Standardization of electrolyte leakage data and a novel liquid nitrogen control improve measurements of cold hardiness in woody tissue

Alisson Pacheco Kovaleski (al.kovaleski@wisc.edu)
University of Wisconsin-Madison https://orcid.org/0000-0002-3287-4963
Jake J Grossman
Swarthmore College

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Article Title

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Authors (*authors contributed equally)

*Alisson P. Kovaleski

Arnold Arboretum of Harvard University, 1300 Centre St., Boston, MA 02131, USA

Department of Horticulture, University of Wisconsin-Madison, 1575 Linden Drive, Madison, WI 53706, USA

Corresponding author: al.kovaleski@wisc.edu

*Jake J. Grossman

Arnold Arboretum of Harvard University, 1300 Centre St., Boston, MA 02131, USA

Biology Department, Swarthmore College, 500 College Ave., Swarthmore, PA 19081, USA

Author Contributions

A.P.K. and J.J.G. jointly and equally conceived of the original project idea, designed the experiment, performed all field and laboratory work, and analyzed resultant data. Both authors contributed to all drafts of the manuscript. Both authors agree to be responsible for contact and ensure communication.

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Abstract

Background. A variety of basic and applied research programs in plant biology require the accurate and reliable determination of plant tissue cold hardiness. Over the past 50 years, the electrolyte leakage method has emerged as a popular and practical method for quantifying the amount of damage inflicted on plant tissue by exposure to freezing temperatures. Numerous approaches for carrying out this method and analyzing the resultant data have emerged. These include multiple systems for standardizing and modeling raw electrolyte leakage data and multiple protocols for boiling samples in order to maximize leakage as a positive control. We compare four different routines for standardization of leakage data and assess a novel control method - immersion in liquid nitrogen in lieu of traditional boiling – and apply them to woody twigs collected from 12 maple (*Acer*) species in early spring. We compare leakage data from these samples using each of four previously published forms of data analysis and boiling vs. liquid

nitrogen controls and validate each of these approaches against visual estimates of freezing damage and differential thermal analysis.

Results. Through presentation of our own data and re-analysis of previously published findings, we show that standardization of raw data against estimates of both minimum and maximum attainable freezing damage allows for reliable estimation of cold hardiness at the species level and across studies in diverse systems. Furthermore, use of our novel liquid nitrogen control produces data commensurate across studies and enhances the consistency and realism of the electrolyte leakage method, especially for very cold hardy samples.

Conclusion. Future leakage studies that relativize data against minimum and maximum leakage and that employ our updated liquid nitrogen control will contribute generalizable, repeatable, and realistic data to the existing body of cold hardiness research in woody plants. Data from studies conducted using a liquid nitrogen (and not a boiling) control can still be compared to previously published data, especially when raw data are standardized using the best-performing approach among those we assessed. Electrolyte leakage of woody twigs emerges as a useful technique for quickly assessing the probability of tissue death in response to freezing in dormant plants. Differential thermal analysis may provide different and complementary information on cold hardiness.

Keywords

Acer, cold hardiness, differential thermal analysis, electrolyte leakage, freezing tolerance, maple

Background

How do plants differ in their cold hardiness, the ability to avoid or tolerate exposure to temperatures below freezing [1]? The capacity to withstand freezing temperatures structures global plant distribution, determining which lineages can radiate into high-latitude or -altitude environments [2,3]. Within these environments, plants that can withstand not just freezing, but extreme cold, are able to realize a larger niche than those more vulnerable to frost or freezing [4,5]. In short, cold hardiness shapes the ecological

dynamics and evolutionary trajectory of temperate plant evolution. Across research domains, researchers require tools for evaluating cold hardiness that can be efficiently repeated across diverse plant lineages while providing interpretable, mechanistic evaluations of cold hardiness traits.

In the years since 1932 when Dexter and colleagues' [6] observation that freezing temperatures destabilize the cellular membrane and accelerate leakage of symplastic solutes out of the cell, quantification of *electrolyte leakage* has emerged as a tool for quantifying cold hardiness. Flint et al. [7] subsequently developed the Index of Injury (*I*) approach, which is calculated through comparison of samples incubated in pure water after being frozen. Values of *I* close to 0 indicate very little disruption of cellular stability and consequently little leakage while higher values indicate greater disruption and more leakage. Over the past 50 years, the measurement of electrolyte leakage from stem and leaf tissues of woody plants adapted to cold climates has remained popular (reviewed in the Introduction of [8]; see [9-11] for more recent examples). Despite this, there exists considerable diversity in the benchtop and analytical approaches employed in electrolyte leakage measurements. We carried out the present work with a particular focus on variability in two areas: the analytical procedures for extraction of critical cold hardiness values from electrolyte leakage data and the control treatment used to relativize freezing damage.

How is cold hardiness determined from leakage?

Since the development of the electrolyte leakage method, practitioners have employed diverse approaches to determine cold hardiness from raw electrolyte leakage data. Typically, procedures follow a three-step approach: absolute electrolyte leakage of a sample is made relative (*R*) by normalizing against electrolyte leakage at an extreme control (typically boiling, as discussed further below), followed by optional adjustment that results in *I,* and curve fitting, which allows extraction of a critical cold hardiness value. Variation in the choices made at each of these steps has produced variation in the ways that leakage data are interpreted in the literature (Fig. 1).

At the first stage of processing electrolyte leakage data, once R is calculated for a given individual across a range of freezing temperatures, it can be transformed into I by "zeroing" against unfrozen control samples and I_{adj} by also "stretching" relative to a theoretical maximum level of damage. In their work, Kreyling et al. [10] opt to do neither; they simply fit curves of R (REL in their notation) vs. freezing temperature across a wide range of temperatures (5°C to -196°C). In this case, R can range from 0 to 100%, but these values are rarely attained. Flint and colleagues' [7] approach is similar, except that R values are zeroed against a negative control kept at room temperature to take into account any electrolyte leakage from samples that might occur due to handling and processing unrelated to freezing (e.g., cut ends of stem pieces), thus becoming I. Curves fit to these data therefore treat freezing damage commensurate with handling and processing damage as "0%" damage by default. Finally, Lim and colleagues [12], popularized an approach that goes one step further, stretching all values of I prior to curve fitting such that a sample's leakage at the lowest temperature tested (often at or below -80°C) is treated as the maximum possible damage for that sample, giving I_{adj}. Curves fit using this procedure contain values of I corresponding to 0% and 100% damage. These three approaches represent a gradient in adjustment of raw electrolyte leakage values ranging from none ("Kreyling") to zeroing against a minimum ("Flint") to both zeroing and stretching to a maximum ("Lim").

Second, curves are fit to one of several curves chosen to model progressive accumulation of damage with exposure to freezing. Historically, most authors have fit data to generalized logistic curves [13–17], but some may use linear models as well [11]. However, while such logistic models always represent damage in response to dropping temperatures as a symmetric, sigmoidal process, actual plant tissues may accrue damage asymmetrically. This is to say, for instance, that marginal increases in damage may occur much more rapidly (over smaller temperature intervals) prior to the inflection point of the sigmoid curve than after it. For this reason, Von Fircks and Verwijst [18] first suggested using the Richards function, which can produce asymmetric, sigmoidal curves, to model I over a range of temperatures. Subsequent work

[8a] has validated this approach, with Lim and colleagues [12] recommending the Gompertz function – a special case of the Richards with one fewer parameter – as the best fit for leakage data. Many subsequent workers have followed their example and modeled leakage data with the Gompertz function [9,19–21]. As such data processed using the Kreyling, Flint, or Lim approach can then be modeled within either a logistic or Gompertz framework.

Third, and finally, a critical value of freezing damage, used to approximate cold hardiness, is extracted from model fit to leakage data. For general logistic models, this usually corresponds to T_{50}, the temperature at which 50% of maximum possible electrolyte leakage is attained, although other damage thresholds can be adopted as well (eg., T_{20}, T_{80}, etc.). Yet modeling of leakage data with the asymmetric Richards and Gompertz curves has also made it possible to extract a potentially more biologically realistic critical value from the resulting fitted curves. Whereas the inflection point of a logistic curve, T_{max}, always corresponds with T_{50}, these two points are decoupled in asymmetric sigmoid curves. As such, T_{max}, the temperature at which the rate of increase in freezing-induced damage is maximized (the temperature of maximum instantaneous slope of the curve), does not have to co-occur with the temperature at which half of the maximum damage possible has been reached. And though T_{max} has been employed in place of T_{50} in the literature [8,12] validation of this choice with reference to other cold hardiness metrics has been limited. As such, differences in the biological realism and thus desirability of the two critical values are still unclear.

What is the best way to standardize measurements of freezing-induced leakage?

Electrolyte leakage data have historically been standardized at the level of the individual sample to control for variability among samples in size and intrinsic (e.g., species- or genotype-dependent) electrolyte leakage capacity. In this work, we also assess the realism and consistency of the most commonly used positive control technique. Typically, all samples are heat-killed [22] following the first

conductivity reading, often through boiling induced by the liquid cycle of an autoclave. Yet, as a control for measurements of cold hardiness, boiling fails to mimic the process of interest: freezing-induced damage. In this vein, at least two groups [8,12] have used conductivity following freezing at -80°C in lieu of boiling as an index of maximum leakage following damage to sampled tissues. However, it has remained unclear whether freezing at these temperatures is actually sufficient to relativize leakage across samples varying in size and anatomy [23].

Furthermore, the temperature at which samples are boiled or heated and/or the time during which this temperature is reached are frequently not specified ([10,14, 24–26], among others). And though boiling at 120/121°C is typical (e.g., [9,17,27]), some [28–31] report using temperatures lower than the boiling point of water to heat kill samples. Yet Deans et al. [13] find that even temperatures above the boiling point (they compare 105° vs. 121°C) vary in their capacity to induce electrolyte leakage. This variability may be especially problematic for woody stem tissue (as opposed to bud or dissected vascular samples), from which leakage may be constrained to cut ends rather than from sample's entire surface area. In short, because electrolyte leakage depends on the temperature of and duration of and time elapsing following heat-killing [13,32], boiling as a control strikes us as a frequent and consequential source of inconsistency in the method.

Our approach

Despite some helpful methodological comparisons [12,13,19], practitioners collecting electrolyte leakage data are still confronted with numerous choices: what type of standardizing control should be employed during data collection? Should data be analyzed using the Kreyling, Flint, or Lim approach to zeroing and stretching? Which critical value should be extracted? And, once such decisions are made, will the resulting estimates of cold hardiness actually predict field plant performance?

In the work presented here, we respond to existing diversity in the methods used to assess cold hardiness through electrolyte leakage by: (i) integrating novel measurements in a panel of 12 maple (*Acer* spp.) species (Fig. 2), and (ii) re-analyzing published data. Our aim is to consolidate existing methods in measuring leakage, validate these methods against other techniques for measuring cold hardiness, and suggest a standard operating procedure in contrast to the variable status quo. We focus on two main domains in which existing practice could be improved. First, we contrast the performance of four main approaches to processing maple electrolyte leakage data: logistic modeling of data adjusted following 1) Kreyling et al. [10], 2) Flint et al. [7], and 3) Lim et al. [12] and 4) Gompertz modeling of data adjusted following the Lim approach. And second, we introduce a novel control procedure of liquid nitrogen immersion and compare its performance to that of the standard boiling control. Our approach addresses four questions:

1) Which of the four approaches to modeling electrolyte leakage data produce estimates of cold hardiness most aligned with those from two other core approaches: visual damage and differential thermal analysis?
2) Does use of a liquid nitrogen control
    a. produce leakage data commensurate with those generated with a boiling control?
    b. improve generalizability of findings across approaches to modeling leakage data?
3) Does re-analysis of previously collected data [10, 11] using data zeroing and stretching and assuming damage commensurate with a liquid nitrogen control provide more realistic estimates of cold hardiness?

In presenting this analysis, we encourage other investigators to continue using electrolyte leakage to measure cold hardiness and facilitate synthesis of existing and forthcoming data despite the use of diverse protocols.

Results

Electrolyte leakage data zeroed and stretched using the Lim approach best approximated visual damage to maple twigs

By design, the four analytical approaches we compared generated variability in curves representing the relationship between electrolyte leakage (*R* or *I*) and freezing temperature (Fig. 3; model parameters given in Additional file 1). Yet critical values extracted using each approach occupy a similar range of values (-12 to -28 ℃; Table 1A) and were generally correlated with each other (Table 2); species that had already broken flower or leaf buds were generally less cold hardy than those that still appeared dormant (Table 1A). Across methods, leakage measurements generated a warmer and somewhat narrower range of critical values relative to visual damage estimates (-17 to -36 ℃) and LTEs (-20 to -38 ℃, Table 1A).

Yet all four approaches to modeling electrolyte leakage did not perform equally well in predicting critical temperatures for freezing damage as measured using these other methodologies (Fig. 4, Table 2). Across a range of critical visual damage levels (20%, 50%, and 80%), T_{50} extracted from general logistic curves fit using the Lim approach best predicted visual damage (T_{50} or T_{80}) and species differences in LTEs. Critical values extracted using the Lim approach with Gompertz (rather than general logistic) curve fitting also performed well, but these require secondary calculation from model parameters, and therefore are less intuitive and less frequently used than general logistic curves. For these reasons, in the following analyses, we present electrolyte leakage data analyzed using the $\text{Lim}_{\text{logistic}}$ protocol: zeroed and stretched data fit to a logistic curve. Critical values for leakage, when determined using this approach, approximate critical values for visually estimated damage despite diversity across species and genotypes (Fig. 5,6).

We found support for the convention of comparing critical temperatures at the point at which 50% of total leakage or visual damage occurs (Fig. 7,8). Critical electrolyte leakage best predicts the temperatures at which between 50 and 80% of visually diagnosed damage occurs, although bias and error between the two assays is lower from 20 to 50% visual damage. Visual damage is accrued at a faster rate than electrolyte leakage (Fig. 5; see values of the *b* parameter in Additional file 1). This explains the

minimization of bias and RMSE for their comparison in the 30-60% range of visual damage. Therefore, a comparison of T_{50} for both electrolyte leakage and visual damage strikes us as an acceptable validation procedure. As shown in the middle panel of Fig. 7, this comparison indicates that electrolyte leakage explains 36% of the variability in visual damage and underestimates T_{50} for visual damage by only 3 °C.

Species-level LTEs show a different, though consistent, pattern of cold hardiness compared to critical temperatures for either electrolyte leakage or visual damage. Ease of exotherm identification in DTA differed between species, which may account for some of the differences from other methods. Ten of the 12 study species experienced LTEs between -34.5° and -38.4°C, just above the theoretical supercooling limit for water (~-42°C; Table 1A). The other two species, *A. caudatifolium* and *A. davidii*, had very high LTEs, indicating ice formation at roughly -20°C. Because of the bimodal distribution of species means in LTEs, there was not a significant relationship between this critical value and T_{50} for electrolyte leakage ($\rho = 0.45$, $p = 0.41$) or T_{50} for visual damage ($\rho = 0.31$, $p = 0.64$).

Data collected using a novel liquid nitrogen control commensurate with those collected using a boiling control

Electrolyte leakage induced by immersion in liquid nitrogen generated an average of 58% of the leakage induced by the standard boiling approach (an autoclave cycle at 120°C) and an average of 184% of the leakage induced by freezing at -80°C. Deming regression of nitrogen-induced leakage based on boiling-induced leakage with a zero intercept yields an allometric equation of

$$Conductivity_{Liquid\ N} = 0.576 \times Conductivity_{Boiling}$$

with two outliers removed and a 95% confidence interval around the slope ranging from 0.557 to 0.595 (Fig. 9). Increased variance made this relationship weaker for physically larger samples (in this case, those with wider diameter), which leaked more electrolytes and had higher conductivity.

Use of a liquid nitrogen control provides more consistent estimates of cold hardiness across different approaches to modeling leakage data

Allometric adjustment of leakage values (*R* or *I*) in which a boiling standard was used to estimate damage that would have resulted from use of a liquid nitrogen standard led to a considerable improvement in the consistency among approaches for converting electrolyte leakage data into estimates of cold hardiness. For instance, when a boiling standard is used, leakage estimated using the Kreyling and the $Lim_{logistic}$ approach are not commensurate above very low levels of damage (Fig. 9B, 10) – with overall bias at 51% and RMSE = 55%. When leakage values are adjusted using the allometric equation presented above to assume a liquid nitrogen standard, leakage estimated using the Kreyling approach increases, creating greater concordance between the two approaches (Fig. 9C) – bias is on average 25% and RMSE reaches 29%. If the two approaches are evaluated in a narrower range, between 25 and 75% of EL based on the $Lim_{logistic}$ approach, bias drops to 6% for the liquid nitrogen standard vs 26% for boiling, and RMSE to 11% and 28% for liquid nitrogen and boiling, respectively.

