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NOTE

Exogenous application of methyl jasmonate alters *Pinus resinosa* seedling response to simulated frost

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Abstract: Physical stresses, such as exposure to cold, can affect plant recruitment, survival, and demography. The ability of plants to tolerate physical stress, however, may depend upon the co-occurrence of other stresses. For example, although plants undergo physiological changes to increase defense against herbivores, it is unknown whether chemical signaling associated with herbivory can alter plant tolerance to cold. We tested the hypothesis that both tissue removal and chemical induction (via exogenous application of 1 mmol/L methyl jasmonate [MeJA]) would alter cold tolerance of seedlings of *Pinus resinosa* Aiton. Application of MeJA resulted in 18% less foliar tissue damage compared with the control plants (estimated via foliar electrolyte leakage) following a simulated frost. Leaf tissue removal similarly limited the extent of cold damage on *P. resinosa* seedlings. Growth in seedlings not treated with MeJA was slower than that of seedlings treated with MeJA following exposure to the simulated frost. Our work indicates that secondary responses via MeJA induction can alter tree seedling response to cold and may (*i*) help explain natural patterns of variation in plant performance, and (*ii*) be leveraged to facilitate agricultural plant tolerance to physical stresses under increasingly variable climate conditions.

Key words: cold stress, cross-talk, electrolyte leakage, winter climate change.

Résumé : Les stress physiques comme l'exposition au froid peuvent affecter le recrutement, la survie et la démographie des végétaux. La capacité des végétaux à tolérer un stress physique peut toutefois dépendre de la présence simultanée d'autres stress. À titre d'exemple, même si les végétaux subissent des changements physiologiques afin d'accroître leur défense envers les herbivores, on ignore si une signalisation chimique associée à l'herbivorie peut modifier la tolérance des végétaux au froid. Les auteurs ont testé l'hypothèse que tant l'ablation de tissu que l'induction chimique (par l'application exogène de 1 mmol/L de jasmonate de méthyle [MeJA]) pourraient modifier la tolérance au froid de semences de *Pinus resinosa* Aiton. L'application de MeJA donnait lieu à une diminution du dommage foliaire de 18 % comparativement aux plants contrôles (estimé par la perte d'électrolytes foliaires) à la suite d'un gel simulé. L'ablation de tissu limitait de la même façon l'étendue des dommages dus au froid chez les semences de *P. resinosa*. Les semences non traitées au MeJA poussaient plus lentement que les semences traitées au MeJA à la suite d'un gel simulé. Le travail des auteurs indique que les réponses secondaires induites par le MeJA peuvent modifier la réponse des semences au froid, et pourraient (*i*) contribuer à expliquer les patrons naturels de variation de la performance végétale et (*ii*) être exploitées pour faciliter la tolérance des végétaux agricoles aux stress physiques dans des conditions climatiques de plus en plus variables. [Traduit par la Rédaction]

Mots-clés : stress par le froid, communication croisée, perte d'électrolytes, changement climatique hivernal.

Introduction

Stressful environmental conditions can determine individual plant fitness and may be particularly important in regions that experience strong environmental stresses or are projected to do so in the future (Gu et al. 2008; Augspurger 2013). However, because exposure to one stress (e.g., herbivory) can affect plant susceptibility to a different stressor (e.g., frost tolerance), understanding a plant's response to one stress will depend on the degree to which different stressors affect each other (Niinemets 2010). Cold can be a barrier to overwinter and spring plant survival, especially for juvenile trees (Schaberg et al. 2008; Drescher and Thomas 2013), and climate projections suggest increased frequency of soil frosts within many North American temperate ecosystems (e.g., Notaro et al. 2011). Given the influence of cold on juvenile tree performance and the potential for different stressors to

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shape a plant's response to cold, studies that evaluate how nonthermal stressors alter seedling's response to cold will be important for understanding future patterns of plant survival (e.g., Wayne et al. 1998).

