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GLOBAL CHANGE ECOLOGY - ORIGINAL RESEARCH

Climatic variation and seed persistence: freeze–thaw cycles lower survival via the joint action of abiotic stress and fungal pathogens

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Abstract Global climate change is altering thermal cycles in soils during late winter, a transition that may directly threaten seed survival via abiotic stress, facilitate infection by soil-borne pathogens, or both. Using field-collected soil and seeds of the perennial bunchgrass *Elymus canadensis*, we tested the hypothesis that soil freeze–thaw events limit survival within the soil through direct effects on seed persistence and amplification of soil pathogen attack using a factorial experiment that manipulated freeze–thaw cycles (constant freeze vs. freeze–thaw) and fungicide addition. Freeze–thaw treatment resulted in lower seedling emergence and delayed emergence time relative to constant-freeze controls. Fungicide-treated soils had greater emergence relative to untreated soils; the lowest seedling emergence was observed in no-fungicide, freeze–thaw-treated soils (<1 %). The strong effects of thermal variability and fungi on seeds were mitigated through interactions at the seed–soil interface, as subsequent experiments showed that fungicide and freeze–thaw treatments alone do not influence dormancy. Our work demonstrates that changes in freeze–thaw events directly limit seedling emergence, delay seedling phenology, and provide opportunities for fungal pathogens to limit seed persistence. As recruitment from seeds is a key determinant of plant population dynamics, these results suggest that climatic variation may generate unique consequences for populations under changing climate regimes.

Keywords Climate change · Soil pathogens · *Elymus canadensis* · Phenology · Emergence

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Introduction

Given that rates of current biodiversity loss and plausible scenarios regarding future climate emphasize increasing climatic variation (Easterling et al. 2000), it is imperative to identify the role that projected climatic variation may play in affecting plant species performance and persistence (Gu et al. 2008; Inouye 2008; Kreyling 2010; Pauli et al. 2013). Climatic variability may alter persistence by increasing abiotic stresses (e.g., Bigler et al. 2006) or by amplifying deleterious biotic interactions, such as parasitism and herbivory (Harvell et al. 2002; Tylianakis et al. 2008). Abiotic and biotic stresses may also interact, exacerbating the influence of climatic variation on plant survival and phenology. For example, in a recent meta-analysis, Jactel et al. (2012) demonstrated that trees experiencing drought stress as a result of increased variability in precipitation suffered greater damage from insects and pathogens than unstressed individuals. As a result, predicting the dynamics of survival or shifts in species phenology under future climate scenarios requires evaluating how climatic variability alters abiotic stress, biotic stress, and the interaction between the two factors.

The effect of climate change on the persistence of seeds and plant recruitment from seeds may have widespread effects on plant populations and communities (Walck et al. 2011). In particular, changes in winter and

spring conditions are a primary mechanism whereby climate change is expected to affect sustained plant recruitment in northern temperate ecosystems (Cannell and Smith 1986; Kreyling 2010; Kreyling et al. 2012a). In addition to changes in mean winter temperature and decreasing snow depth (Dyer and Mote 2006), the number of late winter or early spring soil freeze–thaw cycles that occur annually is expected to increase for many temperate and arctic regions (Henry 2008; Sinha and Cherkauer 2010).

Freeze–thaw cycles and frost events have well-documented wounding and physiological stress effects on mature plants (e.g., Mayr et al. 2003; Cleavitt et al. 2008; Inouye 2008) and may alter species phenology (Inouye 2008). Although less studied, similar abiotic stresses are likely to reduce survival, recruitment, and the timing of life history transitions at early life stages, i.e., seeds and seedlings (e.g., Regehr and Bazzaz 1979; Gu et al. 2008). Climatic variability may also affect biotic stress on seeds and seedlings by providing additional opportunities for soil pathogens, as large metabolic plasticity and rapid generation times may allow pathogens to exploit brief periods of favorable climatic conditions that are functionally inaccessible to seeds and seedlings (Harvell et al. 2002). Additionally, there is considerable potential for variation in freeze–thaw regimes to exacerbate attack by fungal pathogens. Just as direct mechanical damage resulting from ice formation or physiological stress from exposure to extreme cold events influences susceptibility to infection for mature plants (Kreyling et al. 2012b), seeds or seedlings that are physiologically taxed or mechanically damaged by multiple freeze–thaw cycles may be more susceptible to pathogen infection relative to individuals maintained in more stable thermal conditions. Despite the widespread relevance of climatic variability, the important effect of fungal pathogens on seed survival, and the potential for thermal variability to amplify the effect of fungal pathogens on seeds, we lack experimental studies that evaluate the role of thermal variability in pathogen-mediated seed mortality.