Re-analysis of previously published data suggests overestimation of cold hardiness

Re-analysis of raw electrolyte leakage data from [11] underscores the consequences of variation in the major approaches to analyzing data generated using this method. Each of the four approaches we used returned higher critical temperatures - indicating less cold hardiness - for the oak study system than those reported originally by these authors (Table 1). For instance, using the $Lim_{logistic}$ approach, we estimated that unacclimated woody samples accrued 50% freezing damage between -12° and -15°C; originally published estimates ranged from -13° and -20°C. Our estimated range of critical values for acclimated

samples (-21° and -25°C) was also considerably warmer than the originally reported one (-29° and -34°C).

We also observed that, even though Fallon and Cavender-Bares only froze oak woody tissues to a low temperature of -40°C, doing so appeared to cause levels of electrolyte leakage approaching or even exceeding those instigated by boiling (Additional file 2). This contrasts with our findings, in which liquid nitrogen immersion (-200°C) only generated about 58% of the leakage caused by boiling woody tissue from maples. Our figure of 58% maximum leakage comports with those from our re-analysis of Kreyling and colleagues' [10] work, in which the two surveyed maple species showed similar levels of maximum leakage (58% to 68% in March; Additional file 2).

Indeed, our extrapolation of findings from Kreyling and colleagues' [10] work suggests that patterns of maximum freezing-induced leakage likely vary among species and over time (Additional file 2). Notably, we observe that some genera, such as the redwoods (subfamily Sequoioideae) and beeches (*Fagus* spp.), appear to release virtually all of their intracellular electrolytes when immersed in liquid nitrogen (-200 °C), while others, such as the maples and pines (*Pinus* spp.), resist leakage, even when unacclimated to freezing. We also note a phenological pattern in maximum leakage, which, per Kreyling and colleagues' findings, was highest in autumn (November), and lowest at the end of winter (February), before increasing again with springtime deacclimation (March; Additional file 2). This pattern is also observed in some oak species in maximum leakage in [11], where samples collected in the summer had generally higher maximum leakage than in the winter – though the lowest temperature used was -40 °C. These patterns suggest that the electrolyte leakage at a single temperature cannot be used to compare even the same genotype across time when using boiling as a control [33] – but may be possible with a liquid nitrogen control. Differences in maximum leakage among clades also suggest that further research could be performed to understand what portion of electrolytes remains insoluble during freezing at liquid nitrogen temperatures but becomes soluble through boiling.

Neither use of short incubation times nor choice of boiling or liquid nitrogen immersion impairs estimates of cold hardiness

Though it was not the main focus of our experiment, we assessed the sensitivity of our electrolyte leakage measurements to variation in the incubation time elapsing between freezing or autoclaving and measurement and to the choice of boiling (at either 100°C or 120°C) or liquid nitrogen immersion. We found that longer incubation times following experimental freezing (down to -80°C) led to higher sample conductivity, but that there was no relationship between incubation time and conductivity following either autoclaving or liquid nitrogen immersion (Fig. 11A, Additional file 3). In particular, conductivity measured after one to two days of incubation following experimental freezing was statistically indistinguishable, while conductivity continued to increase significantly on days five and seven. On the other hand, post-boiling or post-liquid nitrogen conductivity did not show a significant, linear relationship with incubation time.

This pattern is reflected in critical values for cold hardiness derived from these data (Additional file 4): T_{50} for samples from the same species and assessed using the same standardization protocol varied little regardless of whether they were incubated for one or two days. Estimates of T_{50} derived from samples incubated for five days were generally within a few degrees of those associated with shorter incubations, but, by five days, species-level differences in cold hardiness were no longer reflected in T_{50} (Fig. 11B). Estimates of cold hardiness from samples incubated for seven days became increasingly variable across methods, potentially reflecting microbial growth on samples (Fig. 12). As such, we conclude that measuring leakage for up to two days following experimental freezing does not substantially bias estimates of cold hardiness, while waiting longer for leakage measurements (up to a week in our comparisons) may actually produce biased or unreliable results.

Discussion

We compared late-winter cold hardiness of woody tissue in 12 maple species using our updated electrolyte leakage protocol and validated these measurements against other metrics of cold hardiness. We found general agreement among critical values from four distinct approaches to extracting critical cold hardiness values from electrolyte leakage data, as a result of using relative parameters of fitted curves rather than leakage in absolute terms (i.e., values of *R*). However, we recommend the use of critical values associated with 50% damage obtained using the $Lim_{logistic}$ approach. These critical temperatures correspond well across genotype and species with the temperature at which 50% of twig tissue had experienced cambial browning as determined visually. Finally, we found that liquid nitrogen immersion as a control produces electrolyte leakage data commensurate with that produced with a boiling control, but can be applied more consistently and is better suited to samples with low levels of maximum freezing-induced leakage.

Electrolyte leakage data should be adjusted to account for minimum and maximum damage

In surveying the existing electrolyte leakage literature and developing this protocol, we noted consequential variability in the way that raw leakage data are converted to critical values of cold hardiness. As a result, it is possible to compare cold hardiness among treatment groups or species within a single study, but particular values of *R* or *I* may not be transferable across research groups or study systems. We classified the diverse published protocols into four approaches (Fig. 1) based on whether or not authors standardize raw data based on minimally damaged (unfrozen) or deep-frozen (maximally damaged) controls and on what class of curve is fit to adjusted data (general logistic or Gompertz).

Of the four resultant classes of protocols, we recommend the use of either of the Lim approaches ($Lim_{logistic}$ or $Lim_{gompertz}$), in which all leakage data are converted to $I_{T,adj}$through zeroing against leakage from non-freezing (e.g., 4 °C) and stretching against leakage from deep-freezing (e.g., -80°C) treatments. Though we chose to fit leakage data to a general logistic curve (as in Ouyang et al., 2019a) for reasons of parsimony and ease of interpretation, fitting to a Gompertz curve [12,19–21] generates similar results.

Critical values obtained from this approach best predicted visually observed cambial browning across a variety of damage thresholds (20-80%; Fig. 4), including critical 50% values (Fig. 5) across genotypes and species. (However, we do note that visual browning occurred across a smaller range of temperatures [Table 1]). The values of *R* and *I* we observed also demonstrate the need for the correction of *R* or *I* into $I_{T,adj}$ (i.e., using relativized measurements in the curve estimates): although in *R* and *I*, an absolute value of 50% damage was not reached, a clear plateau is observed in leakage values below -40 °C (Fig. 3). The use of absolute 50% damage (*R* = 50%) as the metric for cold hardiness level would have resulted in all species having cold hardiness > -80 °C in our study, an unrealistic outcome.

Adoption of this approach will likely affect reporting of estimates of cold hardiness derived from electrolyte leakage data. For instance, we found that critical temperatures (LT_{50}) for our maple samples estimated using the $Lim_{logistic}$ approach were highly correlated with ($R^2 = 0.81$), but not identical to, estimates generated through other approaches (Tables 1,2). And application of this approach in our re-analysis of Fallon and Cavender-Bares' [11] findings produced warmer (less cold hardy) estimates of LT_{50} by 1-9°C across species and degree of cold acclimation. We attribute this pattern to Fallon and Cavender-Bares' [11] use of absolute *I* values for estimation of 50% damage (Fig. 1B). However, as discussed previously, plateaus in their data indicate maximum damage may not occur at 100% of boiling leakage. In fact, even at liquid nitrogen temperatures, leakage values can fall far below 100%, and even below 30% (Additional file 2), spuriously suggesting that tissue is hardy to temperatures below -196 °C.

Electrolyte leakage corresponds to visual damage, but not low temperature exotherms

To further explore the biological meaningfulness of electrolyte leakage measurements, we validated critical values from the $Lim_{logistic}$ approach against not only critical values of visual damage, but also LTEs corresponding to the formation of intracellular ice. We found a generally high degree of correlation between damage measured by both leakage and browning (Fig. 4), especially when leakage was between 50 and 80% of its observed maximum (Fig. 7). For instance, across the 12 measured maple species, LT_{50}

(calculated using the $Lim_{logistic}$ approach) was highly and significantly correlated with browning ($\rho = 0.58$, $n = 36$, $p < 0.001$; Table 2). Furthermore, the ranges of these critical values also mostly overlapped, with critical damage occurring between -13° and -32°C (Table 1). As such, we contribute to the evidence that cellular damage measured via electrolyte leakage corresponds to visually apparent damage to plant cambial tissue in response to freezing [8,10,12–14,19,34,35].

On the other hand, we found that critical electrolyte leakage generally occurred at warmer temperatures than did the formation of symplastic ice (the LTE). We documented relatively uniform LTEs ranging from -35° to -38°C for ten species, indicating that these trees could still prevent ice formation in their cells at temperatures far below those they might ever experience in their native ranges. The other two species, *A. caudatifolium* and *A. davidii*, both adapted to relatively warm East Asian climates, had high LTEs (-20°C). Even in these cases, LTEs occurred at temperatures below critical leakage temperatures, suggesting that the two methods capture different aspects of cold hardiness. This finding is consistent with past documentation that LTEs can diverge from critical values for leakage during the growing season but converge when plants are fully cold-acclimated in the winter months [12,24]. As such, we find evidence that visual damage, but not LTEs, should serve as a useful validation of cold hardiness methods made with electrolyte leakage.

Liquid nitrogen as a negative control can enhance the use of the electrolyte leakage approach

We found that electrolyte leakage values derived from a boiling (autoclaved) control and a liquid nitrogen immersion control are highly correlated ($R^2 \sim 80\%$; Fig. 9A). Because freezing samples at ~-200°C by liquid nitrogen immersion is less damaging to woody tissue than boiling, raw leakage values calculated using our new control are roughly 58% of the values calculated using a boiling control. Given all this, and for the sake of transferability of findings, we conclude that values of *R* or *I* calculated using either method can be compared across studies using the linear equation given in Fig. 9. But, since leakage data produced

using either method are linearly related, does the choice of boiling versus liquid nitrogen immersion even matter? Based on our findings, we argue that use of liquid nitrogen bestows four advantages.

First, as surveyed above, there are a wide variety of boiling protocols used in electrolyte leakage studies; variability exists in both temperature and intended duration of boiling, likely hindering generalization of findings. Furthermore, duration of boiling treatments is often not even obvious: it is unclear whether duration of actual exposure to a target temperature or total time in hot water bath is reported (e.g., "killed in a boiling water bath for 20 min" [36]) or an autoclave cycle (which usually also includes higher pressure to prevent boiling over). Liquid nitrogen immersion, on the other hand, exposes samples to a physically constrained range of temperatures (-196 to -210°C) for a much more obvious duration of time (the time of immersion).

Second, a liquid nitrogen control may be more suitable for samples collected from cold-hardy or highly acclimated plants or for any sample with low levels of maximum freezing-induced leakage. Because use of a boiling standard damages samples more than immersion in liquid nitrogen (Fig. 9A), the former approach produces lower values of *I* or *R* across freezing temperatures. As a result, apparent freezing-induced leakage measured using a boiling control often remains low, failing to reach 50% of maximum damage. Low values of *R* or *I*, in turn, are often less commensurate across different approaches to calculating cold hardiness using electrolyte leakage data. For instance, in our comparison of leakage values from the same raw data but calculated using the Kreyling vs. $Lim_{logistic}$ approaches (Fig. 9B, C), the two approaches are only commensurate at low damage levels when data are relativized against boiling. On the other hand, when data are relativized against liquid nitrogen immersion, the two approaches indicate relatively similar damage across a wider range of temperatures, especially for cold-hardy samples like our acclimated maples or the maples and pines in Kreyling and colleagues' [10] study (Additional file 5, Fig. 10). Therefore, use of a liquid nitrogen control or conversion of *R* or *I* to assume a liquid nitrogen control facilitates comparison and synthesis across diverse approaches to measuring cold hardiness using

418 electrolyte leakage. A caveat to this argument is our finding, through re-analysis of Fallon and Cavender-
419 Bares' [11] study of southwestern U.S. oaks, that, when maximum freezing-induced leakage is high, a
420 boiling control can be sufficient for estimation of cold hardiness using electrolyte leakage.
421
422 Third, we note that, as expected, liquid nitrogen immersion provides a more realistic proxy of "maximum
423 damage" to samples than did boiling. Liquid nitrogen immersion (at roughly -200°C) caused a little less
424 than twice the leakage inflicted by freezing at -80°C, which is already a temperature that the vast majority
425 of the world's woody plants never experience in a natural context. As such, freezing through liquid
426 nitrogen immersion seems sufficient to inflict the maximum possible damage that could be expected from
427 this mode of injury on woody samples, while avoiding the excessive treatment afforded by boiling. The
428 choice of boiling versus liquid nitrogen immersion appears to be less important for non-woody samples
429 (e.g., leaves, crowns), though may still be important for more lignified green tissues such as conifer
430 needles [37].
431
432 Fourth and perhaps most obviously, submerging the tubes in liquid nitrogen provides the same physical
433 environment for the samples (ice, compared to boiling liquid water) as they experience when exposed to
434 the damaging stressor of interest. Use of a liquid medium as a control means that the resulting leakage
435 occurring during boiling may be due to other effects other than only temperature.
436
437 **Conclusions**
438 A central recommendation we make for all workers performing electrolyte leakage research is that
439 estimates of cold hardiness using this method should take place across a range of temperatures, and not
440 simply at one freezing temperature of interest. This range must include one temperature above freezing
441 (e.g., 4°C) and one or more temperatures chosen to elicit maximum freezing damage such that a plateau in
442 electrolyte leakage values is observed (e.g., < -80°C and ideally including submersion in liquid nitrogen).
443 If this approach to sampling is observed, all four of the analytical approaches we surveyed are likely to

yield similar critical values of cold hardiness (Tables 1,2). Generally, we found LT_{50}, extracted from data fit to either a general logistic or Gompertz curve following Lim and colleagues' [12] approach, to best approximate visually observed damage to woody tissue.

We highly recommend the use of the liquid nitrogen control we present here in lieu of boiling or autoclaving. This control will be especially useful for very cold hardy woody samples (or physically small ones) coming from, for instance, acclimated temperate or boreal species prior to leaf-out. And though we offer a simple linear equation for estimation of leakage assuming a liquid nitrogen control from leakage data collected using a boiling control (Fig. 9), we suggest some caution in applying this conversion widely, especially outside of the maple genus or to non-woody samples. Application to other systems may benefit from experimental determination of a linear equation relevant to the samples in question.

Finally, we did not expect to find the diversity in maximum freezing-induced electrolyte leakage that we found among species in our own and in re-analyzed studies (Additional file 2). Though this value appears to depend somewhat on the lineage being sampled and on the time of year, it is not clear what underlying mechanism causes such variety in the difference between electrolyte leakage following deep freezing and boiling among woody samples. Future work might explore a connection between this maximum freezing-induced leakage and cold hardiness.

Methods

Study System

The roughly 120 species of the maple genus (*Acer* L.) are distributed widely throughout the northern hemisphere, having diverged from relatives in the Sapindaceae some 60 million years ago and radiated from their center of diversity in eastern China to mesic environments throughout Asia, Europe, and North America (Fig. 2; [38]). The maples are a typical temperate, woody genus, including common forest dominants and rare and subordinate species, with species' native distributions covering areas with mild

winters (Taiwan, Mexico) through very cold ones (Scandinavia, the Amur Valley). In the present study, we focus on twelve maple species spanning the maple phylogeny [39,40] and three principle biogeographic realms colonized by the genus: Asia, North America, and Europe (Fig. 2; Grossman *in review*).

All plant material was collected from mature, healthy trees at the Arnold Arboretum (42°18'26" N, 71°07'13 W", 15-79 m a.s.l) in Boston, MA, USA. The Arboretum has a hot summer continental climate (*cfa* in the Köppen-Geiger system) with a historical mean annual temperature of 9.7 ℃ and mean annual precipitation of 1168 mm. Collections were primarily from accessioned trees in the Arnold's living collections and occasionally from spontaneous individuals growing on the Arboretum grounds. Information about all sampled trees is provided in Additional file 6.

We obtained previously published electrolyte leakage data for six southwestern U.S. oak species (*Quercus* spp.; [11]). We also extracted some data from supplements associated with Kreyling and colleagues' [10] study of diverse woody species at the Ecological Botanical Garden in Bayreuth, Germany.

Electrolyte Leakage Measurements

We developed our electrolyte leakage routine by drawing on published accounts and testing a variety of alternative methods. First, we collected material from three genotypes per species (n=36), completing all sampling on 13 March 2020, noting the phenological stage of each sampled individual. Healthy, one to two year-old stems, typically 3-10 mm in diameter and < 30 cm in length were harvested and placed in sealed plastic bags to prevent desiccation. These were then stored at 4 ℃ to prevent further de/acclimation until sample preparation and testing. Processing consisted of cutting internode stems into 10 mm segments and sealing them in 15mL plastic test tubes with 2 mL of nanopure water (< 18.2 MΩ). For each genotype, 21 segments were prepared. Allocated across 6 freezing temperatures and a control,

these yielded three measurement replicates per genotype. Freezing trials then occurred either 12 or 36 hours after sample preparation was completed; samples were randomized across either the first or second day of trials and stored at 4 ℃ prior to freezing.

