



# Cold and heat tolerances of hybrids for restoration of the endangered *Juglans cinerea* L

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## Abstract

• **Key message** Hybrids had overall intermediate cold and heat tolerances compared to their progenitors, *Juglans cinerea* and *Juglans ailantifolia*. Differences in hybrids were small compared to *J. cinerea* though, indicating that when considering extreme temperature tolerances, hybrids might be used in some restoration circumstances. However, hybrids might exceed their cold tolerance in especially cold areas, such as *J. cinerea*'s northern range limits.

• **Context** Hybridization could incorporate traits for surviving detrimental global changes. *Juglans cinerea*, an endangered North American tree species, can hybridize with non-native *Juglans ailantifolia* Carr. Evidence indicates their hybrids could hold resistance to the fungal disease threatening *J. cinerea*. Consequently, the hybrids are being evaluated for restoration use, but to be effective, they must survive in *J. cinerea*'s distribution. An ecophysiological evaluation could contribute to predicting potential hybrid utilization areas.

• **Aims** To provide a relative comparison of the cold and heat tolerances among *J. cinerea* provenances and between *J. cinerea*, *J. ailantifolia*, and their hybrids.

• **Methods** In the cold test, twigs were subjected to five freeze treatments and resulting damage was estimated using electrolyte leakage. In the heat test, leaflets were subjected to six hot water bath treatments and damage was estimated using chlorophyll fluorescence.

• **Results** Within *J. cinerea*, trees from colder areas exhibited less cold damage than those from warmer areas. Differences in heat damage did not occur among hardiness zones. *Juglans cinerea* exhibited greatest cold tolerance, *J. ailantifolia* exhibited greatest heat tolerance, and hybrids were intermediate.

• **Conclusion** Differences in the cold and heat tolerances of *J. cinerea* and its hybrids were overall minimal, although greater in cold tolerance.

**Keywords** Chlorophyll fluorescence · Electrolyte leakage · Extreme temperature stress · *Juglans ailantifolia* · LT<sub>50</sub>

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**Contributions of the co-authors** Conceptualization: Andrea Brennan, Mercedes Uscola, Robert Joly, Douglass Jacobs; Methodology: Andrea Brennan and Mercedes Uscola; Formal analysis and investigation: Andrea Brennan and Mercedes Uscola; Writing—original draft preparation: Andrea Brennan; Writing—reviewing and editing: Andrea Brennan, Mercedes Uscola, Robert Joly, Douglass Jacobs; Funding acquisition: Andrea Brennan and Douglass Jacobs; Resources: Douglass Jacobs; Supervision: Douglass Jacobs.

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## 1 Introduction

Global change is causing the habitats of many tree species to become unsuitable for survival and reproduction (Parker and Gilbert 2004; Niu et al. 2014). This can be direct, through abiotic change, such as increased extreme climate change events, like heat, drought, and frost (Anderegg et al. 2013; Augspurger 2013; Johnson et al. 2018) or indirect, such as the arrival of non-native invasive species, including pests and diseases (Early et al. 2016). While native species might not be able to deal with these new stressors, other related species might have higher tolerance. Hybridization, or the mating of individuals of two distinct species (Allendorf et al. 2013), could incorporate desirable traits to native species survival, such as disease and pest resistance (Sniezko and

Koch 2017) or abiotic stress tolerances for a changing climate (Hamilton and Miller 2015). Consequently, hybrids are being evaluated as a possible conservation tool (Allendorf et al. 2013; Hamilton and Miller 2015; Jackiw et al. 2015). However, there are many concerns towards using hybrids, including potential invasiveness (Muhlfeld et al. 2014), genetic swamping (Allendorf et al. 2013), and outbreeding depression (Allendorf et al. 2013), which could detrimentally affect the target species and its ecosystem. To serve as an effective substitute for restoration, hybrids must survive and reproduce in the same distribution, while also being ecologically equivalent (Allendorf et al. 2013; Jackiw et al. 2015).

Temperature and water availability are important in determining species distributions (Woodward and Williams 1987). Compared to cold and drought, heat has only recently garnered strong attention through increasing relevance with climate change (Teskey et al. 2015). Even fewer studies have compared both heat and cold tolerances together (but see Burr et al. 1993; Cunningham and Read 2006; Rivero et al. 2001), which allows for delineation of the temperature spectrum that contributes to species distribution limits. Furthermore, evaluation of hybrid ecophysiology compared to their progenitors could contribute to predicting potential hybrid distributions and utilization areas. However, this aspect has not been widely studied, particularly for tree species (Himrane et al. 2004; Crystal and Jacobs 2014; Pinchot et al. 2017).

The butternut (*Juglans cinerea* L.) is a North American tree species for which hybrids are currently being considered to support conservation efforts (Brennan et al. 2020a; Pike et al. 2020). This masting tree produces exceptionally large, energy-rich nuts that are an excellent food source for both wildlife and humans (Schultz 2003), while also holding economic value through, veneer-quality wood (FPL 2010). Unfortunately, butternut canker disease, caused by the fungus *Ophiognomonia clavignenti-juglandacearum* (Nair, Kostichka, and Kuntz) Broders and Boland, has caused rapid declines in *J. cinerea* populations since its discovery in 1967 (Broders et al. 2015). Consequently, the species is now classified as endangered in Canada (Environment Canada 2010) and by the International Union for Conservation of Nature (Stritch and Barstow 2019). Newer tools to preserve *J. cinerea* and the ecological services it provides are needed.