In the work reported in the present paper, we coupled an experimental freeze–thaw regime and fungicide treatment to evaluate the potentially interactive effects of climatic variation and fungal seed pathogens on the dynamics of seedling emergence. We focused on a geographic region where repeated soil freeze–thaw events are rare and where the number of annual freeze–thaw events is expected to significantly increase in the future (Sinha and Cherkauer 2010), as these may be conditions experienced by many temperate systems under future climate scenarios. Specifically, we evaluated the effects of soil freeze–thaw cycles and soil-borne pathogen attack on seedling emergence and the timing of emergence for a native perennial bunchgrass, *Elymus canadensis*. *Elymus canadensis* is distributed across temperate North America and is a common understory component in open forests. *Elymus canadensis* is well suited for our objectives because *Elymus* spp. are typical indicators of the effect of generalist soil pathogens on native seed survival (e.g., Blaney and Kotanen 2001; Beckstead et al. 2010; Meyer et al. 2014) and the persistence of native plant populations (Mordecai 2013). By examining the effect of freeze–thaw cycles on *E. canadensis* emergence and emergence timing, we expected to gain insight into how warming winter conditions may influence recruitment dynamics in forest understories. We hypothesized that *E. canadensis* seeds exposed to multiple freeze–thaw cycles would display lower total emergence and delayed emergence timing. We also hypothesized that pathogenic fungi would exacerbate emergence limitation and have the greatest effect on emergence in soils exposed to freeze–thaw cycles.

Methods

Soil was collected on 16 January 2014 at three sites within the Lakeshore Nature Preserve near University of Wisconsin—Madison (WI, USA). The collection area was open, mixed-deciduous forest with *Tilia americana* (basswood) and *Quercus rubra* (northern red oak) as prevalent tree species. Soil collection sites were selected at random and were >50 m apart to ensure soil collection captured site spatial variability. Soil collection site locations are summarized in Appendix 1 of the Electronic supplementary material (ESM). At each site, snow and leaf litter cover were removed from one 1 m × 1 m plot. Frozen mineral soil was then exhumed to a depth of 3 cm using a hand shovel, broken apart to remove large rocks and roots, homogenized at each site, and then bulked across all three sites in large paper bags. Although visibly frozen, the soil temperature was not recorded at the time of collection. Soil temperatures recorded at 5.1 cm soil depth on 16 January 2014 at the University of Wisconsin Agricultural Research Station in Arlington, WI, USA (~35 km north of field sites) averaged −0.85 °C (range −1.34 to −0.27 °C, http://agwx.soils.wisc.edu), suggesting that soils at our collection depth and in this region were frozen during this period in January 2014. To ensure that the soil did not thaw, it was stored immediately at −25 °C in a chest freezer (W. C. Wood Company, Ottawa, OH, USA). Storage of soil at −25 °C provided a thermally stable environment that limited water loss from the soil and ensured frozen soil integrity was maintained until treatments were applied.

We evaluated the interaction of freeze–thaw cycles and soil pathogen attack within the mineral soil with a 2 × 2 factorial experiment that crossed a freeze–thaw treatment (i.e., constant freeze vs. freeze–thaw treatments) and fungicide application treatment (fungicide+ vs. fungicide−).

Soil was handled in small batches (~200 mL per batch) and processed quickly in a room maintained at 16–18 °C to help ensure that soils remained cold before the treatments were applied. However, if the soils warmed sufficiently to thaw and this influenced soil structure or the composition or activity of the microbial communities, we expected the effect to be consistent across treatment levels. Each soil batch was passed through a 4-mm sieve to remove large organic material (e.g., leaves, roots, large rocks, seeds). Eighty aliquots of 35 mL of frozen soil (21.9 ± 1.2 g dry soil mass; mean ± SE, $n = 3$) were put into separate, sterile 50-mL centrifuge tubes (Fisherbrand®, Thermo Fisher Scientific Inc.). Half of the soil tubes were treated with 1 mL 0.5 % captan fungicide solution (dosage recommended by manufacturer for field application); the other half received 1 mL of the water control. Altering the soil water content towards saturation can influence plant–pathogen dynamics (Cook and Papendick 1972); soils collected for this study were at approximately 56 % of their total moisture holding capacity (B. Connolly, unpublished data; calculated following Brudvig and Damschen 2011), suggesting that the addition of 1 mL of aqueous solution would not approach or exceed the soil's saturation threshold. Captan is a non-systemic fungicide used in ecological studies to exclude major families of soil-borne, seed-decaying fungi (e.g., Blaney and Kotanen 2001), but this fungicide has little effect on endomycorrhizal fungi (Vyas 1988). Other pathogenic agents in the soil (e.g., bacteria, viruses) may also adversely influence seed survival (Agrios 1997), but we did not evaluate the effect of these microorganisms in this study.

Seeds of *Elymus canadensis* were purchased in 2012 from Agrecol Native Nursery (Evansville, WI, USA). The natural seed source for the seeds used in this experiment was in Waushara County, Wisconsin, indicating that the source *E. canadensis* population was adapted to winter climate conditions in south-central Wisconsin. Ten seeds were placed into each 50 mL tube and the tubes were gently inverted five times (~10 s per tube) to thoroughly mix frozen soil and seeds. Seeds were chilled on moist saturation blotters at 1 °C for approximately 8 h prior to use in order to mimic the naturally cold, imbibed state these seeds are likely to be in during winter. All tubes, now with seeds, were returned to −25 °C to simulate soil freezing. Centrifuge tubes assigned to freeze–thaw treatments were removed from the freezer after 15 h and placed in a refrigerator set at 1 °C for 9 h to simulate a soil thaw event. After 9 h the centrifuge tubes were then returned to −25 °C, and this cycle was repeated twice more for a total of three freeze–thaw cycles. There were 20 replicates of each fungicide-freeze treatment combination (see Appendix 2 in the ESM for a summary of the soil temperature regime).