Implementation of costly antiherbivore compounds may represent an important constraint on plant performance (Karban and Baldwin 1997). Recent work with *Arabidopsis thaliana* suggests, however, there may be "cross-talk" in plant signaling such that jasmonates — plant hormones closely associated with herbivore defense (Karban and Baldwin 1997) — can also alter cold tolerance (Hu et al. 2013, 2017). This work raises the possibility that plants might respond differently to cold if first exposed to signals associated with herbivory (e.g., consumption, volatile chemicals from neighboring plants). This question, however, has not been evaluated with study species characteristic of temperate regions where climates are expected to experience the greatest shifts in the future (Gu et al. 2008; Notaro et al. 2011; Augspurger 2013).

We investigated whether plant response to a hormone involved in herbivore damage signaling (i.e., methyl jasmonate [MeJA]) and physical tissue loss affect the response of seedlings of *Pinus resinosa* Aiton to a simulated frost. We included a chemical induction treatment (i.e., exogenous application of MeJA), in addition to actual tissue loss, for several reasons. First, we sought to understand how volatile cues of herbiviory might trigger plant defense and thus contribute to the tremendous variation in defense often observed in natural setting (Karban and Baldwin 1997). Second, including a chemical induction treatment allows us to characterize the role of physiological stress associated with induction in the absence of other potentially compounding stressors introduced by leaf tissue loss, e.g., water stress. Third, examining the role of a plant hormone associated with herbivory is important because the method could readily be applied in the field (e.g., Thaler et al. 1996) if application benefits seedling growth following natural cold stress events.

Pinus resinosa is well-suited for this study because it is widely cultivated for timber production and reforestation, often grows in open (i.e., more frost-prone) sites, has a native range in eastern North American that strongly overlaps with regions experiencing significant increases in freezing stress (Rudolf 1990), and is susceptible to cold-stress events at early ontogenic stages (Connolly et al. 2017*a*). Young *P. resinosa* are also subject to herbivory by invertebrate and vertebrate herbivores (Rudolf 1990) and induce defensive chemistry in response to defoliation (Krause and Raffa 1995). Many Pinaceae display inducible resistance (Karban and Baldwin 1997) and, although direct chemical induction via MeJA has not been previously tested in juvenile *Pinus resinosa*, exogenous application of MeJA on juvenile congeneric pines (i.e., *Pinus pinaster*) increases carbon-based chemical defenses in needles and stem resins (Sampedro et al. 2011; Moreira et al. 2012) and reduces herbivory by insects (Moreira et al. 2009). Given the role chemical induction via jasmonate pathways may play in plant response to cold and the capacity for juvenile *Pinus* spp. to respond to volatile chemical signals, we predict that exogenous application of MeJA will minimize cold damage on *P. resinosa* following exposure to a simulated frost.

Materials and methods

Pinus resinosa seeds were obtained from the Griffith State Nursery (Wisconsin Rapids, WI, USA); the seeds were sourced from populations in northwest Wisconsin and displayed robust, rapid germination (see Connolly et al. 2017*b*). Three *P. resinosa* seeds were sown into each of 144 pots (6.5 cm width × 6.5 cm length × 8.5 cm height) filled with growing medium (Sungro Metro Mix 360, Agawam, Massachusetts, USA). The growth medium was saturated with water, and then pots were placed in a Percival plant growth chamber (Model: E-41L2, Percival Scientific, Perry, Iowa, USA) set at a 15 °C day and 12 °C night temperature regime with a 12:12 h light:dark photoperiod. Germination was monitored daily; we only retained the first emerged seedling in each pot.