Breeding for butternut canker disease resistance is considered an important method for *J. cinerea* conservation (Schultz 2003; Environment Canada 2010). However, similar to the case of American chestnut (*Castanea dentata* (Marsh.) Borkh.; Jacobs et al. 2013), no effective resistance has been found in *J. cinerea* to date, leading to discussion of hybrid breeding (Boraks and Broders 2014; Brennan et al. 2020a; Pike et al. 2020). While black walnut (*Juglans nigra* L.), the only other *Juglans* species co-occurring

with *J. cinerea*, is able to resist butternut canker disease, it does not hybridize with *J. cinerea* (Rink 1990). However, Japanese walnut (*Juglans ailantifolia* Carr.), an exotic species, can hybridize with *J. cinerea* and evidence suggests their hybrids are more tolerant to the disease than *J. cinerea* (Boraks and Broders 2014; Brennan et al. 2020a). Even with increased disease tolerance, the hybrids must possess closely similar ecophysiological tolerances as *J. cinerea* to be an effective replacement. While *J. cinerea* grows in areas where the minimum average temperature can reach  $-40\text{ }^{\circ}\text{C}$  (USDA plant hardiness zone 3; Rink 1990), in the coldest parts of *J. ailantifolia*'s distribution in Hokkaido, Japan (GBIF Secretariat 2019), it rarely drops below  $-20\text{ }^{\circ}\text{C}$  (Japan Meteorological Agency 2012). Thus, while *J. cinerea* and *J. ailantifolia* both experience average high summer temperatures of around  $30\text{ }^{\circ}\text{C}$  in the hottest parts of their distributions, *J. ailantifolia* lives in an overall warmer ecosystem than *J. cinerea*. Recently, Ebrahimi et al. (2020) described higher cold tolerance of *J. cinerea* relative to *J. ailantifolia*. However, we are aware of no study that has compared their heat tolerances. Given this potential disparity in tolerances, it is imperative to understand how hybrids respond to temperature extremes relative to progenitor species. This would be especially important to understand during periods of potential extreme temperature stress, i.e. in the winter, when cold tolerance is highest (Lassoie and Hinckley 1991; Haase 2011), and summer, when heat tolerance is greatest (Vierling 1991; Wahid et al. 2007). Additionally, a deeper inspection by provenance is also needed for *J. cinerea* to allow for potential range-wide variation in adaptations to be uncovered and aid in selection of properly adapted trees (Bischoff et al. 2008).

The objective of this study was to provide a relative, rather than absolute, comparison of the cold and heat tolerances within *J. cinerea* from different USDA plant hardiness zone provenances and between *J. cinerea*, *J. ailantifolia*, and their hybrids. It was hypothesized for *J. cinerea* that the lower the hardiness zone (the colder the area) of the provenance, the greater the cold tolerance, with the reverse hypothesis for heat tolerance. Between species, it was hypothesized that *J. cinerea* would be most cold tolerant, *J. ailantifolia* would be most heat tolerant, and their hybrids would be intermediate. To test these hypotheses, two experiments were conducted using samples from the same set of mature trees.

## 2 Materials and methods

### 2.1 Plant material

For both tests, we selected 35 *J. cinerea* trees, 13 *J. ailantifolia* trees, 12 F1 hybrid trees (*J. cinerea* × *J. ailantifolia*),

and 12 backcross hybrid trees ( $F1 \times J. cinerea$ ; hereon Backcross-*Jc*). Within *J. cinerea* were 5–12 trees (based on availability) from provenances corresponding to USDA plant hardiness zones 4–7 (USDA 2012). Hardiness zones 3 and 8 correspond to the coldest and warmest distribution limit of the species, respectively (Rink 1990; USDA 2012). However, only material from zones 4–7, representing the majority of the *J. cinerea* population, was able to be obtained for the study. Note that two *J. ailantifolia* trees used in the heat test died and therefore, were excluded from the cold test. All plant materials were collected from 7-year or 8-year-old seed-grown trees grown in a *Juglans* species common garden located in two nearby plots in the Hardwood Tree Improvement and Regeneration Center’s Martell Forest at Purdue University (West Lafayette, IN, USA, 40.4313991, -87.0389821). Permission to collect plant material was granted by Mark Coggeshall (USDA Forest Service; prior co-director of the Hardwood Tree Improvement and Regeneration Center). Voucher specimens of this material were not created, but specimens can be retrieved upon request to the authors. Initial morphological identification was conducted by James McKenna (USDA Forest Service) when the plots were established in 2010 and 2011. Immediately prior to running the current study in 2018, samples from all trees were genetically analyzed by Aziz Ebrahimi (Purdue University) to confirm species identity using the nuclear satellite marker methods of Hoban et al. (2008) and chloroplast markers of McClery et al. (2009).