Realistic and biologically relevant soil temperature amplitudes and rates of temperature change are important experimental considerations when conducting soil freeze–thaw studies (Henry 2007). The temperature amplitude and the rate of temperature shift selected for this experiment represent observed values for March and early April conditions for shallow soils without snow cover in mid- to upper-latitude temperate systems (Geiger 1965; Henry 2007). This temperature is also consistent with other soil freeze–thaw studies, i.e., over 30 % of reviewed studies evaluated freeze–thaw effects with similar temperature minima (−18 °C or below) and similar ranges in experimental soil temperature amplitude (Henry 2007). Applying comparable experimental conditions to these studies permits the evaluation of our results within the context of freeze–thaw effects on microbial communities and activity in field-collected soils (e.g., Sharma et al. 2006). The timing of soil collection is also an important experimental consideration for freeze–thaw studies (Henry 2007). We collected soils in mid-January to ensure that the physical composition and microbial communities accurately reflect the status of regional soils prior to the annual transition from winter into spring and the onset of natural freeze–thaw cycles. Our treatments reflect two possible spring soil temperature conditions, one in which soils remain constantly frozen until thawed during spring (i.e., constant freeze) and one similar to projected winter climate conditions in which the seed–soil interface is exposed to multiple rapid freeze–thaw cycles before staying thawed indefinitely (i.e., freeze–thaw treatment).

We focus our analysis primarily on the emergence timing and total proportion emergence of *E. canadensis*, as these are critical determinants of long-term persistence in perennial grasses (Seabloom et al. 2003). After the conclusion of freeze–thaw treatments, all centrifuge tubes were removed from −25 °C and placed in the refrigerator at 1 °C for 8 h. Greenhouse trays lined with greenhouse tray inserts (cell dimensions: 4.9 cm long by 5.7 cm wide by 5.7 cm deep) were filled three-quarters full with Sunshine Redi-Earth peat moss–vermiculite mix (Sun Gro Horticulture, Agawam, MA, USA). Potting media was homogenized prior to addition to trays in order to standardize the effect of any pathogens resident in the media. Experiments in progress, however, indicate that the addition of captan to similar Sunshine Redi-Earth media does not influence the timing of emergence or total emergence for tree species native to northern Wisconsin (B. Connolly, unpublished data), suggesting little to no contribution to seed loss by soil pathogens found in the potting soil. Each centrifuge tube of soil–seed mixture was transferred to an individual cell and covered with 0.5 cm of soil mix. Trays were watered as needed and drained freely to a collection tray to prevent cross-contamination with fungicide and to

mimic water infiltration through the soil profile. Trays were incubated (18/12 °C temperature regime, 10 h photoperiod) in a Percival plant growth chamber (model: E-41L2, Percival Scientific, Perry, IA, USA). Seedling emergence was recorded 2–4 times daily for 20 days. Seedlings were removed once recorded to eliminate competitive inhibition on ungerminated seeds. After 20 days we evaluated the viability of ungerminated seeds and characterized the physical status of seeds to inform possible effects of freeze–thaw treatment and fungi on seed condition (see Appendix 3 in the ESM for the seed viability protocol).

Germination trials using untreated seeds maintained on saturated germination blotters ($n = 20$) were conducted concurrently with seedling emergence trials within the growth chamber to estimate the germination fraction and dormancy of the test seed stock. To evaluate whether freezing treatments affected dormancy, we conducted another germination trial on *E. canadensis* seeds exposed to the two temperature regimes in the absence of soil (see Appendix 4 in the ESM).

We used generalized linear mixed-effects models with a binomial response distribution to evaluate whether freeze–thaw treatments and fungicide treatments influence the proportion of *E. canadensis* emergence and the proportion of flaccid, apparently dead *E. canadensis* seeds. Calculations of total *E. canadensis* germination included only counts of emerged seedlings and exhumed germinants, as no seeds germinated following exhumation (i.e., no exhumed seeds germinated in viability tests). Consistent with the binomial model structure, germination was analyzed as a two-vector response variable where the number of seeds that germinated and the number of seeds that did not germinate were paired for each observation. In all models, freeze–thaw treatment, fungicide application, and the interaction between these treatments were treated as fixed effects. The identity of greenhouse trays holding individual cells was treated as a random effect to accommodate random variation in growth chamber conditions. In order to account for slight overdispersion within the *E. canadensis* germination data, each cell within each tray was also treated as a random effect (Harrison 2014).

Time to emergence for all *E. canadensis* seedlings was evaluated with a linear mixed effects model to determine pairwise effects of fungicide treatment and freeze–thaw treatment on mean germination time. However, because only a single seedling in the freeze–thaw, no-fungicide treatment combination emerged in the course of our experiment, we restricted our analysis to the three treatment combinations that had replication (i.e., the linear mixed model contained one fixed factor with each of the remaining treatment combinations serving as independent levels). Tray identification and insert cell location within tray were treated as random factors. Although our time to emergence data were censored (i.e., not all seeds had emerged at the cessation of the study), high rates of germination led to normally distributed data that satisfied the assumptions for linear models (although our results also do not differ if survival analysis is used, see Appendix 5 in the ESM). All analyses were conducted in R (R Core Team 2014) using the "lme4" package for mixed effects model analysis (Bates et al. 2014), the "lsmeans" package for means comparisons (Lenth and Hervé 2014), the "car" package to construct analysis of deviance tables (Fox and Weisberg 2011), and the "survival" package for survival analysis (Therneau 2014, see Appendix 5 in the ESM). All mixed models used the Kenward–Rogers method of approximation to estimate appropriate degrees of freedom.