Our study emulates when cues of herbivory on juvenile trees are likely to co-occur with the potential for frost damage, i.e., early in the growing season; consequently, treatments were applied on recently-emerged pine seedlings and herbivory treatments and frost treatments were applied, respectively, five hours apart. We evaluated the effect of tissue removal and MeJA application on seedling cold tolerance in a 3 × 2 factorial design. First, seedlings were randomly assigned to one of three herbivory treatment groups: a control group ("control", 2% EtOH in Millipore water), 1.0 mmol/L MeJA ("chemical induction treatment", dissolved in 2% EtOH in Millipore water), and tissue removal ("induction via simulated herbivory"; 30% of needle tissue removed). Control and chemical induction treatments were applied directly to the soil surface in a ring 1 cm around each seedling's stem (modified from Huber et al. 2005; see the Supplementary data[1] for additional details). Seedlings were then randomly assigned to either experience a single moderate freeze event ("cold-stress treatment") or remain at a constant temperature regime ("temperature control"). Freezing trials were replicated in two consecutive sessions, 14 days (n = 116 seedlings) and 20 days (n = 28 seedlings) after the emergence of the final test seedling. Tissue removal treatments were only evaluated during the first freezing session.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2018-0108.

Before each freezing session we recorded pine seedling height ($Ht_{initial}$). Pots were embedded in trays filled with vermiculite to ensure top-down freezing. All of the seedlings were relocated to a Precision BOD incubator (Model 3733; ThermoFisher Scientific, Marietta, Ohio, USA) maintained at 15 °C to expose all of the seedlings to incubator conditions (Connolly et al. 2017*a*). After 1 h, the temperature control plants were returned to the growth chamber. Incubator temperatures were lowered from 15 °C to −8 °C (rate: −5.5 °C/h), were left at −8 °C for one hour, and then warmed to 12 °C (rate: 9.0 °C/h). All of the cold-treated plants were then returned to the growth chamber where the temperature control plants were located.

Sixty-eight seedlings in the first freezing session and all 28 seedlings in the second freezing session were destructively harvested to estimate post-freeze proportion electrolyte leakage (PEL) of seedling needle tissue (see the Supplementary data for the PEL methods). The first freezing session included 13 replicates of MeJA or control application for each freezing treatment, and 8 replicates of the tissue-loss treatment for each freezing treatment; the second freezing session included 7 replicates of MeJA or control application for each freezing treatment.

Forty-eight seedlings in the first freezing session (12 replicates of each MeJA–freeze treatment combination) were left intact to track post-treatment seedling survival and growth. For these 48 seedlings, survival was monitored daily. Two *P. resinosa* seedlings in both the induction treatment and induction control groups (4 seedlings total) died between two and four days after the cold-stress treatment; these individuals were excluded from further analysis. No treatment combination, however, had fewer than 10 replicates for analysis of seedling growth rate. Final seedling height (Ht_{final}) was measured 19 days after freezing. Seedlings were then harvested to measure above and belowground biomass, root length, and lateral root number. Relative seedling height growth rate (GR) was calculated as the difference between the natural logs of initial and final seedling height (i.e., $\ln[Ht_{final}] - \ln[Ht_{initial}]$) divided by the duration of growth time from cold stress treatment to final height measurement (Hunt 1978).

We used general linear models to evaluate the influence of herbivory treatment and cold stress on seedling PEL and GR. Both models included induction treatment, cold stress treatment, and the interaction of these two factors as fixed effects. The model evaluating PEL also included two additional covariates: (*i*) pre-freeze trial seedling height, and (*ii*) freezing session identity. We included seedling height as a co-variate because this metric correlates with seedling age at the time of our freezing treatment and the cold tolerance of coniferous tree seedlings may alter as they grow (e.g., Cochran and Bernsten 1973). Model selection for seedling PEL focused on the main and interactive effects of the induction and cold stress treatments and the main effects of each covariate (Supplementary data, Table S1); higher order interactions were not significant and resulted in greater model AIC. PEL values were logit-transformed prior to analysis (Warton and Hui 2011). We conducted pairwise contrasts to examine the effect of each herbivory treatment within each cold stress treatment. Seedling characteristics (root length, lateral root number, root biomass to shoot biomass ratio [hereinafter "root:shoot"]) were evaluated in independent linear models. Induction treatment, cold stress treatment, and the interaction between these two factors were fixed effects in each of these models. All of the analyses were conducted in R (R Core Team 2017).