## 2.2 Cold tolerance test

Current-year twigs were collected from each tree in late November 2018 (late fall) after leaf senescence. The average minimum temperature for the 3 weeks prior to collection was 2 °C. Under these conditions, trees were assumed to have reached the maximum cold acclimation (Lassoie and Hinckley 1991; Kalcsits et al. 2009; Haase 2011), but unlikely to have experienced frost damage that could confound our results. One to two twigs were collected from the top-west side of each tree, the most exposed crown area that avoided effects from neighbor trees. After harvesting, twigs were moved immediately to the laboratory inside plastic bags with wet paper. Samples were stored overnight at 2.3 °C in a refrigerator to allow for full hydration. Cold tests were carried out the following morning. Two different cold hardiness assays were conducted a week apart. On each date, half the trees per species were analyzed at each temperature (35 trees/temperature/date) (Brennan et al. 2020b).

The cold test was conducted following the methodology described by Haase (2011). Electrolyte leakage (EL) is a standard method used as an indicator of membrane stability for comparing cold tolerance levels of different groups of plants relative to each other, rather than their absolute

cold hardiness values (Earnshaw 1993; Gusta et al. 2009; Haase 2011). Terminal and basal ends of the twigs were discarded and twigs were then cut into 2-cm segments in order to obtain five twig segments per tree. The twig segments were each placed into 20-mL copolymer polypropylene vials (RPI Corp., Mount Prospect, IL) and filled with 13 mL of deionized water. One twig segment per tree was placed in a refrigerator at 2.3 °C as a control. Further twig segments from each tree were tested at the four target temperatures, –14, –22, –30, and –38 °C, in a programmable freezer (40–12, ScienTemp, Adrian, MI). Ultimately, 12–35 twigs/species were used for each temperature (see the “Plant material” section). Beginning at 2 °C, the temperature was decreased at 5 °C hour<sup>-1</sup>. At –2 °C, all samples were shaken for 5 s to promote ice nucleation. The temperature was held constant for 1 h once the target temperature was reached, after which time the vials designated for that target temperature were removed and the temperature continued to decrease to the next target. After removal, samples were placed in a refrigerator at 2.3 °C for 12 h and an additional 24 h at room temperature in darkness to allow for complete thaw.

After thawing, initial conductivity ( $c_i$ ) was measured to assess EL using a conductivity meter (SevenEasy, Mettler Toledo, Columbus, OH). Maximum conductivity ( $c_f$ ) was measured after samples were autoclaved (Medallion, Amsco/Steris, Washington, MO) for 30 min at 121 °C and 100 kPa above atmospheric pressure and 12 h at room temperature for complete electrolyte release. Damage was calculated with the equation:

$$EL = \frac{C_i}{C_f} \times 100(\%) \quad (1)$$

## 2.3 Heat tolerance test

Leaf samples were collected from mid-June to early July 2018. The average maximum temperature was 29 °C for the 3 weeks prior to harvesting. At this point, leaves were assumed to have reached maximum heat acclimation (Vierling 1991; Wahid et al. 2007), but unlikely to have experienced heat damage that could confound results. Leaves selected from each tree were fully mature, without visual damage, and were collected from the top-west side of the tree, the most exposed crown area and which avoided effects of neighbor trees. Each leaf had between 11 and 19 leaflets as these species have compound leaves. Leaves were measured for field maximum photochemical efficiency of PSII [ $(F_v/F_M)_{\text{field}}$ ] in 2–3 leaflets using a fluorimeter (Handy PEA, Hansatech Instruments, Norfolk, UK). Saturating light was provided as red light with a peak at 650 nm and maximum of 600 Wm<sup>-2</sup> intensity (3500 μMol m<sup>-2</sup> s<sup>-1</sup>) and duration of 1 s (Strasser et al. 2000, 2004). Immediately after

harvesting, each leaf was wrapped at the base with a moist paper towel, placed in a plastic bag and transported to the laboratory in an insulated cooler. The leaves were kept in the laboratory overnight in darkness at room temperature. Heat tests were carried out during the following two days. Five heat hardiness assays were conducted between June 12<sup>th</sup> and July 11<sup>th</sup>. In each assay, roughly half the trees per species were analyzed at two temperatures (35–37 trees/temperature), so treatments for each temperature were applied in two separate batches on separate days. Ultimately, 12–35 leaves/species/temperature were used for the highest four treatments (45–54 °C) and half as many leaflets for the lowest temperatures, 30 (as a surrogate for control of the method) and 42 °C, given the low damage and variance (Brennan et al. 2020b).