Freezing trials occurred in a Tenney Environmental (New Columbia, PA, USA) Test Chamber with thermocouple monitoring of temperature using an RDXL4SD data logger (Omega Engineering, Inc., USA). Target temperatures for freezing were: 4 ℃ (controls kept in a growth chamber); -10, -20, -30, -40, and -60 °C (in the Tenney Chamber); and -80 °C (in a separate freezer). Samples in the Tenney Chamber were initially cooled from a storage temperature of 4 to 0 ℃ over thirty minutes and then held at 0 ℃ for an additional thirty minutes. They were then stepped down to -10 ℃ at a rate of -0.33 °C/min and held for one hour at the target temperature. This routine was repeated for -20, -30, -40, and -60 °C with the rate of temperature change between -40 and -60 °C twice that of the rate between other steps. After one hour at -60 °C, samples assigned to a -80 °C target were moved to a separate deep freezer and held there for one to two hours. Samples were removed from the Tenney Chamber or deep freezer and allowed to return to room temperature on the benchtop after being held at their target temperature. Visual inspection showed that water in all tubes froze completely without exogenous ice seeding or inclusion of steel shot to promote ice formation within the first freezing step (-10 ℃). Thermocouple readings indicate that actual sample temperature was on average 1.5 ℃ colder than the target temperature; we use actual temperature reached (e.g., -10.5 in lieu of -10 °C) for analysis.

Following sample thawing, 8 mL of nanopure water were added to each test tube and tubes were shaken at 100 rpm and room temperature for 16 hours. Conductivity of solution in each tube was then read with a conductivity probe calibrated with a low-concentration (0-200 μS/cm) standard (Vernier Software and Technology, Beaverton, OR, USA). Samples were hand-shaken immediately before conductivity measurements; we found that this was essential for accurate readings.

A novel control: Liquid nitrogen immersion

Out of concern with the realism and consistency of the current set of methods used in electrolyte leakage studies, we have developed and employed here an alternative approach, immersing all sample tubes in liquid nitrogen (at least -200 ℃ as indicated by thermocouple readings) for thirty minutes following initial conductivity readings. Samples were then allowed to thaw on the bench and shaken at room temperature and 100 rpm for 16 hours. Final conductivity measurements were then collected as described above.

To validate the use of this new control, a separate set of stem samples (n=176) from all 12 study species collected on 3 and 10 February 2020 was used to carry out the protocol described above. Following freezing in liquid nitrogen, we measured their conductivity, then autoclaved them at 120 ℃ for thirty minutes, loosening tube caps prior to autoclaving to reduce evaporation while preventing overpressurization of tubes. (We autoclaved them at high heat to maximize electrolyte leakage.) We then agitated tubes for 16 hours and measured conductivity, both as described above, allowing for comparison between leakage following liquid nitrogen immersion and boiling.

Previous studies have suggested that frozen samples may not release all diffusible electrolytes for up to a week following freezing and that samples exposed to autoclaving for short periods of time (e.g., 15 min.) will continue to leak electrolytes for ten days or more [13a]. In order to ascertain that our incubation timing (both post-freezing and post-autoclaving or liquid nitrogen immersion) and autoclave intensities were sufficient to capture electrolyte diffusion, we carried out an additional experiment on only two species, *A. caudatifolium* and *A. campestre*. These two maples represented the two extremes of cold hardiness in our main experiments (Table 1), occupy distinct habitats (Fig. 2), and belong to phylogenetically distinct sections [40]. For this additional experiment, we collected stem sections of one genotype of each species (with three measurement replicates per genotype collected) on 29 December 2020 and measured using the same electrolyte leakage protocol described above while varying the amount

548 of time samples incubated after boiling and damage controls (both autoclaving and liquid nitrogen)
549 treatments and the control treatment applied. As such, conductivity of stem segments of both species was
550 measured 1, 2, 5, or 7 days post-freezing and then exposed to either boiling at 100℃ in the Tenney
551 Chamber, autoclaving at 120℃, or liquid nitrogen immersion (N = 504). Segments were then allowed to
552 incubate for the same length of time as their post-freezing incubation and conductivity was re-measured.
553 This experiment crossed incubation time (one day through one week) with control method (moderate
554 boiling, intense boiling, or liquid nitrogen) for two species expected to vary in their cold hardiness,
555 producing 24 factor-level combinations.
556
557 *Validation of Electrolyte Leakage*
558 To validate estimates of cold hardiness from electrolyte leakage, we performed two additional cold
559 hardiness assays on stem tissue from sampled individuals: differential thermal analysis and visual
560 inspection of freezing damage. We briefly describe these procedures below.
561
562 **Differential thermal analysis.** Differential thermal analysis (DTAs; [41–43]) involves cooling plant
563 tissue of interest to progressively lower temperatures while using thermocouples to detect the tissue's
564 release of heat associated with the freezing of water, an exothermic reaction. Extracellular water in stems,
565 buds, etc. freezes at relatively warm sub-zero temperatures, producing a release of heat or high-
566 temperature exotherm (HTE); the freezing of this extracellular water is not considered harmful to
567 acclimated, cold-adapted woody plants. Intracellular water, on the other hand, will generally supercool to
568 much lower temperatures, reaching a limit at about -42 ℃. Freezing of this supercooled water, which is
569 associated with catastrophic damage to affected cells, results in a low-temperature exotherm (LTE),
570 which can be detected through DTA and compared to results from electrolyte leakage assays [8,14,16,24].
571
572 For DTA measurements, carried out on 16 March 2020, we used the same material collected for our
573 electrolyte leakage trials; this material had been stored with proximal ends inserted in water, at 2 ℃ for

the intervening time (3 days). For each genotype (n=36), we cut ten 30 mm segments of the same internodal stem tissue and pooled these measurement replicates into a cell in which they were exposed to Peltier plates, which can detect exotherms and convert their thermal signals to voltage. Samples were then cooled at a rate of -4 °C/hour to -60 °C. Exotherm-associated voltages were collected using a Keithley Multimeter Data Acquisition System (Keithley Instruments, Cleveland, OH, USA). HTE peaks were discarded and LTE peaks were manually curated in Microsoft Excel prior to statistical analysis.

Visual damage inspection. We also visually inspected damage to cortical tissues cells following freezing; this metric serves as an intuitive and holistic, though labor-intensive method for assessing cold hardiness [8,10,14,19,44]. On 16 March 2020, we prepared samples for freezing followed by visual damage inspection from the same material used in other trials. For each genotype (n=36), and each temperature treatment, we cut three 30 mm segments of internodal stem tissue and incubated them in plastic scintillation vials with 2 mL water at 2 °C overnight. On 17 March, we froze these vials using the same freezing routine as the one employed in our electrolyte leakage measurements. One set of vials was kept at 4°C. The others were frozen to -10, -20, -30, -40, and -80 °C. After removal from the freezer, vials were allowed to thaw at 4 °C for 24 hours, then incubated with ends in water, at room temperature for six days. At this time, they were evaluated using a quartile (0%, 25%, 50%, 75% or 100% brown) damage scale.

Data Analysis

All analyses were carried out in R (ver. 3.6.3, [45]).

Treatment of raw electrolyte leakage data. Raw electrolyte leakage at each temperature was normalized into a relative electrolyte leakage (R_T) at temperature T:

$$R_T = L_T / L_k$$

where L_T is the conductivity measured from a sample frozen at T and L_k is the conductivity of the same sample after boiling. R_T is the measurement used for damage estimation based on the Kreyling et al. [10] approach. We then converted R_T to Index of Injury (I_T) for any given temperature T following Flint and colleagues' [7] method such that:

$$I_T = 100 \times \frac{(R_T - R_o)}{(1 - R_o)}$$

Where $R_o = R_T$ for an unfrozen control. In our case, the unfrozen control was kept at 4 ℃. Therefore, $I_T = 0$ at $T = 4$ ℃.

The third method employed was a modified version of Lim and colleagues' [12]. This adjustment builds upon the apparent plateau observed in levels of damage in lower temperatures and assumes maximum damage occurred within the temperatures tested:

$$I_{T,adj} = 100 \times I_T / I_{max} \equiv 100 \times \frac{(R_T - R_o)}{(R_{max} - R_o)}$$

where I_{max} and R_{max} are the maximum unadjusted injury and relative conductivity measured for any genotype (usually attained at either -60 or -80 ℃).

Analysis of electrolyte leakage and visual damage data. Electrolyte leakage values of R_T, I_T, and $I_{T,\,adj}$, and visual damage (VD) were then modeled for each genotype using a four-parameter log-logistic model:

$$R_T, I_T, I_{adj,T}, VD_T = c + \frac{d-c}{1+e^{(b\times((T)-(u)))}}$$

where T is the temperature, e is euler's number, and d, c, b, and u are the parameters estimated: c is the lower limit ($c = 0$ for I_T , $I_{adj,\ T}$, and VD_T); d is the upper limit ($d = 100$ for $I_{adj,\ T}$ and VD_T); b is the slope associated with the logistic function; and u is the inflection point. Here $LT_{50} = u$. For $I_{T,\ adj}$ we also extracted values of LT_{20} and LT_{80} for comparison with $LT_{50.}$ (Additional file 5).

We also modeled $I_{adj,\ T}$ using a Gompertz function and extract both LT_{50} *and* LT_{max} as a critical value of cold hardiness as follows:

$$I_{adj,T} = 100 \times e^{-b\times e^{-k\times T}}$$

$$LT_{max} = \frac{-log(1/b)}{k}$$

where T is freezing temperature, e is euler's number, and b and k are parameters. We fit Gompertz curves for each genotype in our dataset using the *nls* function, whereas log-logistic functions were modeled using the *drm* funditon in the drc package [46].

Critical values of LT_{50} were extracted from the curves obtained from each genotype for each model for comparison of means at a species level. For I_{adj}, values of LT_{20} and LT_{80} were also used. We also calculated the lowest survival temperature (LST) on a species basis for visual damage, which we defined as the lowest temperature at which no stem segment experienced more than 50% damage [8,41]. Species differences for these were analyzed using simple linear regression models and Type I ANOVAs. Tukey's

HSD post-hoc test (*HSD.test*, "agricolae"; [47]) is used to discriminate among groups in cases of significant difference.

Bivariate correlation tests were used to compare values of LT_{20}, LT_{50} and LT_{80} for the three electrolyte leakage models with LT_{20}, LT_{50} and LT_{80} and LST values for visual damage. Further comparisons in terms of correlation, bias, and RMSE were made from LT_{10} to LT_{90} in 10% steps for I_{adj} and VD.

Using raw electrolyte leakage data from a different study comparing acclimated and unacclimated oaks [11], we followed the same protocol described to obtain R, I and I_{adj}. Since there were multiple genotypes within each species, but no repetition within temperatures for each genotype, the control level used for I and I_{adj} was the lowest electrolyte leakage at either 4 ℃ or -5 ℃ within each genotype. The three forms of leakage data were used for log-logistic curve estimations of T_{50}, whereas I_{adj} was used for T_{50} and T_{max} estimations based on the Gompertz function as well.

Comparison of liquid nitrogen and boiling leakage. To compare the values of electrolyte leakage in both types of control, Deming (or "least rectangle") regressions were fit. This type of regression takes into account errors in both axes to find the best fit line. Although we did not include a pure water sample control in the measurements, initial fitting showed a non-significant intercept, and therefore further fitting was done with a zero-intercept model. Regular least-squares R^2 values were calculated in both directions to evaluate fitness of the model, from which a linear allometric relationship was extracted.

Damage estimated based on electrolyte leakage standardized on the liquid nitrogen control was then calculated and fit based on R and I approaches. Fitted values were then compared between values of I_{adj} – for which the control method has no influence – and R and I for both boiling and liquid nitrogen control.

To understand how leakage following boiling compares to that caused by deep freezing across study systems, we compared our maple data to leakage measurements taken on diverse species in two other studies. We extracted additional *R* data at liquid nitrogen temperatures from figures in the supplementary data of Kreyling et al. [10] using a grid with 5% increments. This dataset contained electrolyte leakage for 27 species, 3 genotypes per species, at 3 different points: November, February, and March. Species and time differences were analyzed using simple linear regression models and Type I ANOVAs. Tukey's HSD post-hoc tests were used to discriminate among groups. We also used data from the lowest temperature used by Fallon and Cavender-Bares [11] for similar analyses using species and time (acclimated vs. unacclimated) as variables. Although the Deming regressions used in our data are more appropriate, we used *R* values at liquid nitrogen temperatures and analyzed it using linear regression with species as the explanatory variable for comparison with the other studies.

Impacts of incubation time and control method on electrolyte diffusion. To assess whether electrolyte leakage depended on species identity, control methodology, or incubation time, we built two fixed-effects linear models (using *lm*) of electrolyte leakage measured in our focused study of *A. caudatifolium* and *A. campestre* stem segments. These models both took the form of:

$$L_T, L_K \sim \beta_{Intercept} + \beta_{Temperature} + \beta_{Species} * \beta_{Incubation} * \beta_{Control} + \varepsilon$$

In this analysis, either leakage following freezing (L_T) or exposure to boiling or liquid nitrogen (L_K) was modeled as a linear function of species identity, incubation time (a continuous variable consisting of 1, 2, 5, or 7 days), and control type (autoclaving at 100°C, autoclaving at 120°C, or liquid nitrogen immersion). Temperature of freezing treatment (4°C to -80°C) was included as a covariate as it is expected to affect sample conductivity. Models were fit with all interactions except those with freezing temperature and analyzed using a conservative Type III ANOVA. Differences among factor-level combinations were assessed using Tukey tests, as described above. For further comparison of the impacts of differences in

incubation time and control type on estimates of cold hardiness, critical values (T_{50}) were extracted for each factor-level combination using the $Lim_{logistic}$ approach.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated by the authors during this study (on *Acer* samples) are included in this published article and its supplementary information files. Re-analyzed data provided from other authors are available from the corresponding author on reasonable request and pending permission from the originating author(s). Code and maple data are also available at https://github.com/apkovaleski/EL_Methods.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

APK and JJG jointly and equally conceived of the original project idea, designed the experiment, performed all field and laboratory work, and analyzed resultant data. Both authors contributed to all drafts of the manuscript.

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Tables

Table 1. Phenological condition and cold hardiness of A) maple and B) oak species.

Species	Condition	Critical Values for Electrolyte Leakage					Visual LT_{50}, °C	LST, °C	LTE, °C
		Kreyling	Flint	$Lim_{logistic}$	$Lim_{gompertz}$	$Lim_{gompertz}$			
		LT_{50}, °C	LT_{50}, °C	LT_{50}, °C	LT_{50}, °C	LT_{max}, °C			
A. Maple (*Acer*) data from the present study									
A. saccharum	Dormant	-28^{b}	-28^{b}	-26^{cd}	-26^{cd}	-21^{bc}	-29^{ab}	-33^{ab}	-36^{bc}
A. pseudoplatanus	Dormant	-20^{ab}	-20^{ab}	-22^{bc}	-22^{bcd}	-18^{abc}	-30^{ab}	-33^{ab}	-38^{cd}
A. rubrum	Budbreak	-16^{ab}	-16^{ab}	-17^{ab}	-17^{ab}	-14^{ab}	-18^{a}	-17^{a}	-35^{b}
A. platanoides	Dormant	-25^{ab}	-24^{ab}	-25^{bcd}	-25^{bcd}	-20^{bc}	-27^{ab}	-30^{ab}	-35^{b}
A. campestre	Dormant	-25^{ab}	-25^{b}	-32^{d}	-33^{d}	-26^{c}	-36^{b}	-40^{b}	-37^{cd}
A. hyrcanum	Dormant	-23^{b}	-23^{ab}	-23^{bc}	-23^{bcd}	-18^{abc}	-30^{ab}	-33^{ab}	-36^{ab}
A. tataricum	Bud swelling	-18^{ab}	-18^{ab}	-19^{abc}	-19^{abc}	-14^{ab}	-19^{a}	-20^{ab}	-36^{bcd}
A. tegmentosum	Budbreak	-18^{ab}	-17^{ab}	-18^{abc}	-18^{abc}	-14^{ab}	-17^{a}	-23^{ab}	-36^{bcd}
A. caudatifolium	Budbreak	-12^{a}	-12^{a}	-13^{a}	-13^{a}	-11^{a}	-20^{a}	-20^{ab}	-20^{a}
A. davidii	Bud swelling	-17^{ab}	-18^{ab}	-19^{abc}	-20^{abc}	-16^{ab}	-21^{a}	-27^{ab}	-20^{a}
A. spicatum	Dormant	-20^{ab}	-21^{ab}	-20^{abc}	-21^{abc}	-16^{ab}	-27^{ab}	-23^{ab}	-38^{d}
A. negundo	Dormant	-27^{b}	-27^{b}	-24^{bcd}	-26^{bcd}	-18^{abc}	-20^{a}	-23^{ab}	-36^{bcd}
B. Oak (*Quercus*) data from Fallon and Cavender-Bares (2018)							**Published LT_{50}, °C**		**N**
Q. arizonica	Acclimated	-22^{b}	-21^{bc}	-21^{bc}	-21^{bc}	-18^{b}	-34		39
	Unacclimated	-12^{a}	-12^{a}	-12^{a}	-12^{a}	-10^{a}	-16		44
Q. emoryi	Acclimated	-26^{c}	-26^{d}	-25^{d}	-26^{d}	-23^{c}	-29		12
	Unacclimated	-14^{a}	-14^{a}	-14^{a}	-14^{a}	-11^{a}	-15		10
Q. gambelii	Acclimated	-22^{b}	-21^{bc}	-21^{bc}	-21^{bc}	-18^{b}	-33		17
	Unacclimated	-11^{a}	-11^{a}	-12^{a}	-12^{a}	-10^{a}	-20		16
Q. grisea	Acclimated	-25^{bc}	-25^{cd}	-25^{cd}	-26^{cd}	-23^{c}	Not calculated		2
	Unacclimated	-16^{ab}	-15^{ab}	-15^{ab}	-15^{ab}	-12^{ab}	Not calculated		2
Q. hypoleucoides	Acclimated	-22^{b}	-21^{bc}	-21^{bc}	-21^{bc}	-18^{b}	-23		39
	Unacclimated	-13^{a}	-13^{a}	-13^{a}	-13^{a}	-11^{a}	-13		40
Q. rugosa	Acclimated	-24^{bc}	-23^{cd}	-23^{cd}	-23^{cd}	-19^{bc}	-32		16
	Unacclimated	-13^{a}	-13^{a}	-13^{a}	-13^{a}	-11^{a}	-17		17

Phenological condition at sampling and critical values of cold hardiness for electrolyte leakage, lowest survival temperatures (LSTs), and low temperature exotherms (LTEs) for A) maple species (n=3) and B) oak species-acclimation combinations (sample size and originally published critical values as noted; Fallon and Cavender-Bares, 2018). In cases for which differences among species are significant, superscripts indicate results of a Tukey's HSD post-hoc test ($\alpha = 0.05$). Samples vary in phenological condition at the time of sampling. Maples were sampled at various points ranging from dormancy to post-budbreak in early spring. Oaks were sampled while either cold-acclimated (winter) or unaccclimated (summer).