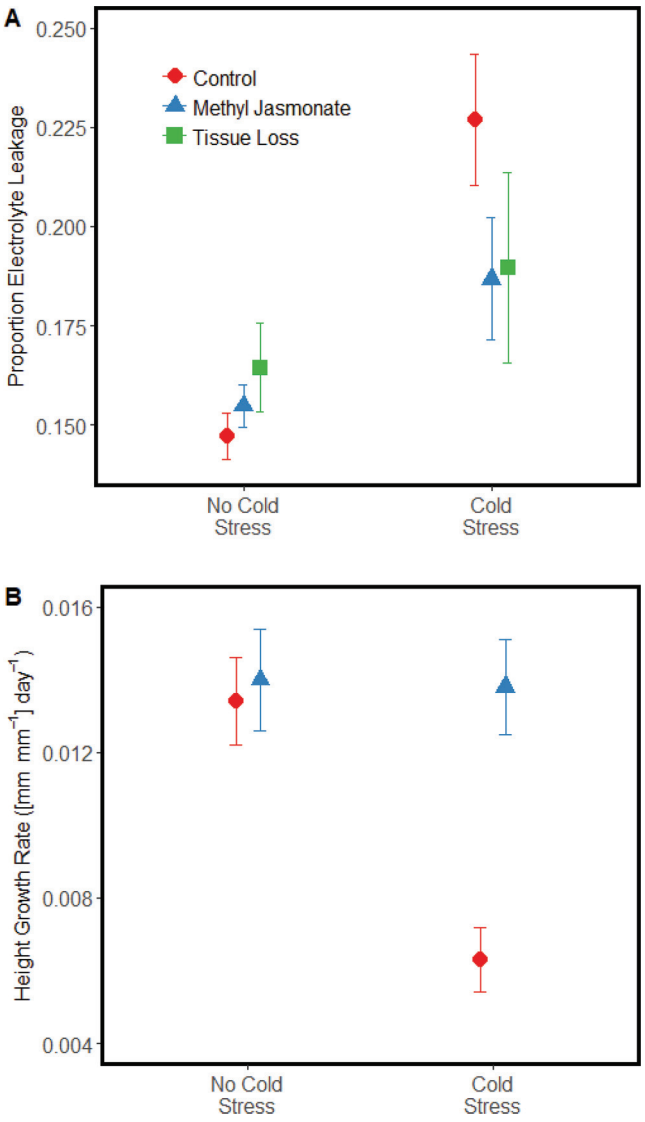
Results

Pinus resinosa seedlings exposed to simulated frost had 33% greater foliar PEL compared with the temperature control seedlings (Supplementary data, Table S1). A significant two-way interaction between temperature and induction treatments indicates the change in PEL differed between induction treatment levels (Supplementary data, Table S1 and Fig. 1A). Within the cold stress treatment, MeJA application resulted in 17.7% lower proportion electrolyte leakage compared with the control seedlings (Fig. 1A; $t = 2.50$, df = 88, $p = 0.014$). Within the cold stress treatment, tissue-loss seedlings displayed a trend of 16.4% lower PEL than control seedlings; however, difference between these two means was not statistically significant at $\alpha = 0.05$ (Fig. 1A; $t = -1.75$, df = 88, $p = 0.084$). We detected no pairwise differences between induction treatment levels in the no cold stress treatment (all *p*-values >0.289). Foliar PEL was greater in shorter *P. resinosa* seedlings (Supplementary data, Table S1 and Fig. S1).