Results

Fungicide application to soils resulted in greater emergence relative to untreated controls ($\chi^2 = 25.71$, $df = 1$, $P < 0.001$, Fig. 1a, see Appendix 6 in the ESM), and a greater proportion of seeds germinated from constant-freeze soils than from soils exposed to freeze–thaw cycles ($\chi^2 = 116.01$, $df = 1$, $P < 0.001$). We found a significant interaction between fungicide addition and freeze–thaw treatment on the proportion of seeds that germinated ($\chi^2 = 6.622$, $df = 1$, $P = 0.010$): fungicide addition to the soil resulted in a greater increase in germination under constant-freeze [68.7 % (no fungicide) to 90.1 % (fungicide addition)] compared to freeze–thaw treatments [0.5 % (no fungicide) to 17.1 % (fungicide addition)].

The germination fraction of untreated *E. canadensis* seed stock was 91.00 ± 1.21 % under our growth-chamber conditions (Fig. 1a, dashed line), suggesting up to 1 in 10 *E. canadensis* seeds was either dead or dormant prior to the initiation of our study. However, because no seeds germinated following exhumation and few exhumed seeds displayed signs of apparent viability (e.g., <18 % of individuals in freeze–thaw treatments), we conclude that the majority of ungerminated seeds were nonviable. This conclusion is further supported by the findings of our ancillary experiments: freeze–thaw treatments in the absence of soil did not affect the proportion of seeds that germinated after 20 ($\chi^2 = 2.320$, $df = 1$, $P = 0.128$) or 31 ($\chi^2 = 0.125$, $df = 1$, $P = 0.724$; Appendix 4 in the ESM) days, or affect mean germination time ($\chi^2 = 0.673$, $df = 1$, $P = 0.412$; Appendix 4 in the ESM). The frequency of flaccid seeds differed between treatments; significantly fewer seeds were flaccid in constant-freeze treatments than freeze–thaw treatments ($\chi^2 = 8.92$, $df = 1$, $P = 0.003$) and in fungicide-addition cells relative to no-fungicide-addition cells ($\chi^2 = 8.70$, $df = 1$, $P = 0.003$), but the frequency of flaccid seeds was not

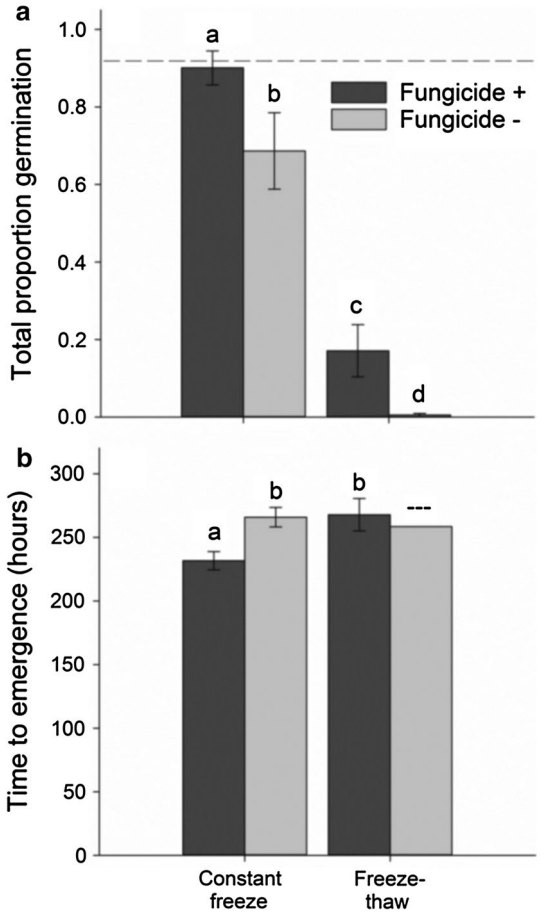

Fig. 1 Effect of freeze–thaw treatment and fungicide application on **a** total proportion of *Elymus canadensis* seeds that germinated ($n = 20$ for each freeze–thaw and fungicide combination) and **b** time to emergence of *E. canadensis* seeds sown in field-collected soil under the four treatment combinations: constant freeze, fungicide present ($n = 152$), constant freeze, no fungicide ($n = 117$), freeze–thaw, fungicide present ($n = 32$), and freeze–thaw, no fungicide ($n = 1$). *Bars* are mean values ± SE. The *dashed line* in panel **a** indicates the proportion of the untreated test seed stock that germinated. Only one individual emerged in the freeze–thaw, no-fungicide treatment combination, so this treatment combination was not included in the analysis (–). The other treatment combinations were analyzed as independent levels. *Different lowercase letters above bars* indicate significant differences at a type I error $p = 0.05$

influenced by any interaction between these factors ($\chi^2 = 0.934$, $df = 1$, $P = 0.334$).