Table 2. Correlations between critical values of cold hardiness based on different approaches.

		Kreyling			Flint			$Lim_{logistic}$			$Lim_{gompertz}$				Visual Damage			
		LT_{20}	LT_{50}	LT_{80}	LT_{20}	LT_{50}	LT_{80}	LT_{20}	LT_{50}	LT_{80}	LT_{20}	LT_{50}	LT_{80}	LT_{max}	LT_{20}	LT_{50}	LT_{80}	LST
Kreyling	LT_{20}																	
	LT_{50}	0.44																
	LT_{80}	-0.1	**0.84**															
Flint	LT_{20}	**0.92**	**0.54**	0.6														
	LT_{50}	0.4	**Identical**	**0.85**	**0.53**													
	LT_{80}	-0.3	**0.88**	**0.99**	0.1	**0.89**												
$Lim_{logistic}$	LT_{20}	**0.89**	**0.6**	0.11	**0.94**	**0.59**	0.17											
	LT_{50}	**0.59**	**0.81**	**0.5**	**0.72**	**0.81**	**0.55**	**0.74**										
	LT_{80}	**0.2**	**0.7**	**0.63**	**0.34**	**0.72**	**0.65**	**0.33**	**0.88**									
$Lim_{gompertz}$	LT_{20}	**0.91**	0.48	-0.2	**0.98**	0.47	0.3	**0.96**	**0.69**	**0.29**								
	LT_{50}	**0.52**	**0.82**	**0.56**	**0.65**	**0.83**	**0.6**	**0.68**	**Identical**	**0.91**	**0.62**							
	LT_{80}	**0.24**	**0.78**	**0.68**	**0.37**	**0.8**	**0.72**	**0.41**	**0.91**	**0.98**	**0.33**	**0.94**						
	LT_{max}	**0.7**	**0.78**	**0.41**	**0.83**	**0.78**	**0.46**	**0.84**	**0.98**	**0.78**	**0.81**	**0.96**	**0.82**					
Visual Damage	LT_{20}	**0.28**	0.28	0.13	**0.34**	0.3	0.16	**0.35**	**0.42**	0.34	**0.34**	**0.4**	0.34	**0.42**				
	LT_{50}	**0.44**	**0.46**	0.22	**0.5**	**0.47**	0.27	**0.51**	**0.6**	**0.49**	**0.49**	**0.58**	**0.5**	**0.61**	**0.85**			
	LT_{80}	**0.46**	**0.49**	0.24	**0.51**	**0.5**	0.3	**0.53**	**0.62**	**0.49**	**0.5**	**0.6**	**0.51**	**0.62**	**0.58**	**0.92**		
	LST	**0.49**	**0.43**	0.14	**0.51**	**0.43**	0.21	**0.54**	**0.59**	**0.46**	**0.52**	**0.57**	**0.47**	**0.61**	**0.48**	**0.8**	**0.89**	
LTE		**-0.31**	**-0.64**	**-0.58**	**-0.31**	**-0.63**	**-0.62**	**-0.36**	**-0.46**	**-0.45**	-0.27	**-0.47**	**-0.48**	**-0.43**	-0.26	**-0.34**	**-0.35**	**-0.28**

Correlations (ρ) between critical values of cold hardiness (°C) for four electrolyte leakage approaches, visual damage (including lowest survival temperature), and low temperature exotherms. Correlations are calculated for maple genotype means (n=36) for all indices except LTEs, for which species means (n=12) are compared to other metrics. We focus on the use of the LT50 critical value extracted using the $Lim_{logistic}$ approach (enclosed with borders). Bolded correlations indicate a significant ($\alpha < 0.05$) bivariate correlation test.

Figure Legends

Figure 1. Schematic comparing different approaches to measuring electrolyte leakage. As described in the main text (Fig. 2), we contrast the Kreyling approach (no minimum or maximum leakage specified, yields *R*) with the Flint approach (minimum but no maximum, yields *I*) and the Lim approach (minimum and maximum specified, yields I_{adj}). A) In the Kreyling approach, the temperature at which a sample accrues 50% of possible damage (T50 for *R*) may not be meaningful; many samples may not reach this "Absolute 50%" damage point. Instead, the temperature at which "Relative 50%" damage is attained may be more meaningful. B) By comparison, values of *I,* in the Flint approach (orange line) are zeroed, although this may not drastically displace the curve relative to a Kreyling curve. C) In the Lim approach, data are forced to reach 100% damage, usually at the coldest temperature employed to freeze samples. Curve shapes may differ and critical temperatures (e.g., T_{50}) corresponding to I_{adj} (extracted from the Lim curve) may or may not differ from those associated with *R* (Kreyling curve). D) Use of a liquid nitrogen control is expected to elevate all leakage values, making, for instance, a Kreyling curve behave more like a Lim curve and improving generalizability among approaches.

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Figure 3. Comparison of four approaches for fitting curves to data representing the relationship between freezing damage and temperature in (A, C, E, G) *A. caudatifolium* and (B, D, F, H) *A. campestre* stem segments (plots for other species provided in Appendix 1). Curves fit to data on a per-genotype (red, blue, and green) and per-species (black curve) basis are fit in each case. Panels show curves fit following the approach of A, B) Kreyling and colleagues' (2015), C, D) Flint et al. (1987), and Lim et al. (1998). Approaches vary, as indicated, in their use of room-temperature (zeroing; C-H) and deep freezing (maximum damage; E-H) controls and reliance on general logistic (A-F) vs. Gompertz (G-H) curves.

Figure 4. A) Validation of critical values from four approaches to modeling electrolyte leakage (as in Fig. 1) against visual estimates of freezing damage. Critical values reflect either 20%, 50%, or 80% electrolyte leakage (rows) or visual damage (columns). The rightmost column indicates lowest survival temperature (LST), the lowest temperature at which stems experienced < 50% damage. Pie wedge size and color indicate correlation. B) 50% electrolyte leakage values using the $Lim_{logistic}$ approach (orange box) best predicted visual damage in the 40-60% damage range.

Figure 5. Damage, as reflected by electrolyte leakage (solid lines) and visual estimates (dashed lines), induced by freezing from -10 to 80°C. Electrolyte leakage is calculated using the $Lim_{logistic}$ approach. Panels represent estimates of damage to particular genotypes. Color-coding indicates species as in Fig. 2; species are also presented in alphabetical order.

Figure 6. Visual cambial damage corresponded to critical cold hardiness estimated from electrolyte leakage data. Values of T50 given here (Table 1) are calculated using the $Lim_{logistic}$ approach.

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Figure 11. A) Electrolyte leakage increased gradually over seven days following experimental freezing (light blue), but not exposure to a boiling or liquid nitrogen control (turquoise), with no evidence of a significant difference when conductivity was measured over the first 48 hours after freezing. Lowercase letters indicate significant differences in conductivity measured at different time points at the 0.05 level based on models reported in Additional file 3 panels A (light blue, a-c) and B (turquoise, d-e). B) As a result of this pattern, estimates of critical values for cold hardiness (T_{50}) are consistent and reflect species differences when samples were incubated for one or two days, but not when they were incubated for 5 or 7 days (Additional file 4).

Figure 12. Stem segments incubated for longer than five days following control treatment (boiling or liquid nitrogen immersion) tended to deteriorate, showing evidence of microbial growth.

Additional files (Additional_files_1–6.xlsx)

Additional file 1. Parameters, error (in parentheses), and pseudo-R2 for models of freezing damage as measured by A-D) electrolyte leakage or E) visual observation of cambial browning. Models are fit to A-C, E) a general logistic curve or D) a Gompertz curve as described in the text.

Additional file 2. Maximum freezing-induced leakage (%) attained across several electrolyte leakage studies. Mean separations reflect outcomes of Tukey's HSD post-hoc tests ($\alpha = 0.05$). A) Data for maple (Acer spp.) species included in the present study. Percentages indicate leakage following immersion in liquid nitrogen divided by leakage following boiling. Superscripts reflect species differences among means. B) Data for oak (Quercus spp.) species from Fallon and Cavender-Bares' (2018) study. Percentages indicate leakage following freezing to -40°C (the lowest freezing temperature used in the study) divided by leakage following boiling. Superscripts reflect differences among means of species-treatment combinations. C) Data for a variety of species assessed during three times of year extracted from supplementary figures from Kreyling et al. (2015). Percentages indicate leakage following immersion in liquid nitrogen divided by leakage following boiling. Two axes of mean separation are presented. Differences among species within the same sampling month are indicated by lowercase letters. Differences among sampling months for a single species are indicated by uppercase letters. Color coding (red to blue) highlights species and temporal patterns in maximum freezing-induced leakage in the Kreyling et al. data.

Additional file 3. Results from Type III ANOVA of linear models assessing the consequences of species (A. caudatifolium vs. A. campestre), control type (boiling at 120C, boiling at 125 C, or liquid nitrogen immersion), and incubation time (1, 2, 5, or 7 days post-treatment) on A) conductivity after experimental freezing and B) conductivity after control treatment. Temperature of experimental freezing was included as a covariate.Samples were collected on 29 Dec. 2020.

Additional file 4. Critical cold hardiness (T50, degrees C) estimated using the Limlogistic approach for two species, A) Acer caudatifolium and B) A. campestre with control type (boiling at 120C, boiling at 125 C, or liquid nitrogen immersion) and incubation time (1, 2, 5, or 7* days post-treatment) varied. Samples were collected on 29 Dec. 2020.

Additional file 5. Species means of critical temperatures at which tissue accrued 20% and 80% damage as measured with electrolyte leakage and calculated using the Limlogistic approach. In cases for which differences among species are significant, superscripts indicate results of a Tukey's HSD post-hoc test ($\alpha = 0.05$).

Additional file 6. Description of all sampled plants. Genotypes of 10 species are all accessioned individuals in the Arnold Arboretum's living collections, as detailed below. Samples of A. negundo were collected from three spontaneously occurring plants found in the Arboretum's Bussey Brook Meadow. Samples of A. platanoides were similarly collected from three spontaneous individuals growing on the Arboretum's Weld Hill parcel.

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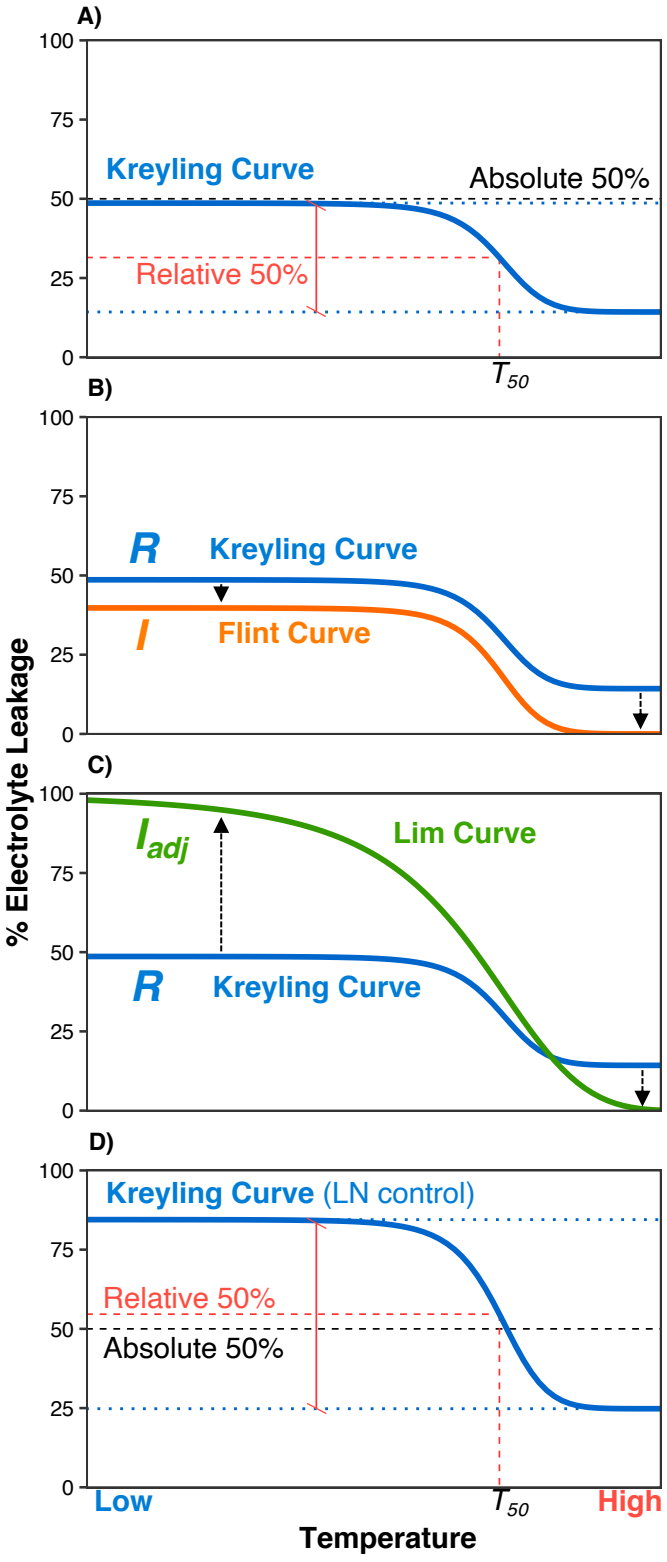

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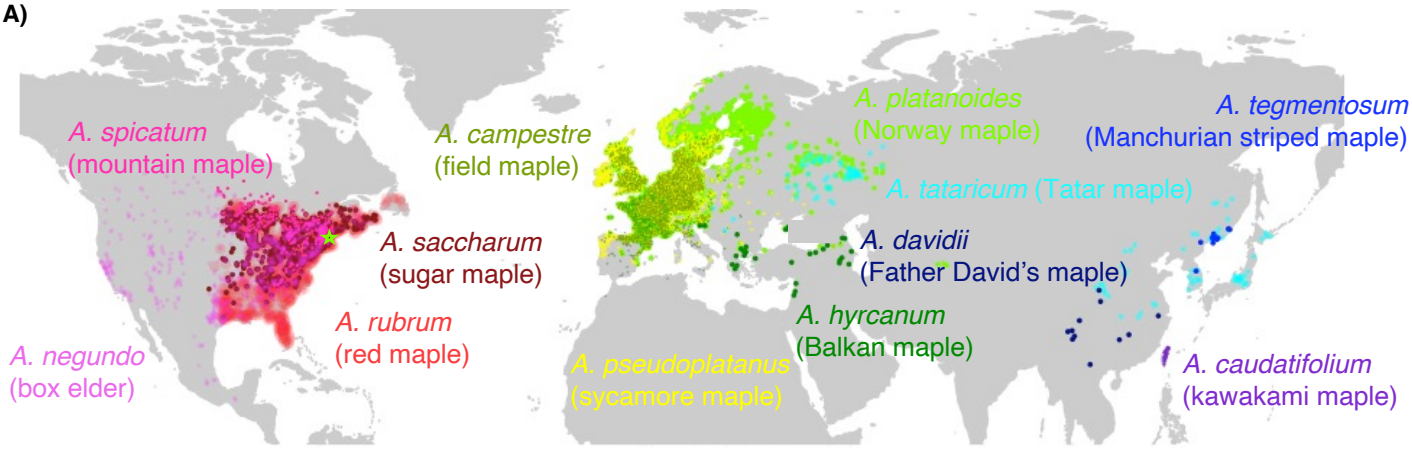