Rate of seedling growth was slower for seedlings from the cold stress treatment, but this effect depended on the induction treatment level (Supplementary data, Table S1). Within the induced control, *P. resinosa* seedlings exposed to cold-stress grew 53.8% slower than conspecifics not exposed to cold stress (Fig. 1B; $t = -4.07$, df = 40, $p < 0.001$). The growth rate of MeJA-induced *P. resinosa* seedlings did not differ between cold-stress and the no cold-stress treatments (Fig. 1B; $t = -0.15$, df = 40, $p = 0.885$).

The roots of *P. resinosa* seedlings in the cold-stress treatment were 10.7% shorter than the roots of seedlings from the temperature control (Supplementary data, Fig. S2), but this trend of shorter roots in cold-stressed seedlings was not statistically significant ($p = 0.094$; Supplementary data, Table S2). There were approximately 1.5 fewer lateral roots (19.6% fewer) on seedlings from the cold stress treatment compared to seedlings from the temperature control (Supplementary data, Table S2 and Fig. S2). MeJA induction treatment resulted in significantly lower

Fig. 1. The effect of cold stress treatment and induction treatment (non-induced control, induced via methyl jasmonate, and induced via simulated herbivory [i.e., loss of 30% of the leaf tissue]) on (A) foliar proportion electrolyte leakage (n = 20 for each control and chemical induction treatment level for each cold stress level; n = 8 for simulated herbivory for each cold stress level) and (B) seedling height relative growth rate for recently-emerged *Pinus resinosa* seedlings (induction control and induction treatment only, n = 12 for each induction treatment level for each cold stress level). Symbols represent the mean ± SE. [Colour online.]



root:shoot than the induction control (Supplementary data, Table S2, Fig. S2); root:shoot in our MeJA induction treatment was 17.0% lower than the root:shoot for the induction control.

Discussion

Variation in temperature regimes are an inextricable component of climate change and can represent a significant barrier to plant survival and growth (Kreyling 2010; Hufkens et al. 2012; Connolly and Orrock 2015). Our ability to understand how shifts in temperature regime will influence plant populations relies on understanding how multiple different stresses interact to shape an individual plant's survival and growth. Here, we demonstrate that a plant hormone commonly associated with herbivory can alter the response of recently emerged *Pinus resinosa* seedlings to a simulated frost (Figs. 1A and 1B); actual tissue loss generated a similar trend for PEL (Fig. 1A). By linking cues commonly associated with herbivore attack to shifts in cold tolerance in recently-emerged seedlings, our work presents the possibility that the effects of herbivory, in addition to directly limiting plant survival, might also indirectly alter plant response to physical stressors.

Woody plant seedling survival is highly variable. Herbivory and overwinter mortality are primary determinants of tree seedling survival in temperate forest systems (e.g., Cleavitt et al. 2014). While an increasing number of studies demonstrate the direct effects of cold on tree seedling survival (Schaberg et al. 2008; Drescher and Thomas 2013) or show that sublethal exposure to cold events can influence the magnitude of herbivory on tree seedlings (Connolly et al. 2017*a*), this study illustrates how cues commonly associated with herbivory can increase the cold tolerance of tree seedlings. Physiological pathways described in a model plant species corroborate our results; endogenous synthesis of jasmonates is tightly linked with cold acclimation in *Arabidopsis thaliana* and exogenous application of jasmonates increases *A. thaliana* cold tolerance regardless of plant cold acclimation status (Hu et al. 2013, 2017). Our work suggests that elicitation of the jasmonate pathway by exogenous application of MeJA may mirror plant physiological response to actual tissue loss (Fig. 1A) and play a role safeguarding tree seedling performance (i.e., growth) immediately following cold stress (Fig. 1B). Cold stress on tree seedlings varies across the landscape (e.g., Cleavitt et al. 2014) and a possible implication of our results is that chemical cues associated with herbivory (e.g., volatile signals from neighboring seedlings that have been attacked, Karban and Baldwin 1997, Heil and Karban 2010) may help differentially structure tree seedling defense to physical stressors (i.e., frost events) across the landscape. Plants also induce in response to other herbivore-related cues (e.g., herbivore saliva [Ohse et al. 2017]; kairomones [Orrock et al. 2018]), suggesting other mechanisms whereby herbivores may indirectly alter plant response to abiotic extremes. These ideas, however, await further testing.

