future dynamics of native versus introduced species may benefit from studies that explore freeze–thaw events that are predicted to increasingly characterize the future climate (Allstadt et al. 2015).

For native tree species, freeze–thaw events either had no effect (*A. saccharum* mortality was consistently high regardless of treatment; Fig. 1), or freeze–thaw events had a small positive effect (*P. strobus*) on survival. The positive effect of freeze–thaw effects on *P. strobus* is consistent with the generally northern distribution of this species, as well as consistent with studies that suggest that tissues of *P. strobus* from more northern latitudes are more tolerant of damage from freezing (Mergen 1963). Interestingly, our results contrast a previous study that found little effect of freeze–thaw events on *P. strobus* seed germination and growth (Connolly et al. 2017). However, the difference between the previous study and ours may be informative: the previous study used only a single freeze–thaw event and found no effect of freeze–thaw on *P. strobus* germination, whereas our study used multiple freeze–thaw events. Taken altogether, these studies suggest that the effect of freeze–thaw events on *P. strobus* may depend upon the number of freeze–thaw events that occur in a given region, as well as ecotypic variation in freezing tolerance and seed stratification regime (Mergen 1963).

In finding a significant effect of freeze–thaw events on survival of two widespread invasive shrub species that have deleterious effects on the richness of understory plant communities and survival of woody saplings (Collier et al. 2002; Knight et al. 2007; Orrock et al. 2015; McNeish and McEwan 2016), our results suggest that increases in freeze–thaw events could help reduce invasive shrub recruitment. However, the ultimate effect freeze–thaw events on plant populations, and communities will depend upon many additional factors, including the baseline viability and survival of native seed species, the effect of other natural enemies (e.g., bacterial and viral pathogens, granivores, and herbivores), the availability of suitable microsites, and levels of total seed production. For example, although we have documented significant differences in seed survival of two introduced shrub species due to freeze–thaw exposure, the overall survival of one native tree species, *A. saccharum*, was uniformly low and much lower than other species. Stated another way, even with the deleterious effect of freeze–thaw treatments, survival of introduced shrub seeds was always greater than survival of *A. saccharum* (Fig. 1); in natural settings, this difference in seed mortality might be further compounded by low viability of native tree seeds, as *A. saccharum* viability can be less than 20% (Houle and Payette 1991). Moreover, although freeze–thaw events significantly reduced invasive shrub seed survival (and *L. maackii* in particular), both *R. cathartica* (Knight et al. 2007) and *L. maackii* (Orrock et al. 2015) are capable of prodigious seed production, potentially outweighing any reduction in individual seed survival. Deleterious effects of freeze–thaw events on seed survival may also be offset by other plant traits. For example, leaves of *L. maackii* are resistant to frost damage in early spring (McEwan et al. 2009). As a result, the ultimate effect of freeze–thaw events on the number of seedlings that recruit from seed may depend not only on the fraction of seeds that survive, but also the number of seeds produced and the longevity of reproductive adults.

Seed mortality is an important consideration for the formation of seed banks, where viable seeds in the soil germinate over successive seasons and years. Our results support previous studies suggesting that *A. saccharum* is unlikely to form long-lived seedbanks (Houle and Payette 1991), as most seeds in our study did not survive (Fig. 1) and the majority of seeds that did survive (56%) also germinated. However, over 70% of surviving *R. cathartica* seeds and over 90% of the surviving seeds of *L. maackii* and *P. strobus* in our study consisted of dormant seeds (i.e., viable, ungerminated seeds). Our results suggest that freeze–thaw events may reduce seed banks for *R. cathartica* and *L. maackii* by increasing seed mortality, and may also contribute to observations that seed banks of *R. cathartica* are quite limited (Schuster et al. 2023). Although freeze–thaw events may decrease the size of *P. strobus* seed banks by increasing *P. strobus* survival (Fig. 1), fungi have also been found to affect the viability of dormant *P. strobus* buried in outdoor settings (O'Hanlon-Manners and Kotanen 2006), and past research has also shown that *P. strobus* subjected to freeze–thaw events as seeds are more susceptible to herbivory as seedlings (Connolly et al. 2017).

Conclusions and future directions

In finding highly variable responses of seed of four woody species to freeze–thaw events, our findings highlight how recruitment of some woody species (but not others) may be significantly impacted by increased climatic variability. Moreover, our study illustrates how predicting changes in the diversity and function of future forests will require additional studies to determine the ultimate effect of increasing freeze–thaw events on native and introduced plant species. Although our study provides important data regarding the effect of freeze–thaw events on survival of native and introduced species, the inference gleaned from our study is inherently limited to the four species we examined and the methods we used (e.g., soils collected from a single geographic locality). Future studies that explore whether seed tolerance to freeze–thaw events is predictable based upon seed traits (e.g., morphology, physiology), the timing of seed collection, the areas where soils are collected, or the source population where seeds are collected will be important for further characterizing the effect of increased freeze–thaw events on plant seeds, populations, and communities (Saatkamp et al. 2019).

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Data availability

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Competing interests

The authors declare no competing interests.

Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/cjfr-2024-0276.

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