B)

Species	Section	Region	Distribution	Habit
A. saccharum	Acer	Eastern N. America	Wide	Dominant
A. pseudoplatanus	Acer	Europe	Wide	Dominant
A. rubrum	Rubra	Eastern N. America	Wide	Dominant
A. platanoides	Platanoidea	Europe	Wide	Dominant
A. campestre	Platanoidea	Western Europe	Wide	Subdominant
A. hyrcanum	Acer	S. Europe/Asia Minor	Narrow	Shrub/Understory
A. tataricum	Ginnala	Pan-Asian	Wide	Ruderal
A. tegmentosum	Macrantha	East Asian	Narrow	Understory
A. caudatifolium	Macrantha	East Asian	Narrow	Understory
A. davidii	Macrantha	East Asian	Wide	Understory
A. spicatum	Spicata	Eastern N. America	Wide	Understory
A. negundo	Negundo	Pan-North American	Wide	Riparian

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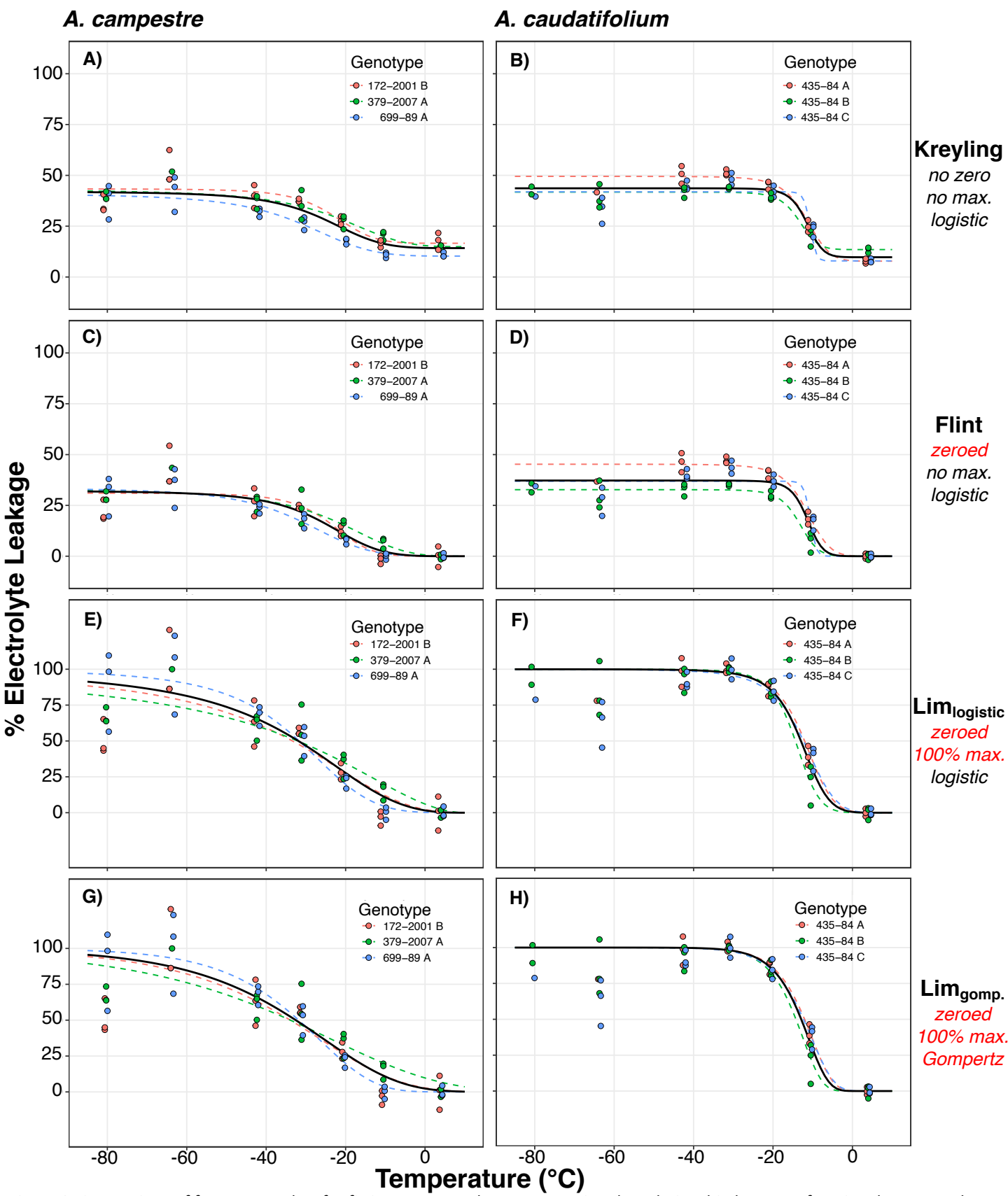

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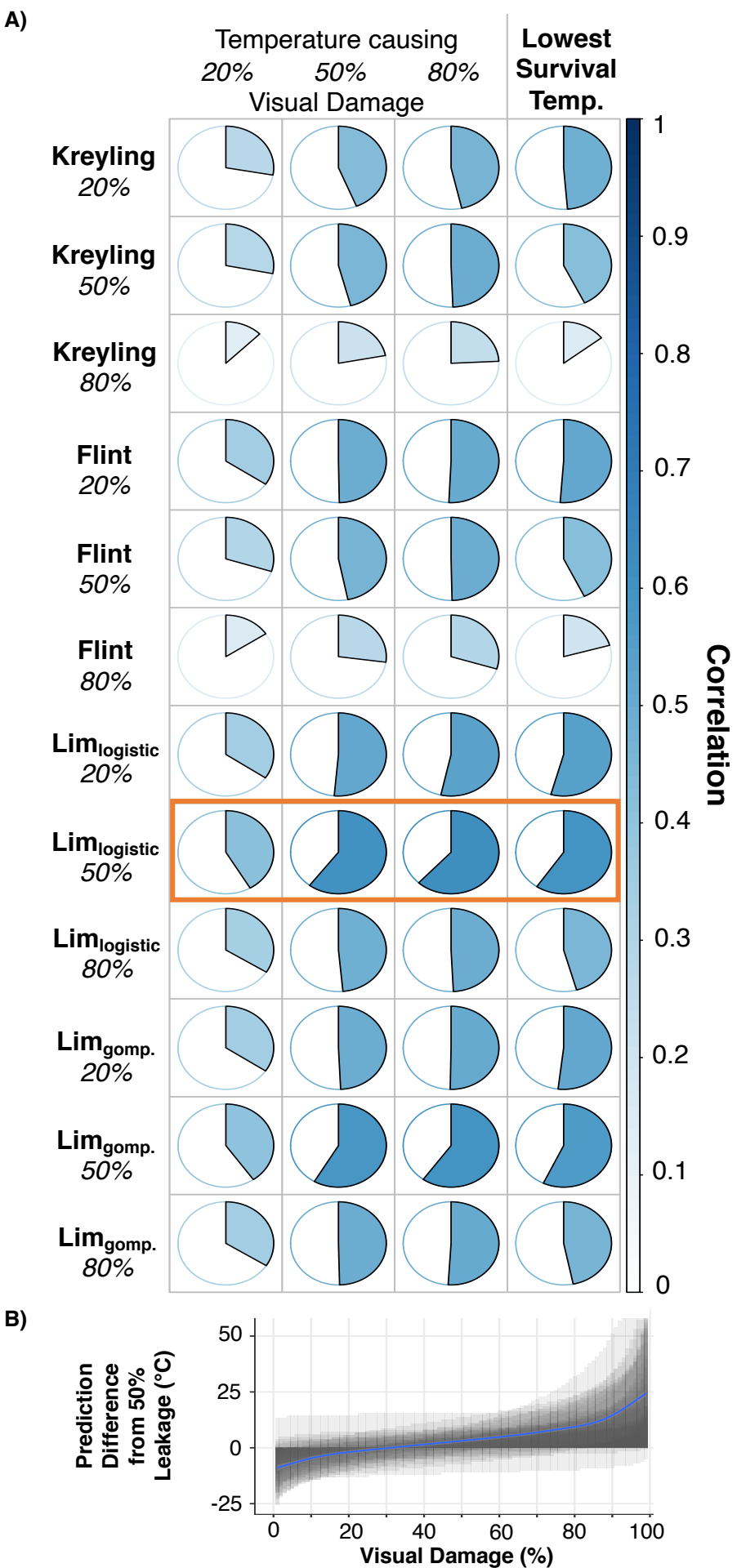

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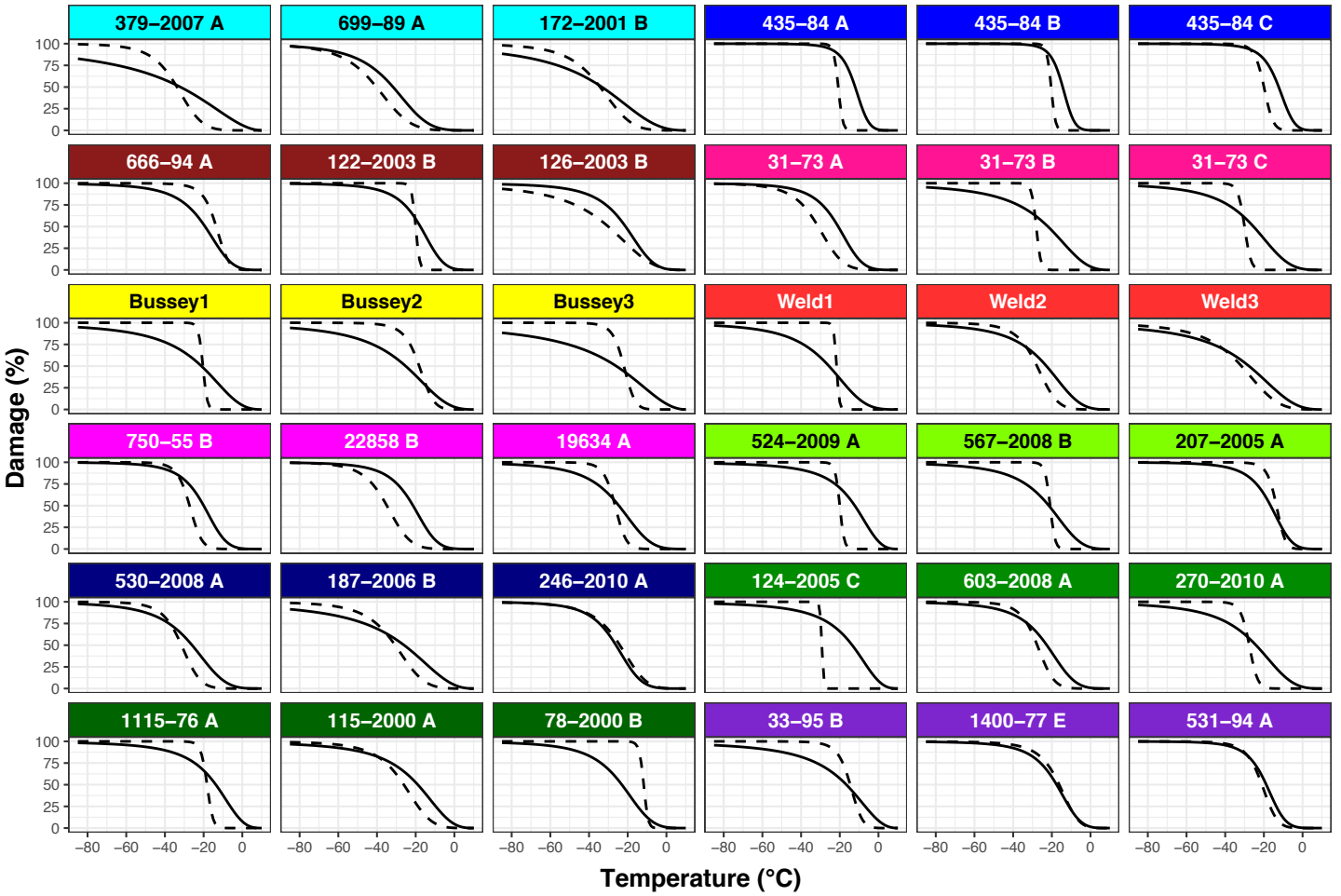

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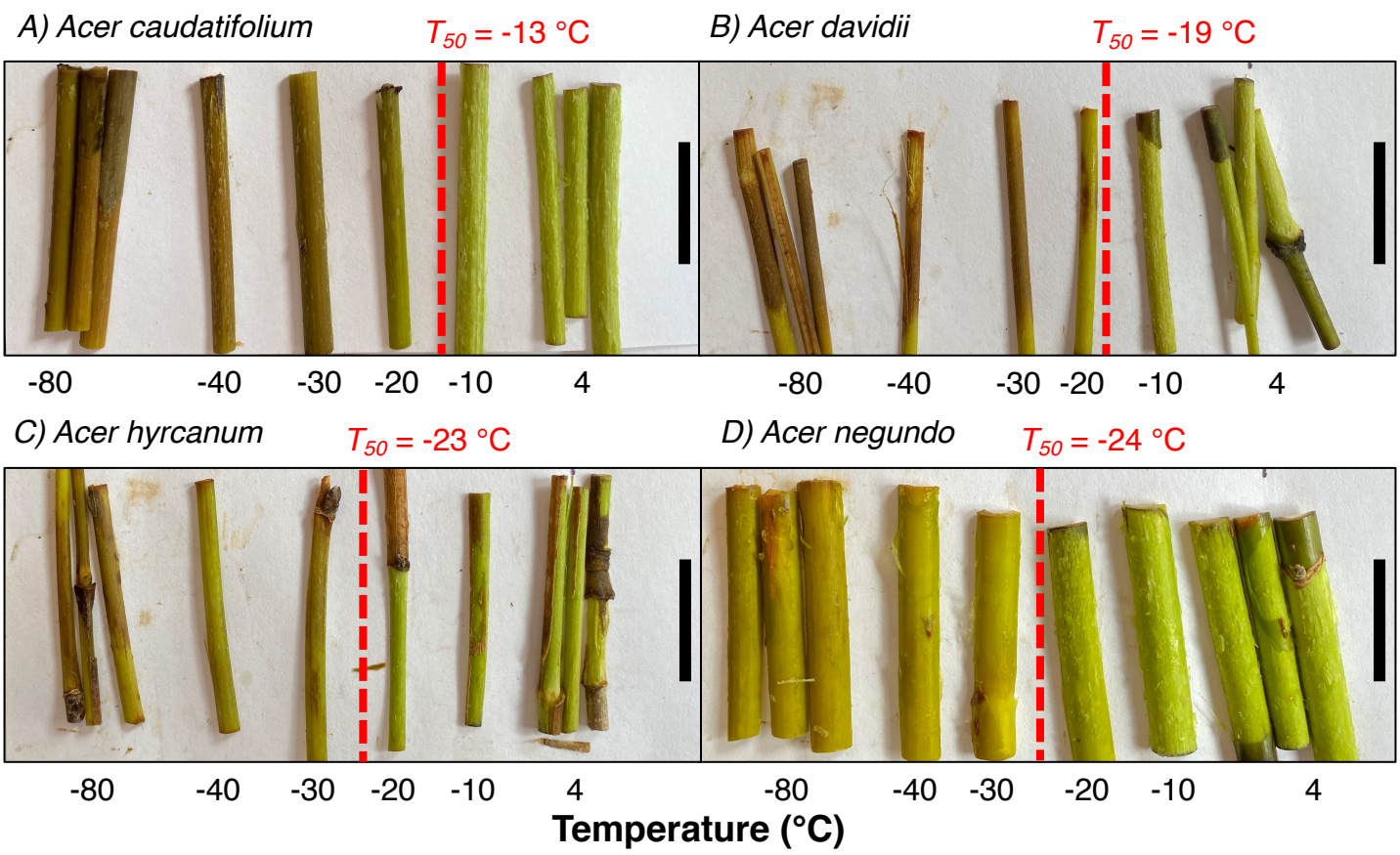