Heat treatments were applied using the hot water bath method (Marias et al. 2016). Chlorophyll fluorescence is an indicator of the maximum photochemical efficiency of PSII ( $F_V/F_M$ ) and similar to EL with cold tolerance,  $F_V/F_M$  is a standard method for comparing the relative, rather than absolute, heat tolerances of different groups (Bilger et al. 1984; Marias et al. 2016; O’Sullivan et al. 2017). Apical and bottom leaflets from each leaf were discarded and five leaflets were selected from each tree. Selected leaflets were measured for initial maximum photochemical efficiency of PSII [ $(F_V/F_M)_{pre}$ ] following 24 h of dark acclimation.  $(F_V/F_M)_{pre}$  measurements were compared to  $(F_V/F_M)_{field}$  measurements to confirm that leaflet responses to treatments could be attributed to heat stress;  $F_V/F_M$  did not vary more than  $0.013 \pm 0.005$  on average from field to pre-test conditions. Leaflets were wrapped with wet paper and aluminum foil and sealed inside a heat durable, watertight plastic bag to prevent samples from touching the hot water (Kreeb 1990). Leaflets were then immersed for 30 min in a water bath (89,032–220, VWR, Radnor, PA) at one of the target temperatures: 30 (control), 42, 45, 48, 51, or 54 °C (each tree was represented at each temperature by one leaflet sample). During each bath, the temperatures of two randomly selected leaflets were continuously monitored with a thermocouple (1312-EN-01, Professional Instruments, Hong Kong). Following the water baths, the wraps were eliminated, and leaflets were placed in 20-mL plastic vials with 3 mL of deionized water and kept at room temperature and darkness for 24 h. Maximum photochemical efficiency of PSII was measured 24 h later [ $(F_V/F_M)_{post}$ ]. Reduction in  $F_V/F_M$  was calculated with the equation:

$$F_V/F_M \text{ reduction} = (F_V/F_M)_{pre} - (F_V/F_M)_{post} \quad (2)$$

## 2.4 Statistical analysis

All data analyses were conducted in R v. 3.4.3 (R Core Team 2019). Inclusion of the treatment batch variable did

not improve model fit in the initial analyses and thus was not included in the final models. Hierarchical nonlinear regression was used to analyze the cold differences in cold tolerances between species (species model) and hardiness zone provenances (*J. cinerea* model) with R packages “nlstools” (Baty et al. 2015), “lme4” (Bates et al. 2015), and “arm” (Gelman and Hill 2007). A four-parameter logistic model (Pinheiro and Bates 2000) nested for each species was used:

$$EL = a + \frac{b - a}{1 + e^{\left(\frac{c - T}{d}\right)}} (\%) \quad (3)$$

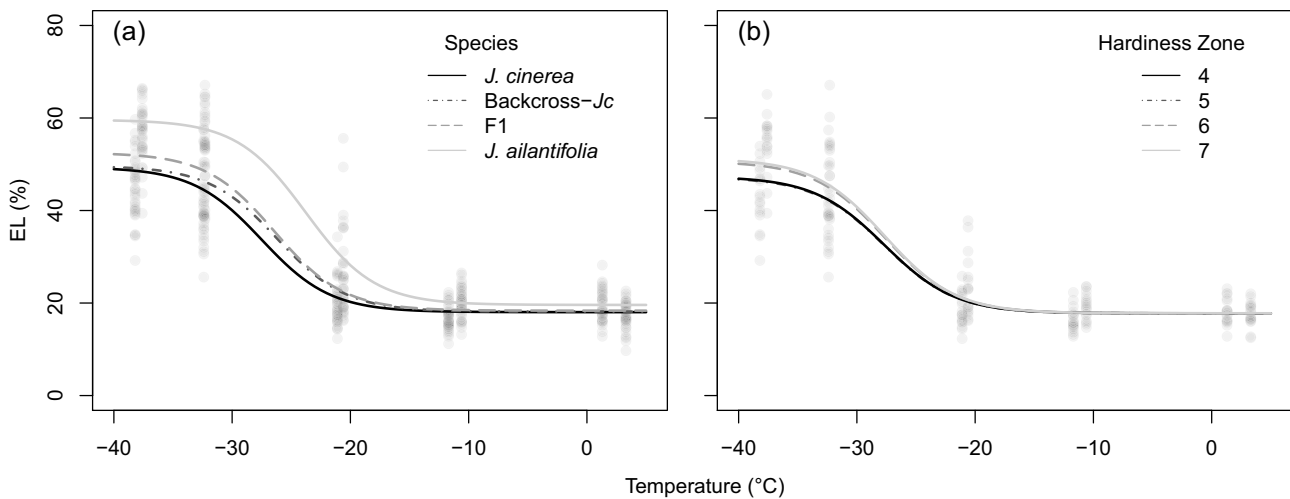
where  $a$  is the left horizontal asymptote (equivalent to maximum cold damage),  $b$  is the right horizontal asymptote (equivalent to minimum cold damage),  $c$  is the inflection point of the linear section of the curve (equivalent to the  $LT_{50}$ ),  $d$  is the inverse of the slope (equivalent to the inverse cold damage rate by temperature), and  $T$  is the temperature. Differences are indicated by the absence of overlapping SE.

The data from the heat test did not conform to a logistic curve so linear mixed models were used with individual tree nested in species (species model) or hardiness zone (*J. cinerea* model) as a random effect using R package “nlme” (Pinheiro et al. 2019). Post hoc tests using Tukey’s HSD were conducted using R package “emmeans” (Lenth et al. 2019). Significance level was established at  $\alpha = 0.05$ .  $F_V/F_M$  reduction data was logit-transformed to meet the assumption of normally distributed errors. The variance in the response variable increased as the temperature increased and so was fixed within each treatment temperature to improve model fit.