In documenting the interplay between sublethal herbivory and tolerance of cold stress in tree seedlings, our results have timely implications given projected increases in cold damage on plant populations (Kreyling 2010; Marino et al. 2011; Hufkens et al. 2012). Climate change will likely increase the frequency of untimely cold events (e.g., "false springs"; Gu et al. 2008; Marino et al. 2011), and the effect may be particularly devastating

for new growth in woody plant species (Niinemets 2010; Hufkens et al. 2012; Augspurger 2013). Our work suggests that tissue loss may be one factor controlling woody plant cold tolerance, and, consequently, herbivores may play a cryptic role shaping plant response to changing winter conditions. However, the effects of herbivory on plants under changing winter conditions are likely to be complex (Penczykowski et al. 2017), suggesting further work is needed to determine if herbivory-mediated changes in woody plant cold tolerance are influential under field conditions.

Our study may also help inform strategies for mitigating cold damage on juvenile trees under certain open nursery conditions. Production of coniferous tree seedlings is subject to limitation by frosts occurring in late summer, autumn, or spring, particularly in northern temperate forests where seedlings are often grown outdoors (Colombo et al. 2001). Seedlings in our study acquired tolerance to cold rapidly (<5 h) after exogenous application of MeJA, suggesting that, given warning of imminent frosts, nursery managers may be able to use jasmonates to safeguard conifer seedlings from frost damage. National weather advisories typically issue freezing warnings within 24 h of the estimated frost events. Timely application of MeJA may reduce sublethal frost damage to bare root stock transplants and permit faster post-transplant seedling growth, but this recommendation awaits testing under field conditions.

Our study corroborates the work of others (Huber et al. 2005; Schaberg et al. 2008) indicating that soil freezing and methyl jasmonate application can alter juvenile tree root structure and growth (Supplementary data, Fig. S2). Tree seedlings root architecture and growth are critical determinants for resource acquisition and anchorage within the soil — limitations on root architecture imposed by soil freezing or application of volatile chemical may directly influence tree seedling's ability to acquire necessary resource, weather physical forces that could uproot or displace seedlings, or form root associations with microbial mutualists (e.g., mycorrhizal fungi). It is important to note that jasmonates are involved in several important plant development process (e.g., seedling development, root growth; Wasternack and Hause 2013), and that while these plant hormones can play large role in plant-herbivore interactions, the role of jasmonates in influencing plant performance and reproduction should also be considered in experimental outcomes. Further in situ evaluation of MeJA application and soil freezing on root growth dynamics of conifer seedlings will help inform the utility of these proposed methods.

Conclusions and future directions

Signals associated with herbivory may be an important and under-considered component of plant ecology. For example, the value of volatile chemicals as a means of inducing cold tolerance may decrease as the growing season progresses and seedlings develop (Supplementary data, Fig. S1). Future studies considering how ontogeny relates to environmental information use by plants will help us understand how the importance of herbivory cues change throughout a plant's life (Orrock et al. 2015). Pairing remote sensing data measuring forest health and stress (e.g., Hufkens et al. 2012) with patterns of herbivore movement across the landscape (e.g., Fortin et al. 2005) may allow us to make large-scale evaluations of potential associations between landscape-level patterns of herbivory and stress tolerance in juvenile trees. Finally, signals associated with other biotic interactions (e.g., pathogen attack, mutualisms) may also influence the capacity of plant to tolerate subsequent abiotic limitations (Fujita et al. 2006). Understanding how biotic cues structure plant tolerance to physical stressors will improve estimates of plant growth under changing climate conditions and may inform novel means of mitigating physical stress for economically valuable species by leveraging innate plant responses to biotic cues.

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