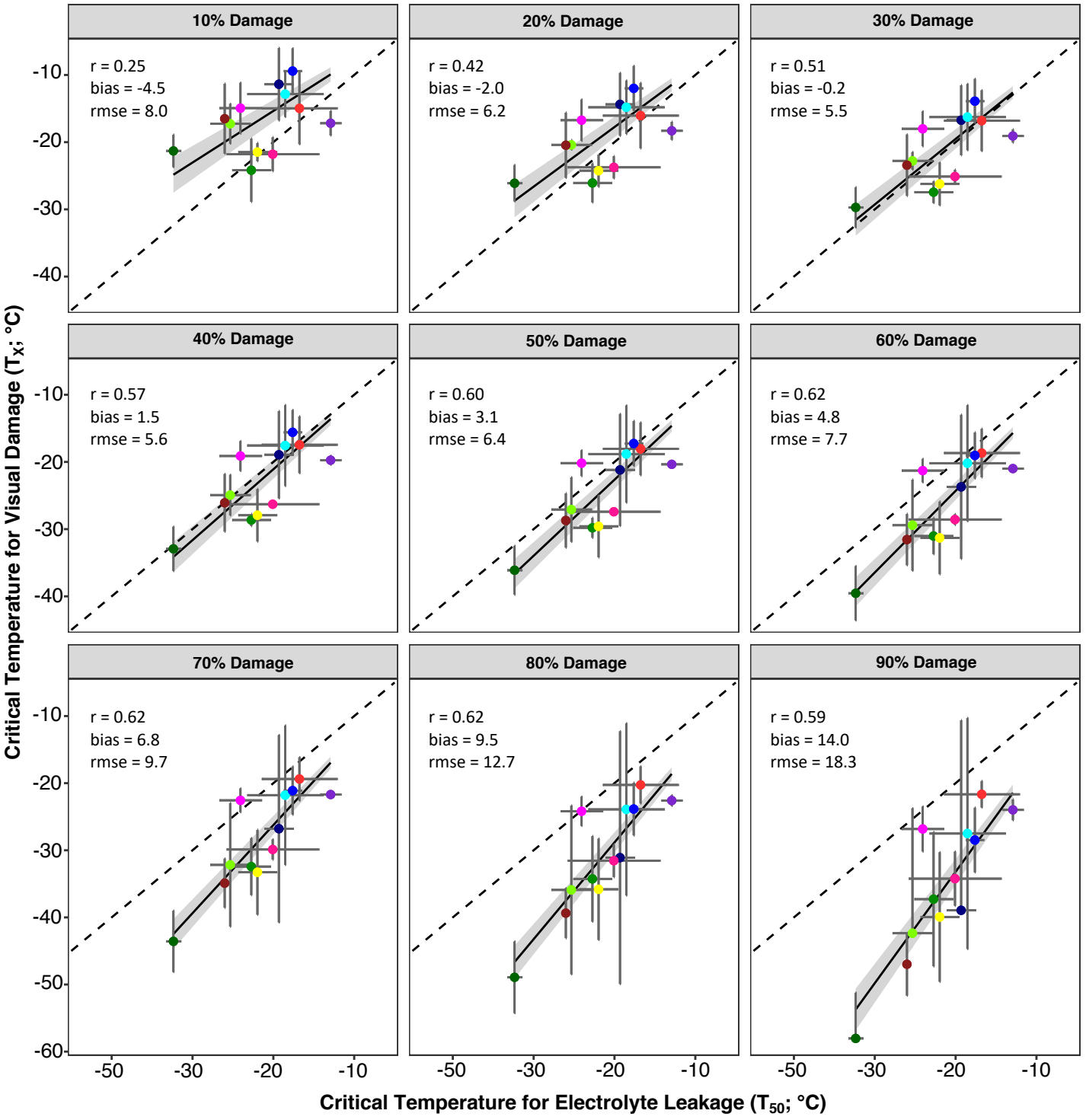

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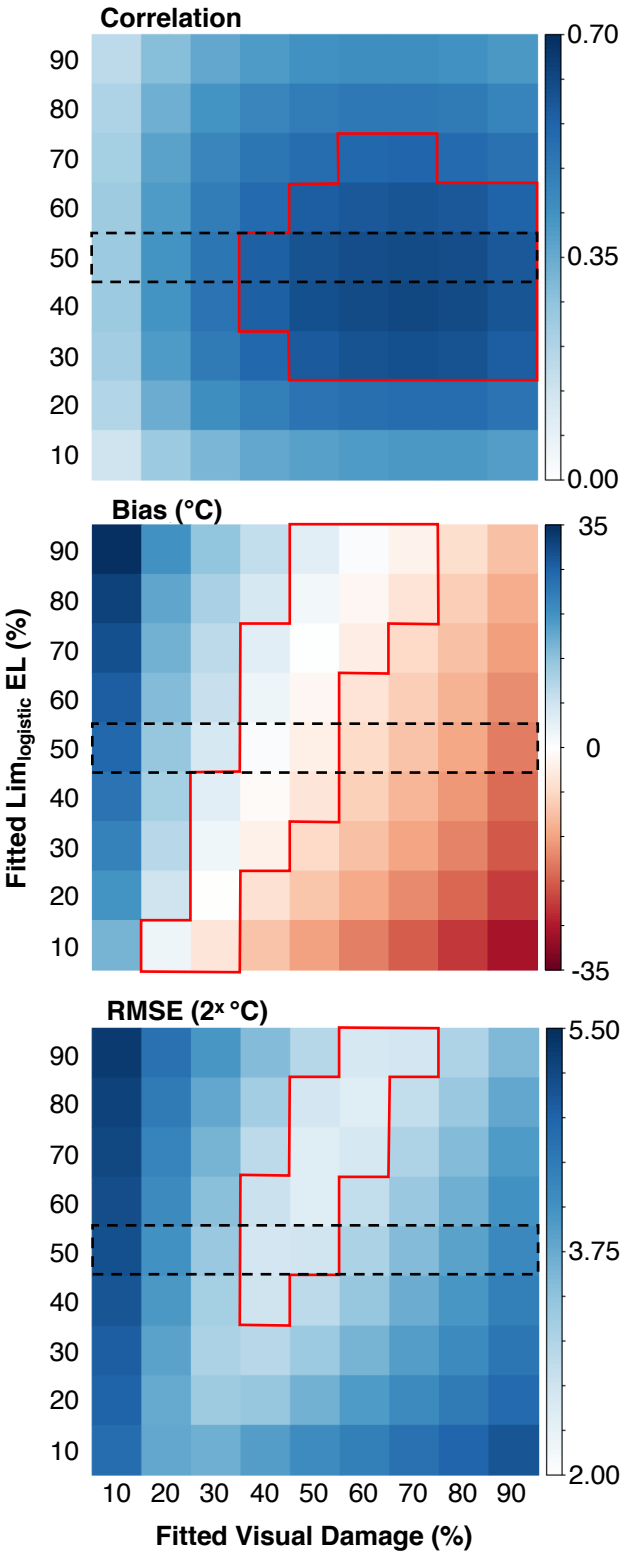

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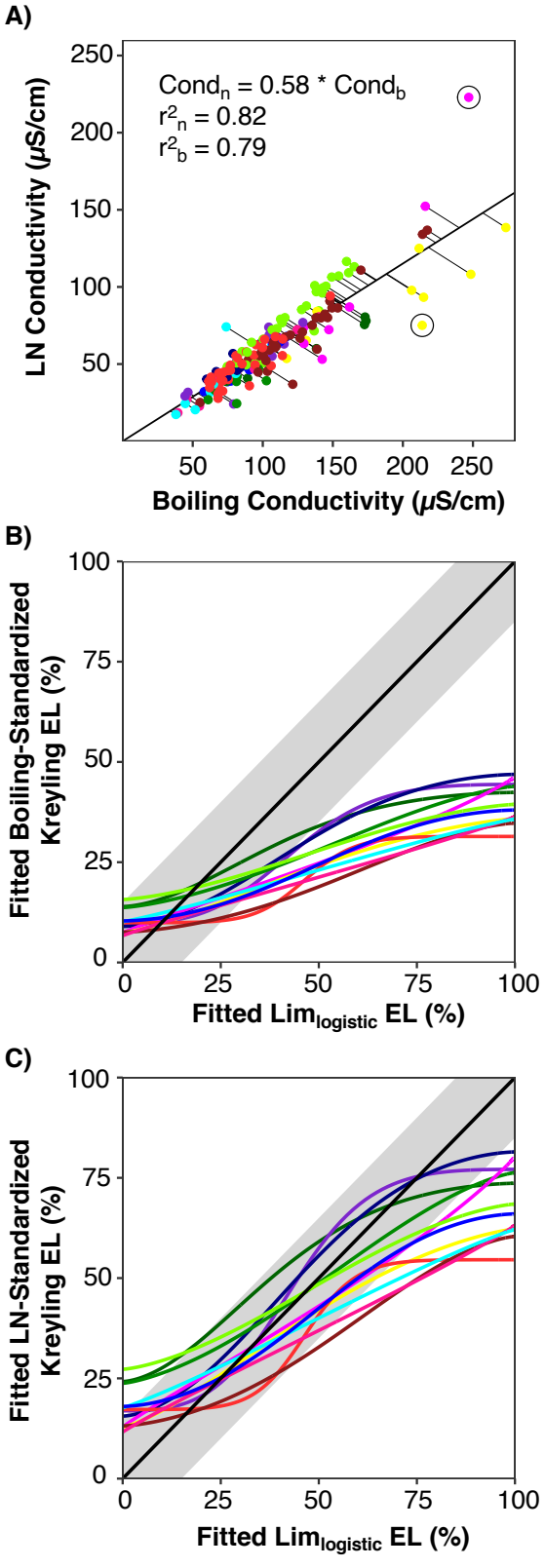

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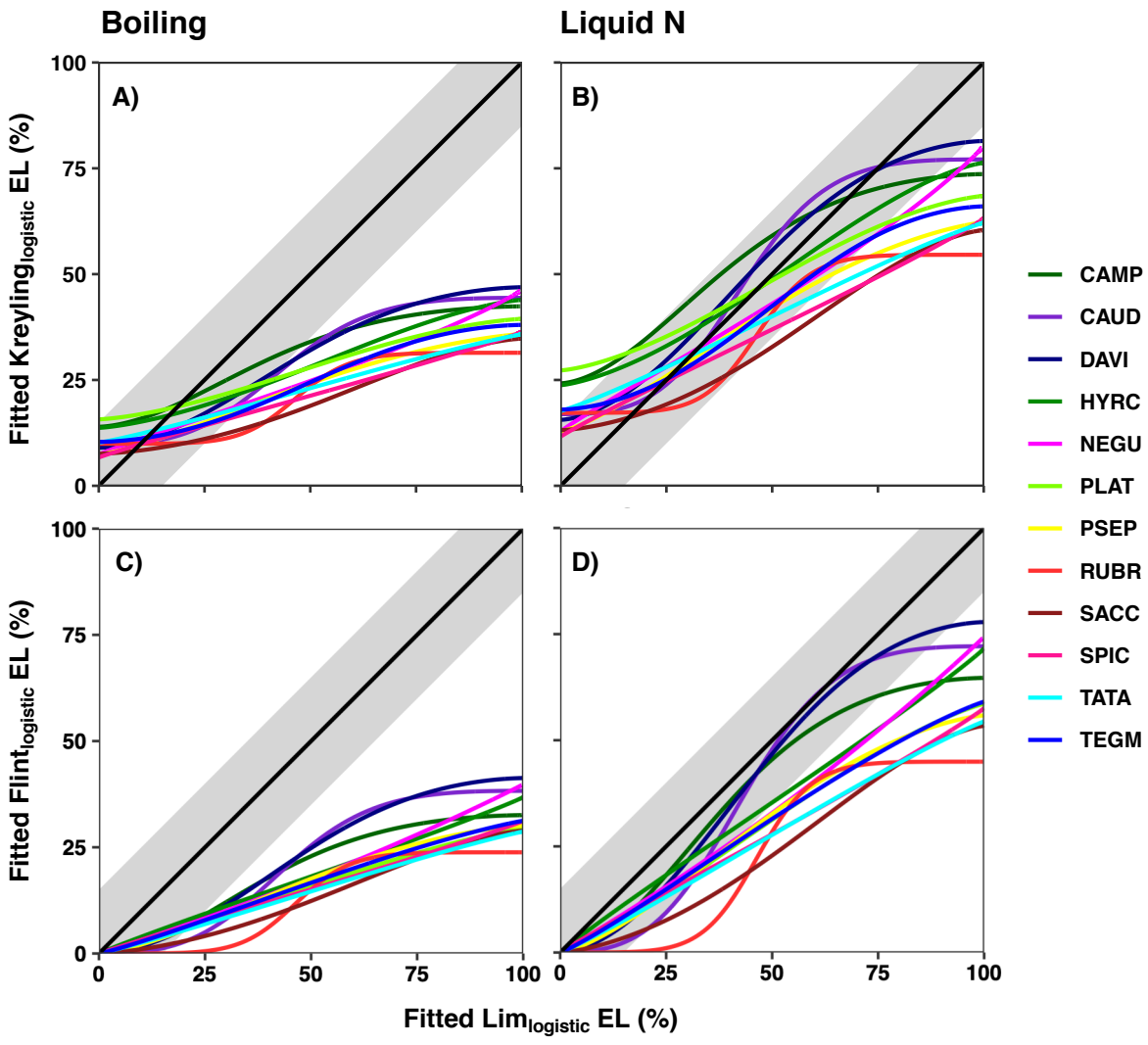

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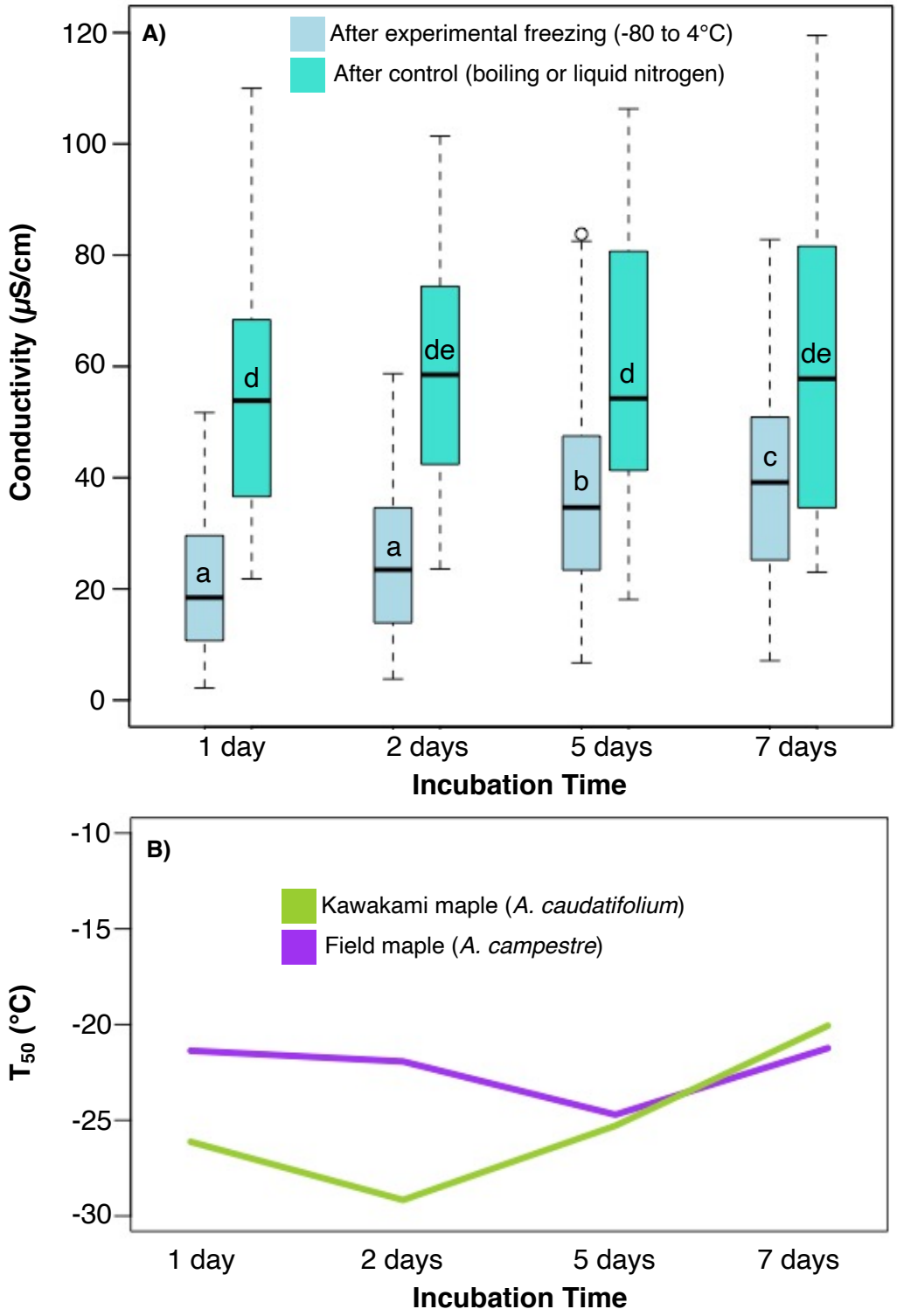
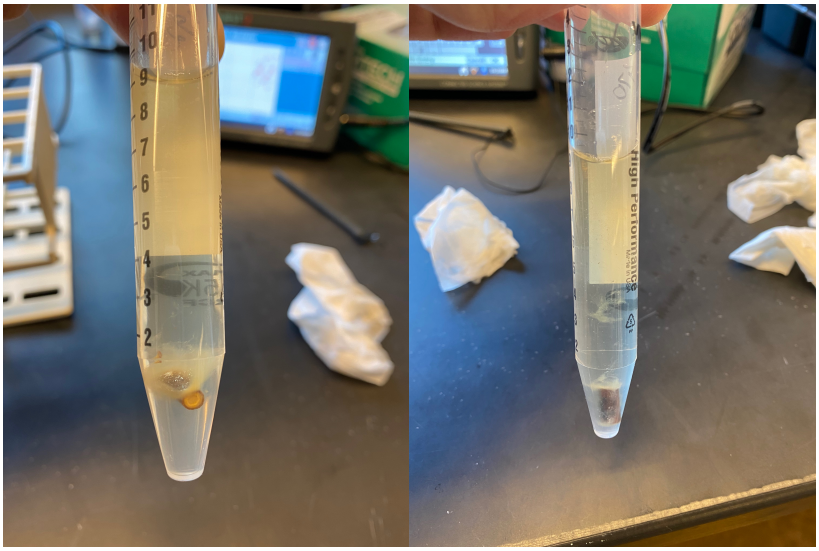


Figure 11. A) Electrolyte leakage increased gradually over seven days following experimental freezing (light blue), but not exposure to a boiling or liquid nitrogen control (turquoise), with no evidence of a significant difference when conductivity was measured over the first 48 hours after freezing. Lowercase letters indicate significant differences in conductivity measured at different time points at the 0.05 level based on models reported in Additional file 3 panels A (light blue, a-c) and B (turquoise, d-e). B) As a result of this pattern, estimates of critical values for cold hardiness (T_{50}) are consistent and reflect species differences when samples were incubated for one or two days, but not when they were incubated for 5 or 7 days (Additional file 4).