Captan fungicide can delay the germination of some species (Mitschunas et al. 2009), but we saw no indication that captan fungicide application significantly delayed *E. canadensis* emergence relative to no-fungicide controls (Fig. 1b). Mean time to emergence was affected by the treatment level ($\chi^2 = 19.27$, $df = 2$, $P < 0.001$); time to emergence was significantly shorter in constant-freeze, fungicide-application treatments relative to either constant-freeze, no-fungicide-application ($t = 3.99$, $df = 39$, $P < 0.001$) or freeze–thaw, fungicide-application ($t = 2.84$, $df = 72$, $P = 0.016$) treatments. Average emergence times did not differ between constant-freeze controls without fungicide and freeze–thaw treatments with fungicide ($t = 0.14$, $df = 78.7$, $P = 0.989$).

Discussion

The persistence of plant populations depends upon successful survival and reproduction despite adverse winter and early spring climates brought about by global warming (Inouye 2008). Here, we demonstrate that one direct physical effect of winter warming—the increase in soil temperature variability—delays the timing of seedling emergence and lowers total seedling emergence for a common native perennial bunchgrass, *E. canadensis*. Additionally, our work suggests that variability in soil temperature may provide increased opportunities for fungal pathogens to additively limit *E. canadensis* emergence (Fig. 1). Our work suggests that, through the joint action of abiotic stress and fungal pathogens, changes in climatic variability, in addition to changes in mean climatic conditions, may be an important consideration for understanding seed persistence and plant recruitment in northern temperate plant communities.

In our study, *E. canadensis* seedling emergence was 78.9 % lower in freeze–thaw treatments relative to constant-freeze, fungicide-addition treatments, indicating that projected increases in freeze–thaw cycle frequency may directly reduce the seed germination fraction. Because *E. canadensis* is a common component of forest and prairie systems in temperate regions and *E. canadensis* recruitment may be strongly limited by seed survival (Tilman 1997), our findings suggest that the thermal variability within the soil is a strong abiotic stress that is capable of severely restricting the establishment of this widespread species in temperate plant communities. Our results also show that the timing of emergence for *E. canadensis* seedlings was affected by the freeze–thaw treatment. In fungicide-addition pots, freeze–thaw-treated *E. canadensis* seeds displayed a pronounced lag in mean time to emergence relative to seedlings in constant-freeze controls (i.e., 37 h delay, Fig. 1b). These results corroborate recent studies showing that shifts towards extremes in climate variability, particularly during winter or early spring, can alter plant phenology (Inouye 2008). Shifts in seedling germination and emergence time, even for periods as short as 24 h, can alter the order in which individual seedlings access available resources and influence biomass accumulation (Ross and Harper 1972; Verdú and Traveset 2005; Orrock and Christopher 2010) and winter-mediated shifts in phenology may directly lower individual survival, productivity,

and reproductive capacity (e.g., Inouye 2008). Collectively, our results suggest that climatic variability may have unappreciated consequences for the dynamics of recruitment from seeds in *E. canadensis* populations within temperate regions through direct effects on individual seed survival and by altering the timing of critical life-history transitions. In particular, seed dispersal and subsequent survival will be important for plant populations to track shifting climate regimes (Hampe 2011), but greater seed loss due to novel and deleterious abiotic conditions may increase the importance of other slower and more spatially restricted methods of recruitment (i.e., vegetative propagation), possibly placing severe reproductive constraints on plant populations under novel climate conditions.

Our results provide an important perspective on the potential role of climatic variability, compared to changes in mean climate, to affect pathogen-mediated plant mortality. Specifically, we found that projected increases in one expression of climatic variability (i.e., freeze–thaw cycles) work additively with soil pathogen attack to generate significant reductions in total *E. canadensis* emergence (Fig. 1a). Our finding that more flaccid, apparently dead, seeds were collected from freeze–thaw treatments than frozen controls suggests that the additive effect of freeze–thaw treatments and fungi arose because freeze–thaw events may compromise seed coat integrity (e.g., Bell and Amen 1970). Physical forces exerted on seeds by proximate soils during freeze–thaw cycles (e.g., expansion, contraction, shearing, heaving) or the nucleation of ice crystals around soil particles or within the seed may fracture or rupture the seed coat and generate avenues of infection for soil pathogens. Future experiments that explicitly evaluate these mechanisms will be critically important for understanding the mechanistic links between freeze–thaw treatments and attack by fungal pathogens.

Warming trends and increased climate variability may favor plant pathogen growth (Harvell et al. 2002), and microbial activity can increase following soil freeze–thaw regimes (Sharma et al. 2006), suggesting that there is a greater likelihood that microbes—possibly plant pathogens—will interact with seeds in the soil under the new climate conditions. Additionally, pathogenic fungi can respond quickly to nearby hosts. For example, sporangial germination of some plant pathogens found in the soil (e.g., *Pythium ultimum*) can occur rapidly (1.5–4 h), with extensive mycelial growth and infection of seeds occurring in <24 h (Stanghellini and Hancock 1971a, b). Consequently, delayed germination times caused by thermal variability (Fig. 1b) may also increase seed mortality by providing a longer period of time for rapidly growing fungal pathogens to act (Baskin and Baskin 1998; Dalling et al. 2011).