## 3 Results

### 3.1 Cold tolerance

Electrolyte leakage greater than that of the control began at  $-14$  °C (Fig. 1). After this point, the species/hybrids began to differentiate, with greatest separation occurring at  $-38$  °C, the lowest temperature tested. Among the four parameters defining the shape of the logistic function, the largest difference in cold damage among *Juglans* species was observed for parameter  $a$  (Table 1). Parameter  $a$  explained 19% of the variance of the model while the remaining parameters, taken together, explain less than 5% of the variance (0.9, 2.6, and 0% of the variance for  $b$ ,  $c$ , and  $d$  parameters respectively). Parameter  $a$ , which defines the asymptotic maximum damage reached at the lowest temperature tested, indicated that *J. cinerea* sustained the lowest maximum damage, while *J. ailantifolia* suffered the greatest. The F1 hybrids reached maximum damage at levels intermediate to those of their progenitors and were statistically different



**Fig. 1** Electrolyte leakage (EL) measured on excised twig segments of **a** *Juglans* species and their hybrids and **b** USDA plant hardiness zone provenances within *J. cinerea* following five low temperature

treatments from them. Damage sustained by the backcross-*Jc* hybrids was closer to that of *J. cinerea* than to the F1 hybrids and no differences were observed between the backcross-*Jc* hybrids and the *J. cinerea* progenitor.

Variation was also observed in the species model for parameter *c* (equivalent to the  $LT_{50}$ ). Fitted values of parameter *c* revealed that the  $LT_{50}$  occurred in the same manner as that of parameter *a* (maximum damage), with maximal values predicted for *J. ailantifolia* and minimal values for *J. cinerea*, respectively, while intermediate values were noted for both the backcross-*Jc* and F1 hybrids, which did not differ from each other. Little to no differences were seen in parameters *b* (minimum EL) or *d* (inverse rate of cold damage by temperature) between *Juglans* species. Among *J. cinerea* hardiness zones, only parameter *a* explained

sufficient variance to generate statistical differences (5.2% of variance explained for parameter *a* and less than 0.05% for the remaining parameters). Trees with provenances in hardiness zones 4 and 5 sustained lower maximum damage than those from zones 6 and 7.

treatments relative to maximum EL. Each point represents the EL (%) of an individual twig sample. Lines represent a logistic function fit for each species/zone

### 3.2 Heat tolerance

At 42 °C and below, there was little to no reduction in  $F_V/F_M$  (Fig. 2) and  $F_V/F_M$  values did not decrease below 0.729 for any species or hardiness zone group at these temperatures (Appendix, Table 2). Above 42 °C, the higher the temperature, the greater the reduction in  $F_V/F_M$  in all the species ( $\chi^2 = 2291.12, P < 0.0001$ ). However, there were no differences in  $F_V/F_M$  reduction between 45 and 48 °C. All *Juglans*

**Table 1** Estimated parameters ( $\pm$ SE) of a fitted logistic function for electrolyte leakage (EL) and temperature across *Juglans cinerea* provenances (USDA plant hardiness zones) and *Juglans* species/hybrids. Parameter *a* indicates maximum EL (%), *b* indicates minimum EL (%), *c* indicates the  $LT_{50}$  (°C), and *d* indicates the inverse rate of cold

Model	Parameter			
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
Global	52.76 $\pm$ 2.43	18.55 $\pm$ 0.78	-26.02 $\pm$ 1.04	2.91 $\pm$ 0.37
<i>Juglans cinerea</i>	49.37 $\pm$ 0.33 A	18.03 $\pm$ 0.07 A	-27.51 $\pm$ 0.12 A	2.91 $\pm$ 0.00
Hardiness zone 4	47.35 $\pm$ 0.36 a	17.78 $\pm$ 0.02	-27.68 $\pm$ 0.03	2.99 $\pm$ 0.00
Hardiness zone 5	47.20 $\pm$ 0.46 a	17.77 $\pm$ 0.02	-27.69 $\pm$ 0.04	2.99 $\pm$ 0.00
Hardiness zone 6	50.62 $\pm$ 0.29 b	17.77 $\pm$ 0.01	-27.65 $\pm$ 0.02	2.99 $\pm$ 0.00
Hardiness zone 7	51.20 $\pm$ 0.32 b	17.78 $\pm$ 0.01	-27.64 $\pm$ 0.03	2.99 $\pm$ 0.00
Hybrids	-	-	-	-
Backcross- <i>Jc</i>	49.63 $\pm$ 0.56 AB	18.14 $\pm$ 0.12 A	-26.18 $\pm$ 0.21 B	2.91 $\pm$ 0.00
F1	52.49 $\pm$ 0.56 B	18.35 $\pm$ 0.12 A	-26.40 $\pm$ 0.21 B	2.91 $\pm$ 0.00
<i>Juglans ailantifolia</i>	59.60 $\pm$ 0.59 C	19.59 $\pm$ 0.13 B	-23.78 $\pm$ 0.22 C	2.91 $\pm$ 0.00

damage by temperature. Upper case letters indicate differences among species. Lower case letters indicate differences within *J. cinerea* provenances. The global model utilized all possible data sets simultaneously to arrive at a single model that best fits all species/hybrids

species had the same response to temperature as indicated by an absence of any significant species  $\times$  temperature interaction ( $\chi^2 = 6.35$ ,  $P = 0.10$ ). Species was a significant predictor ( $\chi^2 = 15.63$ ,  $P = 0.001$ ), with *J. ailantifolia* experiencing less reduction in  $F_V/F_M$  than *J. cinerea* ( $P = 0.0002$ ) and F1 hybrids ( $P = 0.01$ ). The F1 hybrids were not different from the backcross-*Jc* hybrids ( $P = 0.78$ ) or *J. cinerea* ( $P = 0.98$ ), and the backcross-*Jc* hybrids were not statistically different from *J. cinerea* ( $P = 0.44$ ) or *J. ailantifolia* ( $P = 0.18$ ).