Figure 12. Stem segments incubated for longer than five days following control treatment (boiling or liquid nitrogen immersion) tended to deteriorate, showing evidence of microbial growth.



Figures

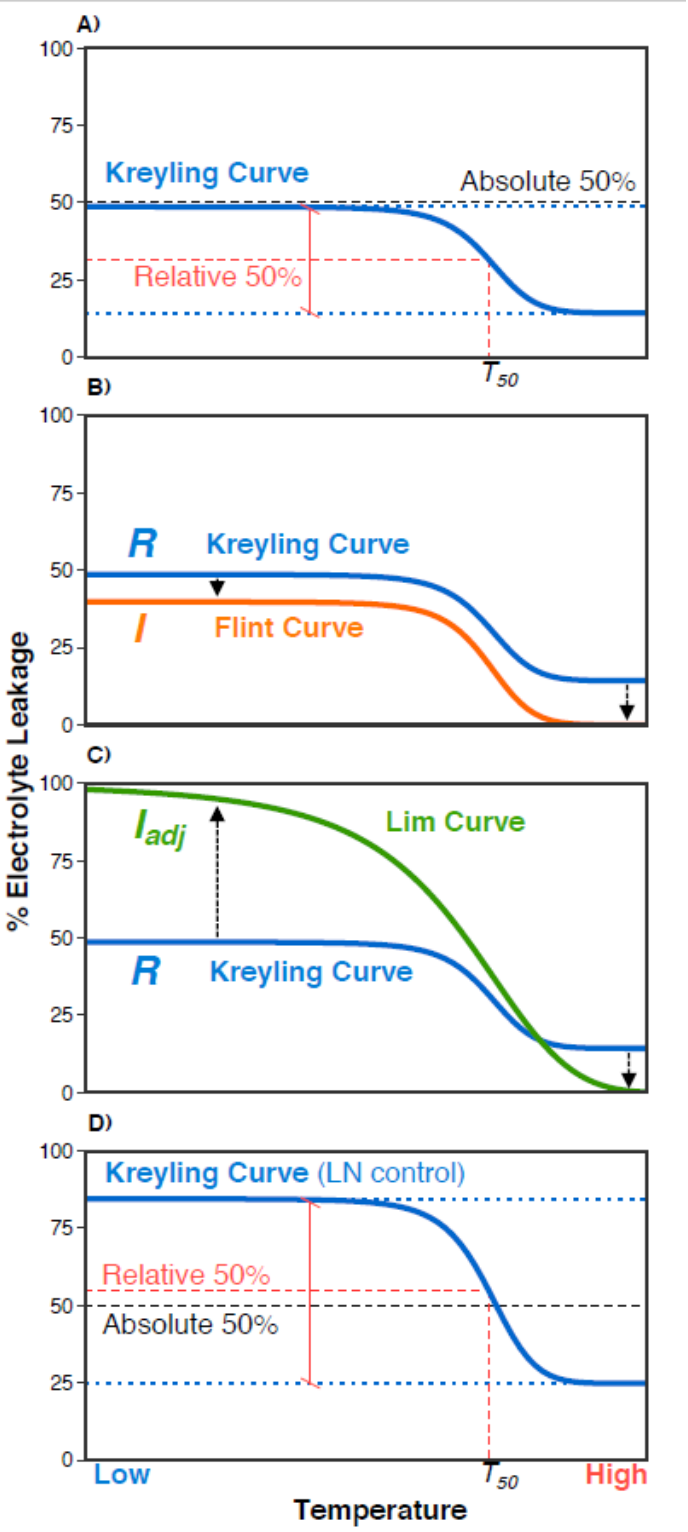
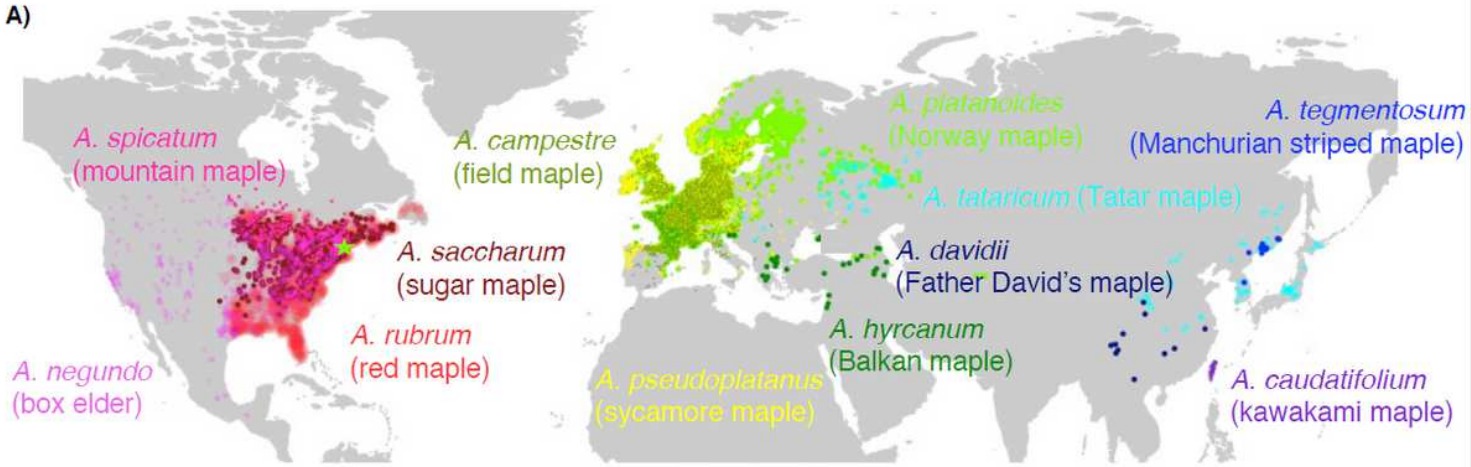


Figure 1

Schematic comparing different approaches to measuring electrolyte leakage. As described in the main text (Fig. 2), we contrast the Kreyling approach (no minimum or maximum leakage specified, yields R) with the Flint approach (minimum but no maximum, yields I) and the Lim approach (minimum and

maximum specified, yields Iadj). A) In the Kreyling approach, the temperature at which a sample accrues 50% of possible damage (T50 for R) may not be meaningful; many samples may not reach this “Absolute 50%” damage point. Instead, the temperature at which “Relative 50%” damage is attained may be more meaningful. B) By comparison, values of I, in the Flint approach (orange line) are zeroed, although this may not drastically displace the curve relative to a Kreyling curve. C) In the Lim approach, data are forced to reach 100% damage, usually at the coldest temperature employed to freeze samples. Curve shapes may differ and critical temperatures (e.g., T50) corresponding to Iadj (extracted from the Lim curve) may or may not differ from those associated with R (Kreyling curve). D) Use of a liquid nitrogen control is expected to elevate all leakage values, making, for instance, a Kreyling curve behave more like a Lim curve and improving generalizability among approaches.



B)

Species	Section	Region	Distribution	Habit
A. saccharum	Acer	Eastern N. America	Wide	Dominant
A. pseudoplatanus	Acer	Europe	Wide	Dominant
A. rubrum	Rubra	Eastern N. America	Wide	Dominant
A. platanoides	Platanoidea	Europe	Wide	Dominant
A. campestre	Platanoidea	Western Europe	Wide	Subdominant
A. hyrcanum	Acer	S. Europe/Asia Minor	Narrow	Shrub/Understory
A. tataricum	Ginnala	Pan-Asian	Wide	Ruderal
A. tegmentosum	Macrantha	East Asian	Narrow	Understory
A. caudatifolium	Macrantha	East Asian	Narrow	Understory
A. davidii	Macrantha	East Asian	Wide	Understory
A. spicatum	Spicata	Eastern N. America	Wide	Understory
A. negundo	Negundo	Pan-North American	Wide	Riparian

Figure 2

Schematic comparing different approaches to measuring electrolyte leakage. As described in the main text (Fig. 2), we contrast the Kreyling approach (no minimum or maximum leakage specified, yields R) with the Flint approach (minimum but no maximum, yields I) and the Lim approach (minimum and maximum specified, yields Iadj). A) In the Kreyling approach, the temperature at which a sample accrues 50% of possible damage (T50 for R) may not be meaningful; many samples may not reach this “Absolute 50%” damage point. Instead, the temperature at which “Relative 50%” damage is attained may be more meaningful. B) By comparison, values of I, in the Flint approach (orange line) are zeroed, although this

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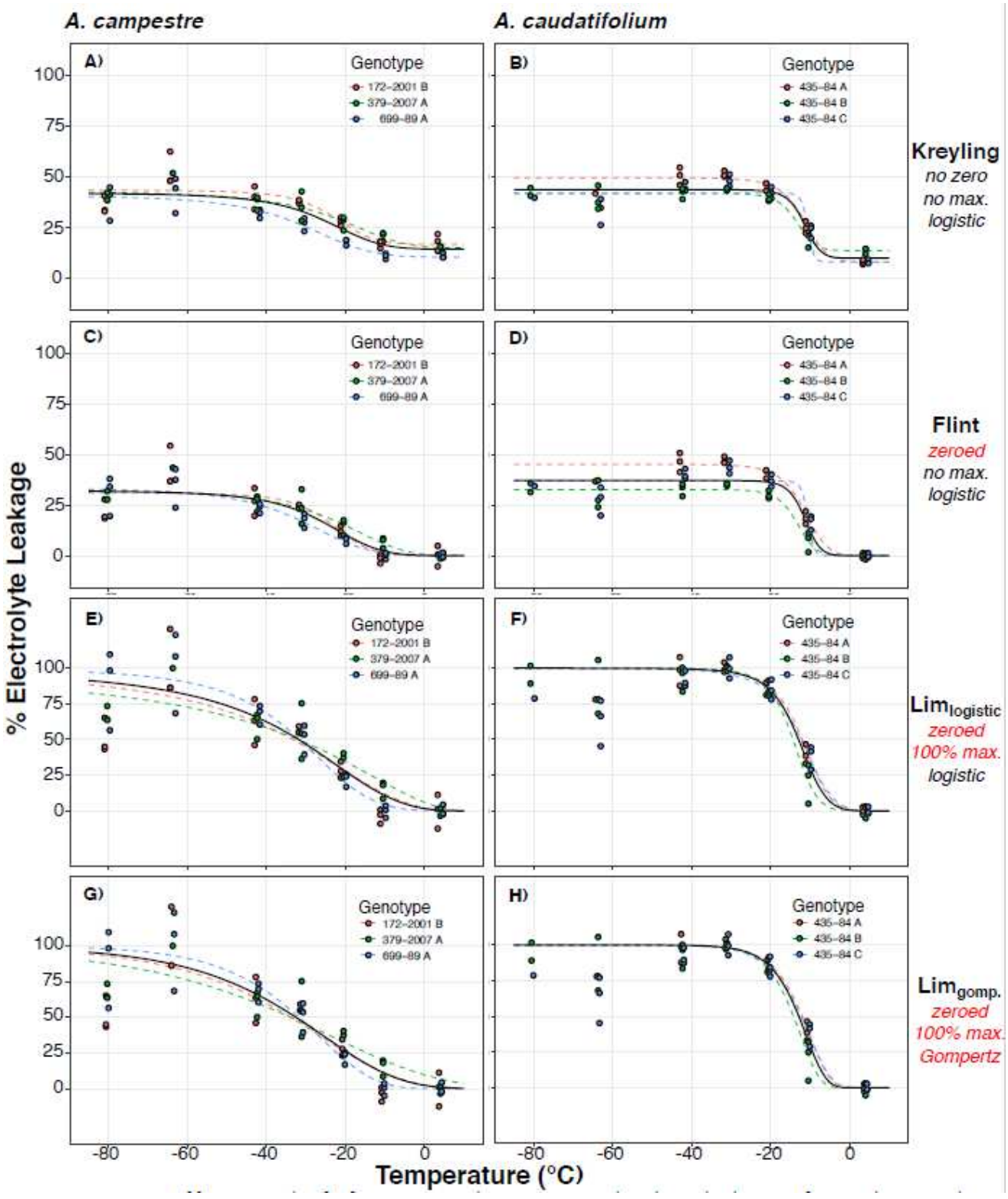

Figure 3

Comparison of four approaches for fitting curves to data representing the relationship between freezing damage and temperature in (A, C, E, G) A. caudatifolium and (B, D, F, H) A. campestre stem segments (plots for other species provided in Appendix 1). Curves fit to data on a per-genotype (red, blue, and green) and per-species (black curve) basis are fit in each case. Panels show curves fit following the approach of A, B) Kreyling and colleagues' (2015), C, D) Flint et al. (1987), and Lim et al. (1998). Approaches vary, as

indicated, in their use of room-temperature (zeroing; C-H) and deep freezing (maximum damage; E-H) controls and reliance on general logistic (A-F) vs. Gompertz (G-H) curves.

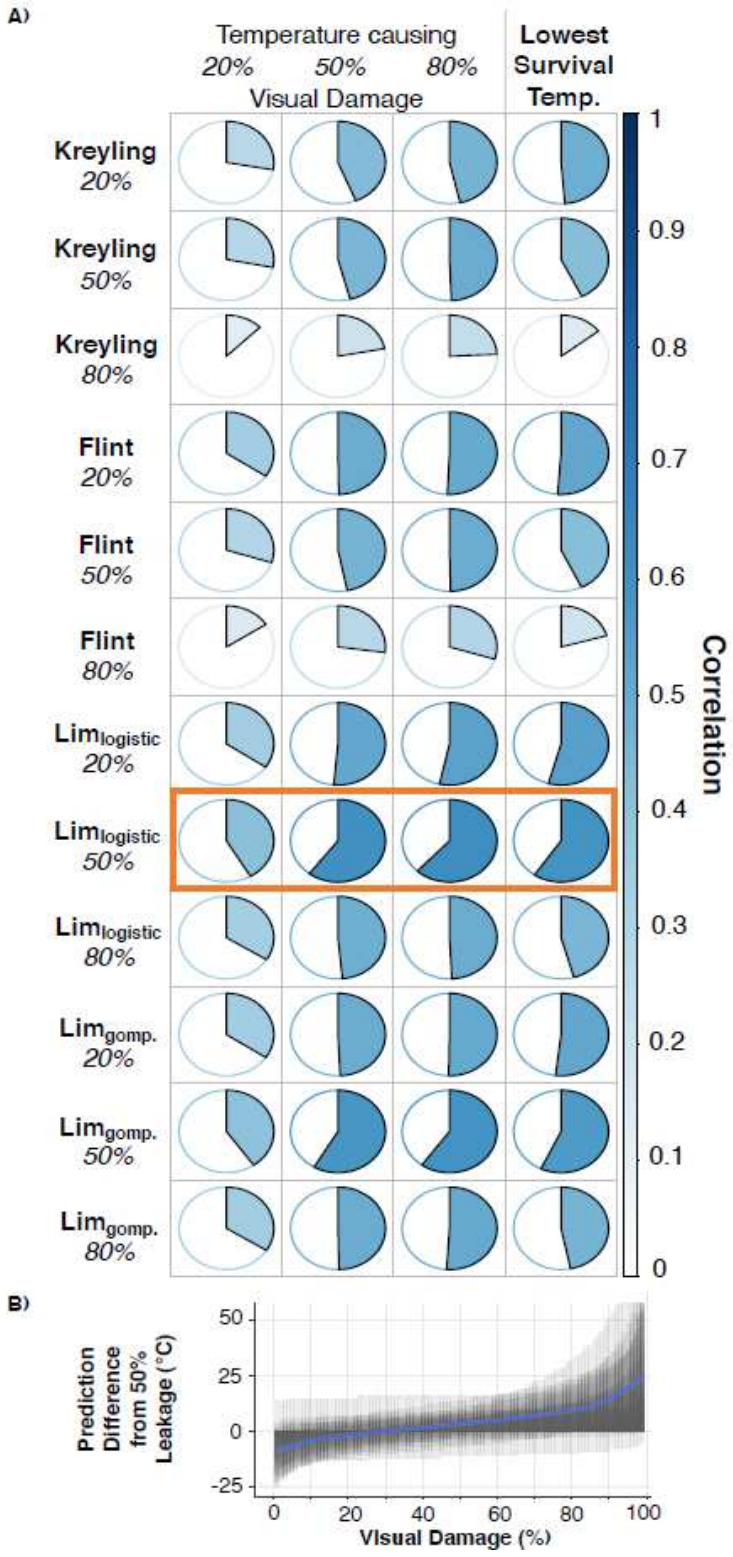

Figure 4

A) Validation of critical values from four approaches to modeling electrolyte leakage (as in Fig. 1) against visual estimates of freezing damage. Critical values reflect either 20%, 50%, or 80% electrolyte leakage (rows) or visual damage (columns). The rightmost column indicates lowest survival temperature (LST),

the lowest temperature at which stems experienced < 50% damage. Pie wedge size and color indicate correlation. B) 50% electrolyte leakage values using the Limlogistic approach (orange box) best predicted visual damage in the 40-60% damage range.