Attack by fungal pathogens in the soil can influence plant population dynamics (Kirkpatrick and Bazzaz 1979; Crist and Friese 1993) and community structure (Olff et al. 2000), and current empirical efforts to characterize the effect of climate change on plant pathogen interactions and seed persistence in natural systems rely primarily on testing average increases or decreases in one or more environmental parameters (e.g., Leishman et al. 2000). However, shifts in climatic variation are hypothesized to play a predominant role in promoting plant pathogen infections in agricultural systems (Coakley et al. 1999; Scherm 2004), and our study suggests that increases in climatic variability, in addition to average changes in climatic conditions, may also regulate plant–pathogen interactions in natural plant communities.

Implications and future directions

Entire plant populations are likely to be affected by winter climate change (Inouye 2008; Kreyling 2010). Consequently, accurate predictions of climate change effects—in particular the role of climatic variability—on plant demography require a thorough understanding of how both abiotic stressors and the severity of biotic interactions will influence plant survival, recruitment potential, and the timing of critical life-history stages. Our work may have important implications beyond the population dynamics of *E. canadensis*, since many terrestrial plant species are limited by seed recruitment (Turnbull et al. 2000), temperate plant species vary widely in their susceptibilities to fungal seed pathogens (Leishman et al. 2000; Blaney and Kotanen 2001; Beckstead et al. 2010), and germination timing influences individual performance, population persistence, and community composition (Dyer et al. 2000; Orrock and Christopher 2010). The potential for increased seed mortality and changes in germination timing we observed in *E. canadensis* may be important in the context of climate change, as successful dispersal and subsequent recruitment from seeds will be required for populations of native plants to track changing climatic conditions. Large-seeded perennial grasses (such as *E. canadensis*) are often common, influential components of plant communities. Because these species often have short-lived or transient seed banks (Thompson and Grime 1979; Baskin and Baskin 1998), seed survival and germination within the first year following dispersal is an important demographic transition in the establishment of perennial grass populations (Seabloom et al. 2003). Other pathogenic agents (e.g., bacteria, viruses) may likewise lower perennial grass fitness (e.g., Egli et al. 1975; Malmstrom et al. 2005), but it is currently unclear how these microorganisms will influence seed survival under changing climate conditions. Future work characterizing how the viability and germination timing of multiple species is affected by climatic variability in the presence and absence of different soil

pathogens (i.e., fungi, bacteria, viruses) and under field conditions will help to (1) identify how these factors will influence community structure, (2) increase the potency of land management plans (such as assisted migration) focused on mitigating the effects of global climate change by identifying key abiotic and biotic contributors to seed loss, and (3) parameterize species distribution models that incorporate both abiotic and biotic determinants of natural recruitment.

Author contribution statement JLO and BMC conceived and designed the experiments. BMC performed the experiments. JLO and BMC analyzed the data and wrote the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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ELECTRONIC SUPPLEMENTAL MATERIAL

Appendix 1. Soil collection site locations and elevations

Appendix 2. Freeze-thaw temperature treatment description and profiles

Appendix 3. Summary of seed viability assessment protocol

Appendix 4. Protocol and results regarding temperature treatment effects on *E. canadensis* germination fraction and seed dormancy in the absence of soil

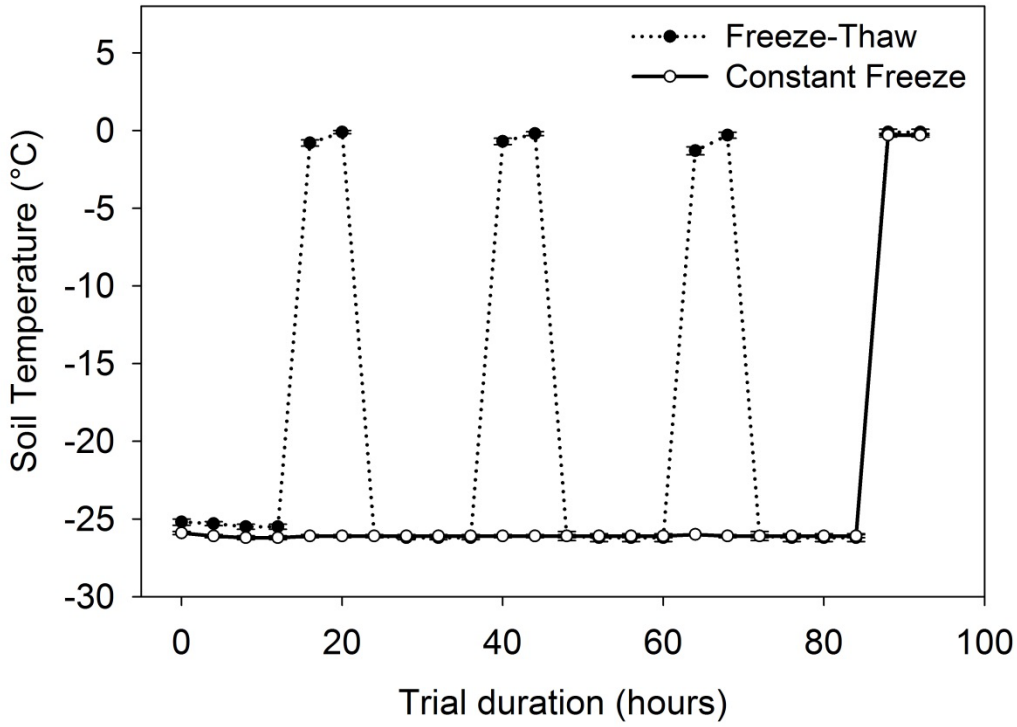
Appendix 5. Survival analysis results for *E. canadensis* emergence timing

Appendix 6. Generalized linear mixed effects model results for total proportion germination for *E. canadensis*

Appendix 1. Table summarizing soil collection site latitude, longitude, and elevation.