Likewise, in the *J. cinerea* provenance model, the same response of increasing damage to increasing temperature was observed ( $\chi^2 = 2671.02$ ,  $P < 0.0001$ ), with the exception that no differences in  $F_V/F_M$  reduction were observed between 45 and 48 °C, as in the full species model. No hardiness zone  $\times$  temperature interaction was evident either ( $\chi^2 = 2.91$ ,  $P = 0.41$ ). Additionally, hardiness zone was not a significant variable for predicting heat damage ( $\chi^2 = 3.86$ ,  $P = 0.28$ ).

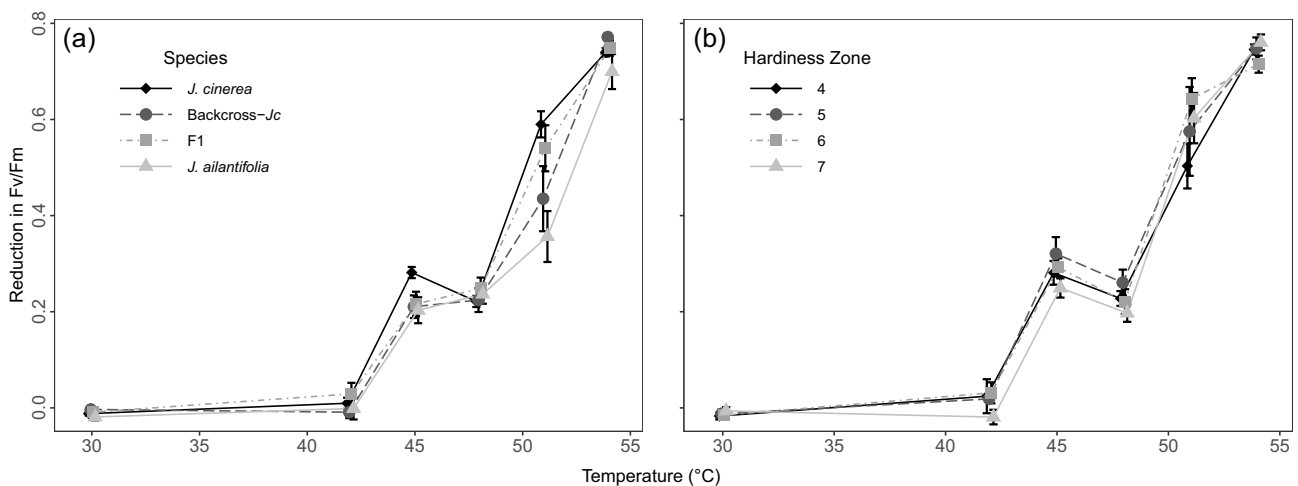
## 4 Discussion

### 4.1 Cold tolerance

Consistent with our hypotheses, provenances of *J. cinerea* from colder areas (lower hardiness zones) exhibited greater cold tolerance than those from warmer areas when assessed by EL in current-year twigs. This result was similar to that reported for Persian walnut (*Juglans regia* L.), where individuals from colder provenances sustained less damage following cold treatments (Guàrdia et al. 2013) and performed better on colder sites (Hemery et al. 2005) than individuals from warmer provenances. Similar results have also been reported in other species, such as white ash (*Fraxinus americana* L.; Alexander et al. 1984) and maritime pine (*Pinus*

*pinaster* Aiton; Corcuera et al. 2011). Differences in cold tolerance among *J. cinerea* provenances reported here were small. The only difference was in the maximum damage at the lowest temperature tested (parameter *a*), which differed by only 4.00% among provenances (Table 1). Specifically, provenance differences were between the two lowest temperature hardiness zones (4 and 5) versus the two highest temperature hardiness zones (6 and 7), rather than between each hardiness zone individually. Thus, cold tolerance as measured by EL was stronger in *J. cinerea* trees originating from colder provenances more broadly, but not necessarily at the finer scale of individual hardiness zone.