Site Name	Latitude	Longitude	Elevation (m)
Muir 1	43.076521	-89.403352	281
Muir 2	43.076758	-89.404136	274
Muir 3	43.076276	-89.403970	283

Appendix 2. Freeze-thaw treatment temperature description and profiles.



Appendix 2, Figure 1. Two thermal profiles for soils in freeze controls (A) and freeze-thaw treatment (B). Soil temperature regimes in freeze-thaw treatment soil and constant freeze soils were estimated using iButtons (n=5 for each temperature treatment). Control freeze soils were -26.1 ± 0.1 °C (mean ± SE); freeze-thaw treatment soils were -26.0 ± 0.2 °C (mean ± SE) in the freezer and -0.6 ± 0.3 °C (mean ± SE) under refrigeration. The rate of temperature change between freeze and thaw treatments was approximately 7 degrees Celsius per hour.

Appendix 3. Seed viability assessment protocols.

In order to test the viability of apparently dormant seeds, ungerminated seeds were recovered using a sieve after 20 days of incubation in the seedling emergence trials. Viability of collected seeds was estimated with germination assays followed by the firm test (Borza et al. 2007). Recovered seeds were surface-sterilized in 3 successive 1-minute washes in 70% ethanol, 10% hypochlorite, and sterile deionized water, respectively. Germination tests were conducted in sterile plastic petri dishes (100 x 15mm Petri dishes) on saturated germination blotters (8.57cm diameter; Anchor Paper Company, Saint Paul, Minnesota, USA) for ten days; germination dishes were maintained at ~25°C. Seeds were scored as germinated if both radicle and hypocotyl emerged > 3 mm from the caryopsis. Fungal growth occurred infrequently on seeds during germination tests, but seeds were spaced equidistant within the petri dish to minimize cross-contamination. If fungi grew rapidly from a seed (e.g., mucoraceous fungi), the seed was immediately removed from the petri dish and placed in an identical petri dish and monitored the remainder of the germination trial.

Following germination trials, each ungerminated seed was externally evaluated to check for an intact seed embryo and the endosperm was depressed with a pair of blunt-nosed forceps to check for rigid structure (i.e., the "firm" test). Rigid seed structure following imbibition can be a strong indicator of a viability (Borza et al. 2007), consequently, we classified the remaining ungerminated seeds into apparently viable seeds (i.e., those seeds with an intact embryo and firm endosperm) and apparently non-viable seeds (i.e., those seeds that either entirely or partially lacking an embryo and/or lacking a turgid endosperm). Importantly, chill injury can increase plant cell leakage (Wang 1990) which may decrease seed turgidity (Woodstock 1988); consequently, following germination trials, we recorded the frequency of flaccid apparently dead

seeds in order to estimate the contribution of freeze-thaw action on post-trial seed condition.

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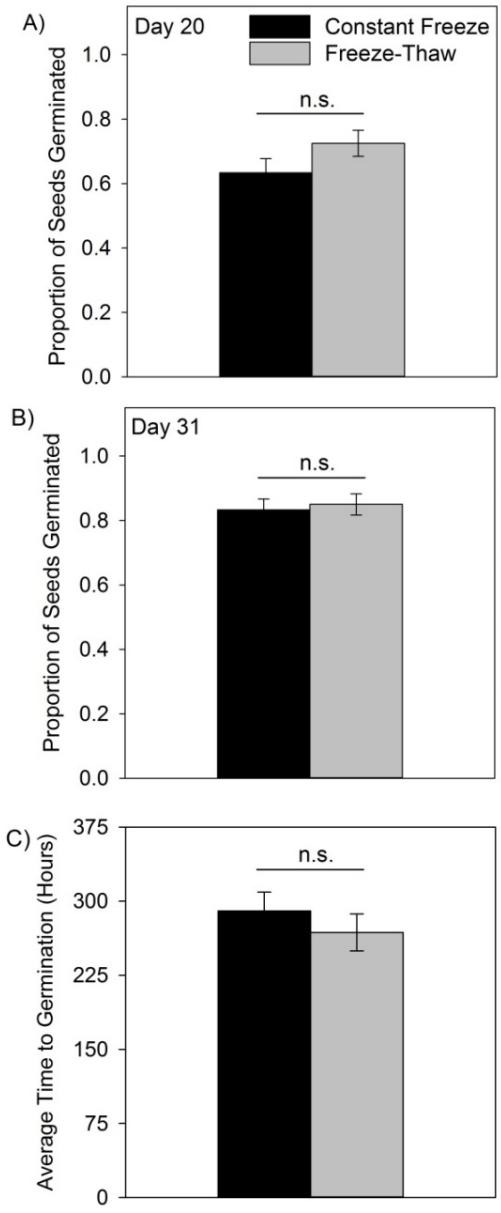
Appendix 4. Temperature treatment effects on *E. canadensis* germination fraction and seed dormancy in the absence of soil.