The variation in cold tolerance between *Juglans* species was greater than that seen within *J. cinerea*. The main differences were in the  $LT_{50}$  and maximum damage reached at the lowest temperature. Thus, results suggest that there were likely earlier activated and more effective cold tolerance mechanisms in the most cold-tolerant species in the study. Consistent with the hypotheses, *J. cinerea*, the species native to the coldest areas, exhibited the greatest cold tolerance while *J. ailantifolia* exhibited the least. Also, as hypothesized, hybrids exhibited cold tolerance intermediate to their parent species. Furthermore, although the difference in  $LT_{50}$  for either of the hybrid types relative to *J. cinerea* was statistically significant, the magnitude of the difference was small ( $\sim 1$  °C), while the difference with *J. ailantifolia* was higher (2–3 °C). Consistent with other studies, this suggests that cold tolerance might be a heritable and possibly dominant trait, because the response of both hybrid types was closer to that of the most cold-tolerant parent, *J. cinerea*. These results align especially closely with those reported by Ebrahimi et al. (2020) in a cold hardiness evaluation of seven *Juglans* species and their hybrids, including *J. cinerea* crossed with *J. ailantifolia* and *J. regia*. When less cold-tolerant species were crossed with more cold-tolerant species,



**Fig. 2** Reduction in  $F_V/F_M$  measured on excised leaflets of **a** *Juglans* species and their hybrids and **b** USDA plant hardiness zone provenances within *J. cinerea* following six high temperature treatments relative to pre-treatment levels. Data are means and SE

their hybrids had intermediate cold tolerance, but most often closest to the cold hardiest progenitor. The dominance of cold tolerance in hybrids has also been observed in other genera, such as *Pinus* L. (Lu et al. 2007), *Populus* L. (Deacon et al. 2019), and *Pseudotsuga* Carr. (Rehfeldt 1977).

## 4.2 Heat tolerance

The hardiness zone where material originated was not a predictor of heat tolerance as with cold tolerance. This could be because USDA plant hardiness zones are designated based on the average annual extreme minimum temperature (USDA 2012). While, more broadly, species native to colder places tend to have lower heat tolerances than those from warmer areas (Cunningham and Read 2006; O'Sullivan et al. 2017), the relationship between cold and heat tolerances seems to be more complex than simply being inverse of each other. For example, Marias et al. (2016) found no difference in thermotolerance between seedlings from different populations within ponderosa pine (*Pinus ponderosa* Lawson and C. Lawson) and *Pseudotsuga menziesii* and suggest this could be due to seedling growth and acclimation in the same environment.

While an inverse relationship between heat and cold tolerance was not seen between provenances of *J. cinerea* in this study, it was observed between *Juglans* species. In accord with the hypotheses, *J. ailantifolia* exhibited greatest heat tolerance, *J. cinerea* had the least, and the hybrids were intermediate. Several studies have also reported intrageneric heat tolerance differences in tree species, such as *Quercus* L. (Hamerlynck and Knapp 1994) and *Picea* Mill. (Zhang et al. 2018). The  $LT_{50}$  for high temperature was above 49 °C in all species included in the current study. This is a similar value to that of other temperate species, as well as species in other types of ecosystems, such as tropical (Cunningham and Read 2006) or desert (Curtis et al. 2014). Additionally, the biggest differences in heat tolerance among species were found above 48 °C, although differences were small. Both facts together suggest that high temperatures have a limited effect on species distribution.

An unexpected finding in the heat tests was the near constancy of  $F_v/F_M$  reduction between the 45 and 48 °C treatments, which was consistent across all species, provenances, and treatment batches. However, this maintenance of  $F_v/F_M$  values between 45 and 48 °C was more pronounced in *J. cinerea*. Even though this effect is not commonly described, it has also been found in other species. For instance, Weng and Lai (2005) reported that  $F_0$  values (basic fluorescence) increased in response to increasing temperature. While for some species like *Ipomea aquatica* Forssk. (water spinach), this increase was progressive, others like *Oryza sativa* L. (rice) and *Pachira aquatica* Aubl. (money tree) exhibited nearly static  $F_0$  measurements for a part of their curves,

despite rising temperatures. Although a direct explanation of this result was not found in the existing literature, it is reasonable to speculate that one or more rapidly induced physiological mechanisms may have been activated and effective from around 45–48 °C, depending on the species. For example, a study of mustard seedlings (*Sinapis alba* L.) found that rapid rises in salicylic acid by more than 400% occurred within 30 min of a 1-h long heat treatment at 45 °C (Dat et al. 1998). Salicylic acid is a plant hormone and signaling molecule linked to the production of antioxidants and heat shock proteins (among other biochemical factors) and is thus known to provide thermal protection against heat shock (Larkindale and Knight 2002; Snyman and Cronjé 2008).

## 4.3 Implications of heat and cold tolerance for restoration

Heat tolerance was not correlated with cold tolerance within *J. cinerea*. However, *J. cinerea*, the most cold-tolerant species, was also the least heat-tolerant species and the reverse pattern occurred in *J. ailantifolia*. Thus, at the interspecific level, cold and heat tolerance were negatively correlated. Furthermore, species differences in cold tolerance were larger than in heat tolerance. Although both measures provide useful information on the nature of genetic adaptation to source environment, differences in cold tolerance indicate a stronger link to the distribution of the species and provenances than did differences in heat tolerance. Additional work, particularly using additional metrics, is needed to fully explain this outcome. Electrolyte leakage has long been used to evaluate cold tolerance (Lassoie and Hinckley 1991; Earnshaw 1993; Haase 2011) and chlorophyll fluorescence has similarly been a powerful tool to assess heat tolerance (Bilger et al. 1984; Marias et al. 2016; O'Sullivan et al. 2017). However, in each case, these tests measure only one response on part of the plant and only provide a relative comparison, rather than delineating specific cold or heat tolerance levels. Consequently, these methodologies do not allow us to determine the real temperature thresholds for species, though comparative analyses of species are still highly informative for establishing a comparative framework among species. Future evaluations that utilize the whole plant and additional assays of stress indicators could provide a more realistic assessment to better inform the relationships among cold and heat tolerances and distribution.

Based on our results for *J. cinerea*, matching plant material to site might be best accomplished by considering the low, rather than high, temperatures experienced in a provenance. Differences in the temperature tolerances of hybrids were small compared to *J. cinerea*. It is acknowledged, however, that materials from the very northern and southern extremes of the range (USDA zones 3 and 8) were not included in the study. Nonetheless, the minor differences in

cold and heat tolerance between the hybrid and *J. cinerea* suggest that the hybrids might be planted successfully in a similar distribution as this progenitor. However, care should be taken when planting the hybrids in the colder, northern extremes due to the ecological importance of *J. cinerea* there. Because hybrid heat tolerance was similar to *J. cinerea*, the hybrids could likely tolerate the heat of the southern extremes of its progenitor's range. Furthermore, given the temperature rise projected under climate change for the southern portion of *J. cinerea*'s distribution (Kunkel et al. 2013), it was critical that no heat tolerance loss was observed in the hybrids.

Additionally, hybrids have shown intermediate traits compared to progenitors in other aspects. Specifically, *J. cinerea* × *J. ailantifolia* hybrids have less drought tolerance than drought-tolerant *J. cinerea* and less flood tolerance than flood-tolerant *J. ailantifolia* (Crystal and Jacobs 2014). In a vegetative and adaptive trait analysis of the same species, hybrids were found to vary widely, with only some being able to occupy the same space as *J. cinerea* (Crystal et al. 2016). Taken together, the results of ecological studies indicate that the hybrids may not be able to fully fill the niche of their *J. cinerea* progenitor. Thus, it is ultimately recommended that if these hybrids are to be used for restoring *J. cinerea*, hybrid families should be screened for their ecophysiological tolerances and matched to the climate of the target planting areas, particularly for the northern extremes. Hybrid ecophysiology is one of the many aspects that need to be considered if they are to be used to restore a threatened species (Allendorf et al. 2013; Jackiw et al. 2015). A full recommendation on hybrid use for restoring *J. cinerea* cannot be made without also evaluating other characteristics, both ecological and economical, such as reproductive potential, invasiveness, and wood quality (Allendorf et al. 2013; Woodcock et al. 2017).

## 5 Conclusion

Although *J. cinerea* cold tolerance varied by provenance, differences were relatively small, suggesting that provenance be considered at broader geographical scales for the species. However, no differences were detectable in heat tolerance of *J. cinerea* from different provenances. Thus, at the intraspecific level, heat and cold tolerance did not correlate. Differences in tolerances were both larger in magnitude and negatively correlated at the interspecific level. *Juglans cinerea* was most cold tolerant, *J. ailantifolia* was most heat tolerant, and their hybrids were intermediate. In general, differences in cold tolerance were larger than for heat tolerance, indicating that low temperatures were better able to explain species distribution patterns. The hybrids were closer to the most cold-tolerant

progenitor (*J. cinerea*). Interestingly, common to all species, provenances, and treatment batches evaluated, results suggest the activation of heat tolerance mechanisms above 45 °C. Overall, differences in cold and heat tolerances of the hybrids were small compared to *J. cinerea*, indicating that when considering extreme temperature tolerances, hybrids could be used in some circumstances for restoration. However, hybrids might exceed their cold tolerance in areas with especially low temperatures, such as in the northern limits of *J. cinerea*'s range.

## Appendix

**Table 2**  $F_V/F_M$  and standard error (SE) measured on excised leaflets across *Juglans cinerea* provenances (USDA plant hardiness zones) and *Juglans* species/hybrids following control temperature (°C) water baths

Species	Temperature	$F_V/F_M$	SE
<i>Juglans cinerea</i>	30	0.800	0.001
Hardiness zone 4	30	0.800	0.002
Hardiness zone 5	30	0.797	0.004
Hardiness zone 6	30	0.803	0.002
Hardiness zone 7	30	0.800	0.000
<i>Juglans cinerea</i>	42	0.745	0.006
Hardiness zone 4	42	0.731	0.021
Hardiness zone 5	42	0.754	0.012
Hardiness zone 6	42	0.751	0.005
Hardiness zone 7	42	0.738	0.008
Backcross- <i>Jc</i> hybrids	30	0.799	0.004
Backcross- <i>Jc</i> hybrids	42	0.758	0.006
F1 hybrids	30	0.801	0.003
F1 hybrids	42	0.729	0.016
<i>Juglans ailantifolia</i>	30	0.801	0.002
<i>Juglans ailantifolia</i>	42	0.746	0.016

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**Data availability** The datasets generated and analyzed during the current study are available from the Purdue University Research Repository: <https://doi.org/10.4231/9SSA-3890>.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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