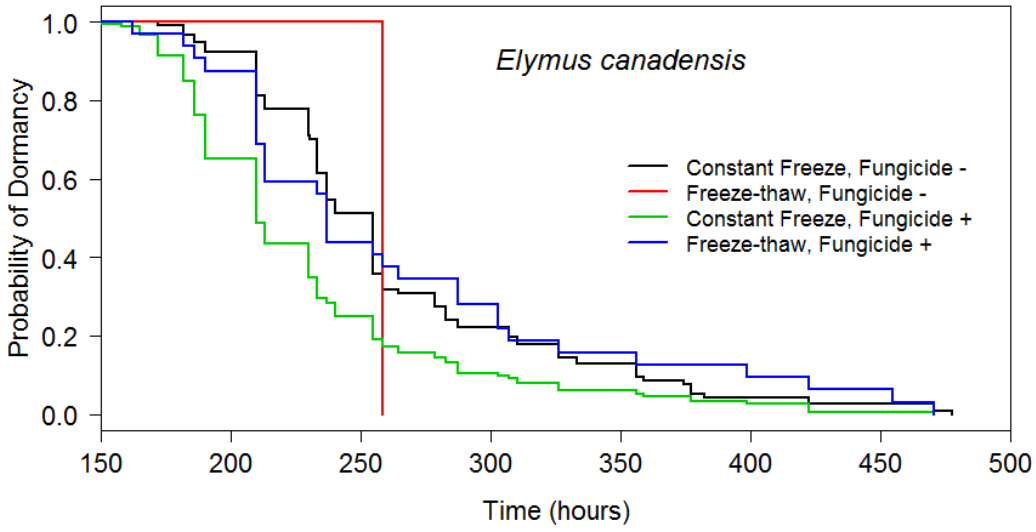
In order to evaluate the effect of temperature treatments (constant freeze vs. freeze-thaw treatments) on *E. canadensis* germination fraction and seed dormancy in the absence of soil, 10 seeds were placed in each 50 mL centrifuge tube, then exposed to the freezing treatments (n = 12 tubes per treatment), and maintained on saturated germination blotters in the growth chamber for 31 days. We analyzed the effect of freezing treatment on *E. canadensis* dormancy in the absence of soil using a generalized linear model with a binomial response distribution with 1) total proportion germination after 20 days (i.e., the duration of the seedling emergence study), 2) total proportion germination after 31 days (i.e., the duration of the seed stock germination trial), and 3) mean germination time as response variables. All analyses were conducted in R (R Development Team, 2014).



Appendix 4, Figure1. Mean proportion germination of *Elymus canadensis* seeds subjected to constant freeze and freeze-thaw treatments without field-collected soil; mean proportion germination does not differ between freezing treatment levels at Day 20 (**A**) or Day 31 (**B**). Mean time to germination (**C**) of *Elymus canadensis* seeds subjected to freeze control and freeze-thaw temperature regimes in a sterile microfuge tube without field-collected soil; mean time to germination does not differ between freezing treatment levels in the absence of soil.

Appendix 5. Survival analysis results

Survival analysis was conducted using the time to emergence data collected for *Elymus canadensis*. Time to emergence data were censored (i.e., not all seeds had germinated) and we used the Cox proportional hazard analysis in program R (R Core Team 2014; package "survival", Therneau 2014) to evaluate main effects (fungicide application, freeze-thaw treatment) and the interaction of these main effects. Fixed effects results (see below Appendix 5, Table 1) were generated with package "car" (Fox and Weisberg 2011).



Appendix 5, Figure 1. Emergence curves generated from Cox's proportional hazards analysis using fungicide application and freeze-thaw treatment as fixed effects. To present this "time-to-event" data the *y*-axis represents the probability that a seedling would remain dormant (i.e., not emerged). Each event decreases the probability that an individual in a treatment combination will remain dormant; the more rapid the decline in the curve, the more quickly seedlings germinated and emerged.

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Therneau T (2014) A Package for Survival Analysis in S. R package version 2.37-7 [URL:http://CRAN.R-project.org/package=survival]

Appendix 5, Table 1. Chi-squared analysis results for censored *Elymus canadensis* time to emergence data evaluating main effects (fungicide application, freeze-thaw treatment) and the interaction of the two main effects. Bolded *P*-values indicate significant effects at a Type I error = 0.05.

Factor	Chi-Square Value (χ^2)	D.F.	*P*-value
Fungicide (FNG)	19.20	1	**<0.001**
Freeze-thaw Treatment (FRTH)	8.95	1	**0.003**
FNG × FRTH	0.22	1	0.643

Appendix 6. Generalized linear mixed effects model results for total proportion germination for *E. canadensis* in a 2 × 2 factorial experimental crossing of fungicide application (Fungicide + vs. Fungicide -) and a cold temperature treatment (Constant freeze vs. Freeze-thaw). Bolded *P*-values indicate significant effects at a Type I error = 0.05.

Factor	Chi-Square Value (χ^2)	D.F.	*P*-value
Fungicide (FNG)	25.71	1	**<0.001**
Freeze-thaw Treatment (FRTH)	116.02	1	**<0.001**
FNG × FRTH	6.62	1	**0.010**