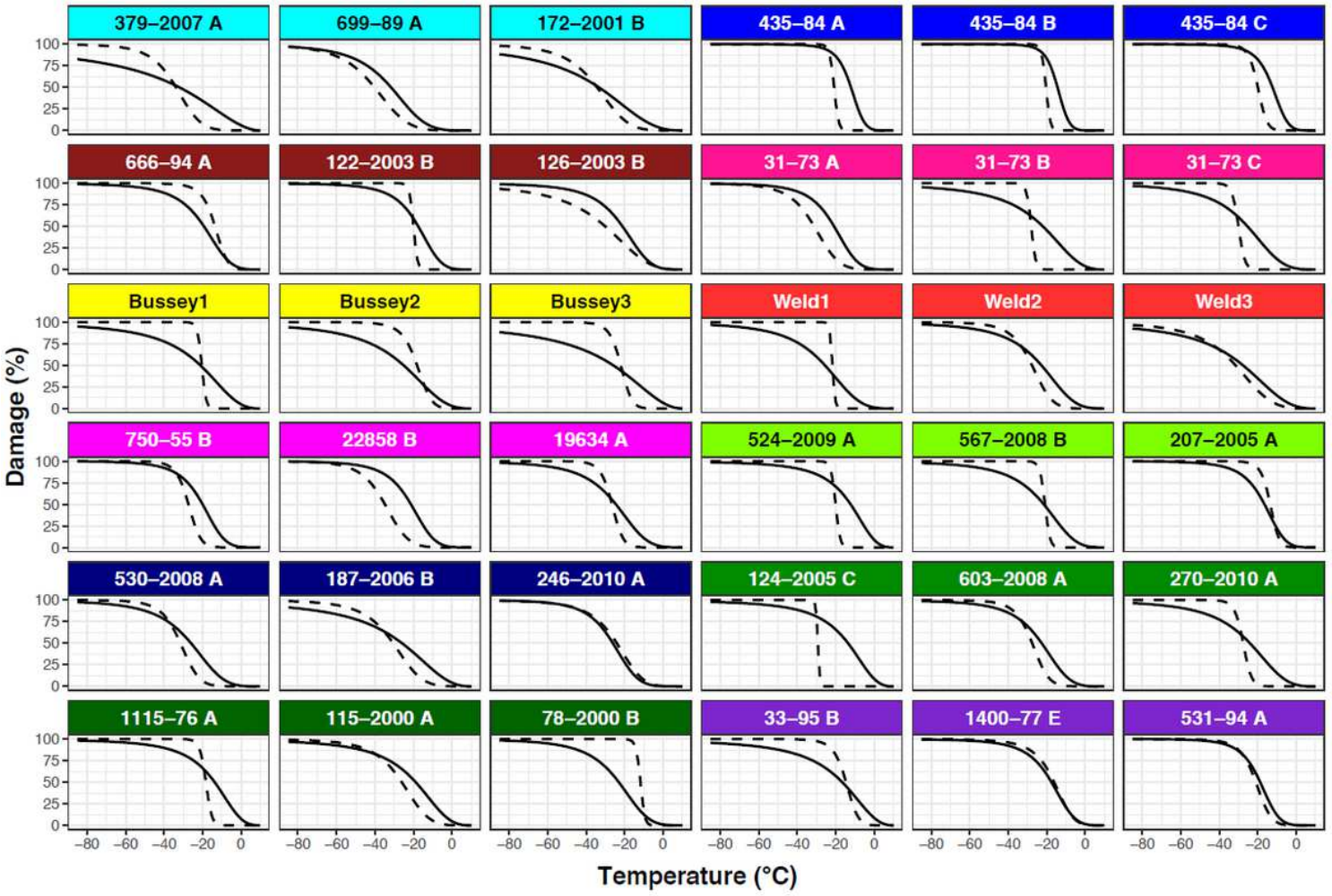

Figure 5

Damage, as reflected by electrolyte leakage (solid lines) and visual estimates (dashed lines), induced by freezing from -10 to 80□. Electrolyte leakage is calculated using the Limlogistic approach. Panels represent estimates of damage to particular genotypes. Color-coding indicates species as in Fig. 2; species are also presented in alphabetical order.

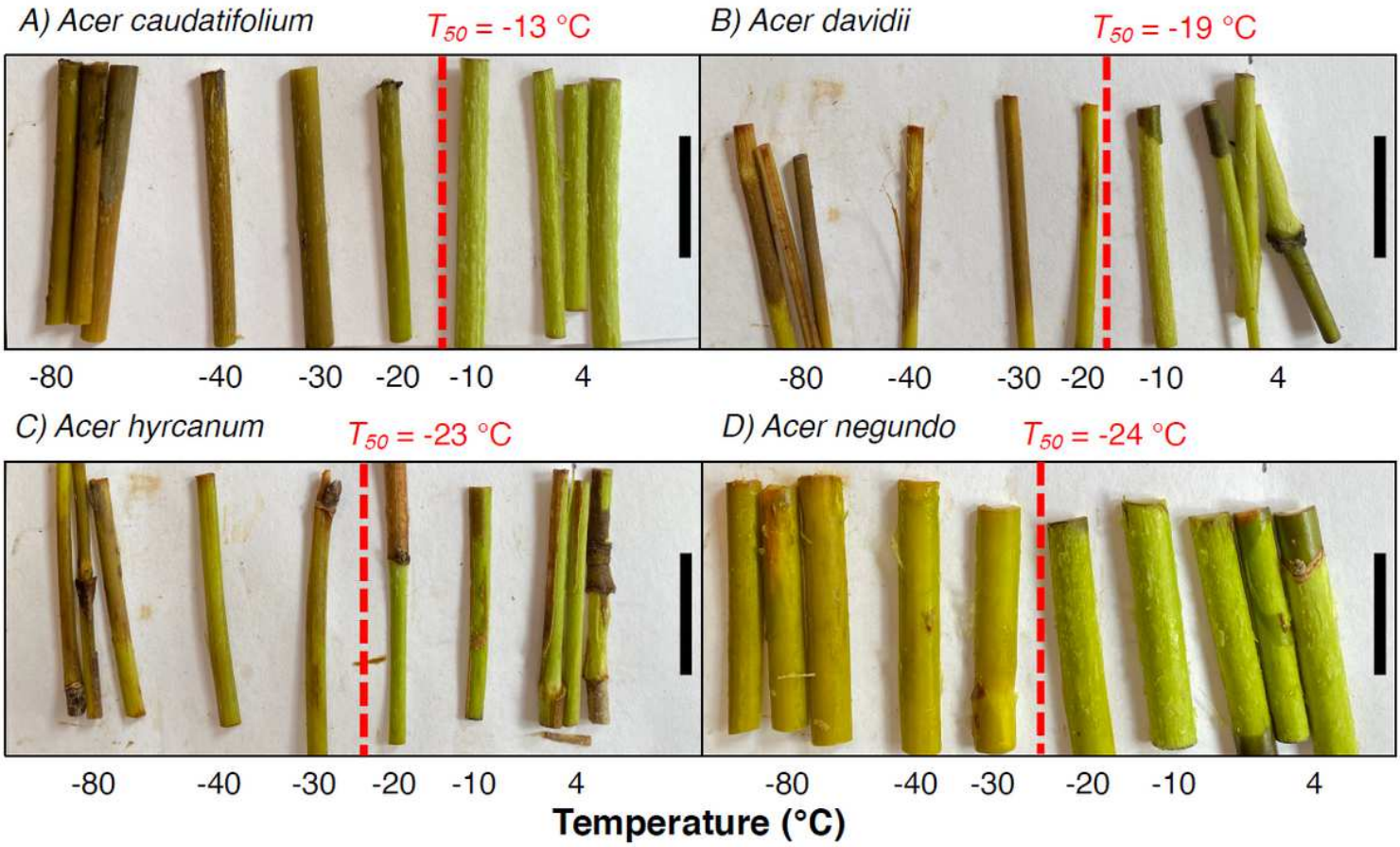

Figure 6

Visual cambial damage corresponded to critical cold hardiness estimated from electrolyte leakage data. Values of T50 given here (Table 1) are calculated using the Limlogistic approach. Representative stem samples following freezing are shown for A) Acer caudatifolium, B) A. davidii, C) A. hyrcanum, and D) A. negundo. Scale bar = 0.5 cm.

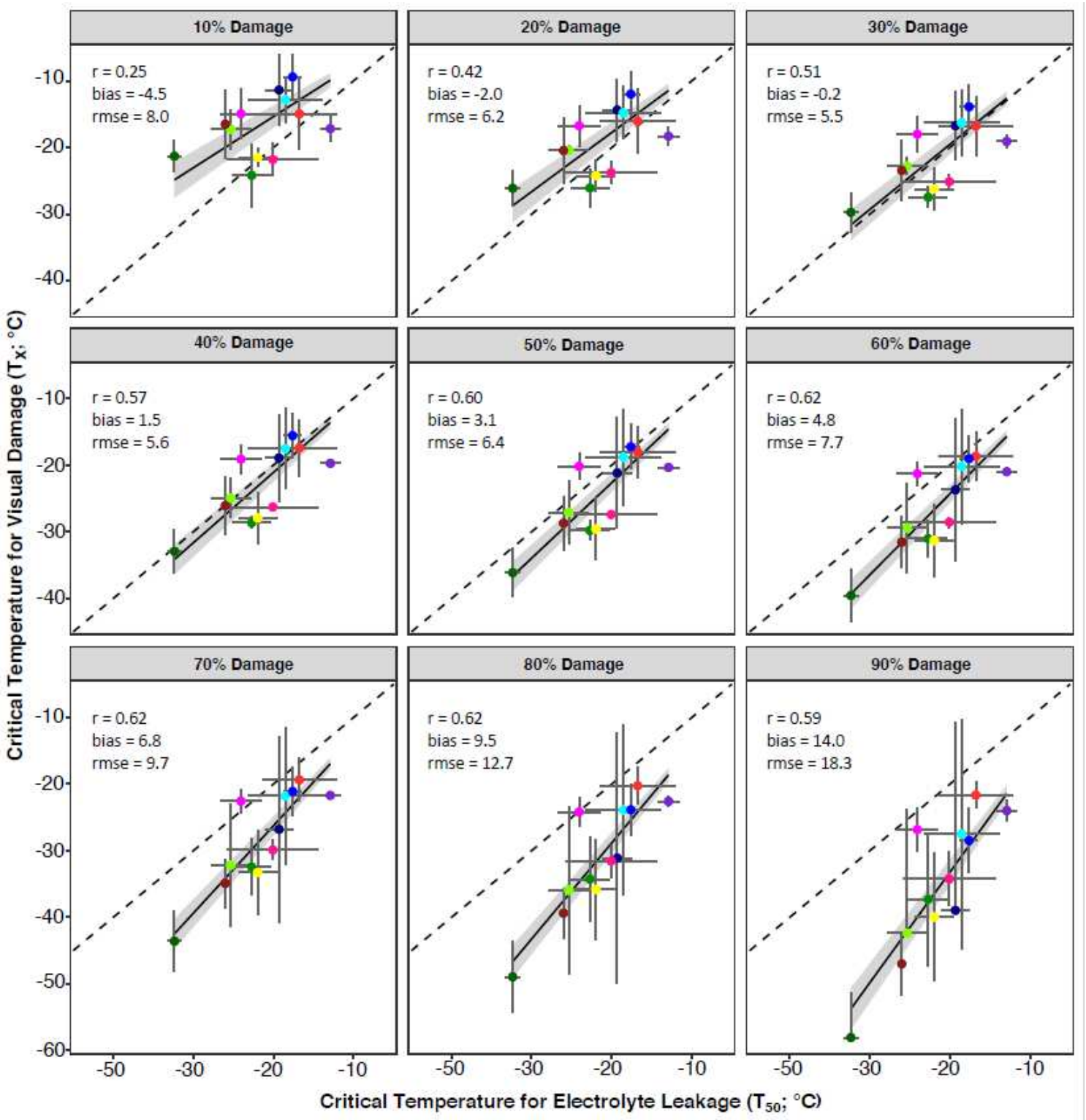

Figure 7

Critical electrolyte leakage (estimated using the Limlogistic approach) best approximates 50% visual damage when leakage is between 50 and 80%. Bias, though, is lowest from 20 to 50% leakage. Color-coding indicates species as in Fig. 2. Error reflects variation among genotypes of a given species.

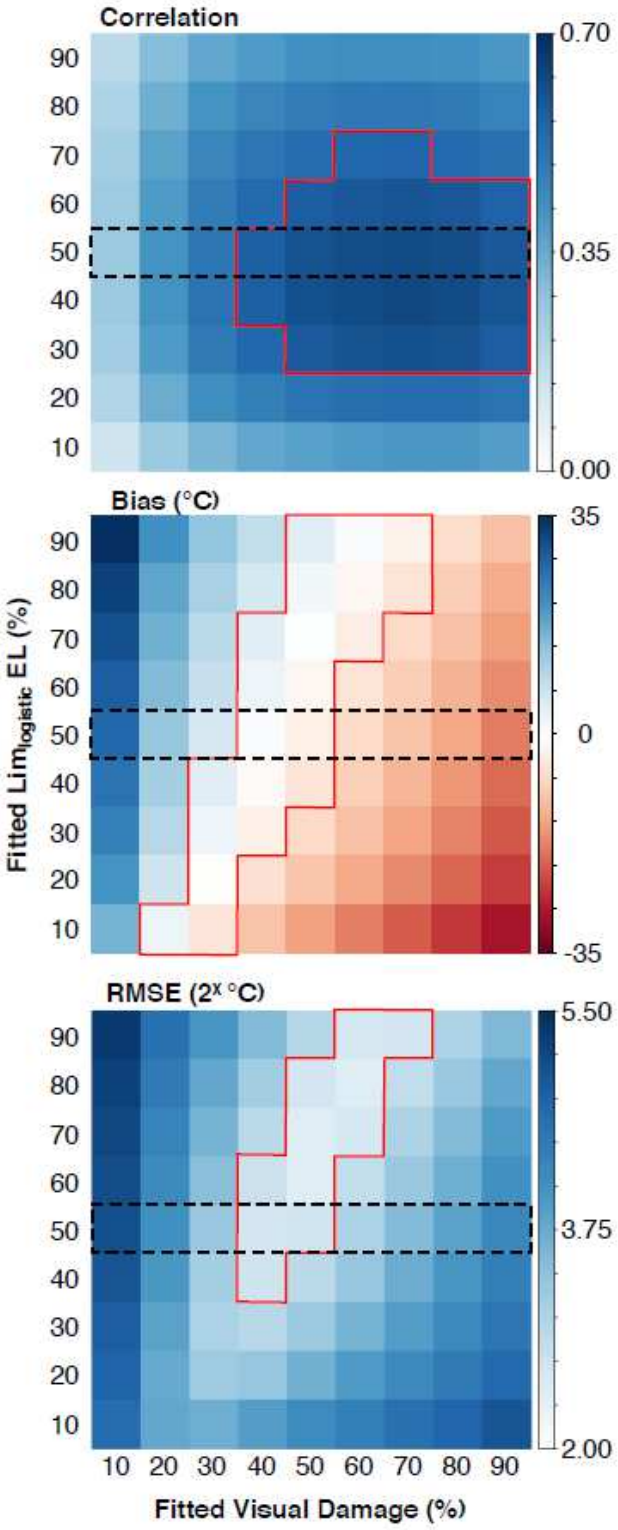

Figure 8

Fitness characteristics of the relationship between fitted values of electrolyte leakage and visual damage. Red contour delimits the area where: A) Correlation is greater than 0.55; B) Bias < abs (5); and RMSE < 7 . Dashed rectangle delimits data used in Figure 7.

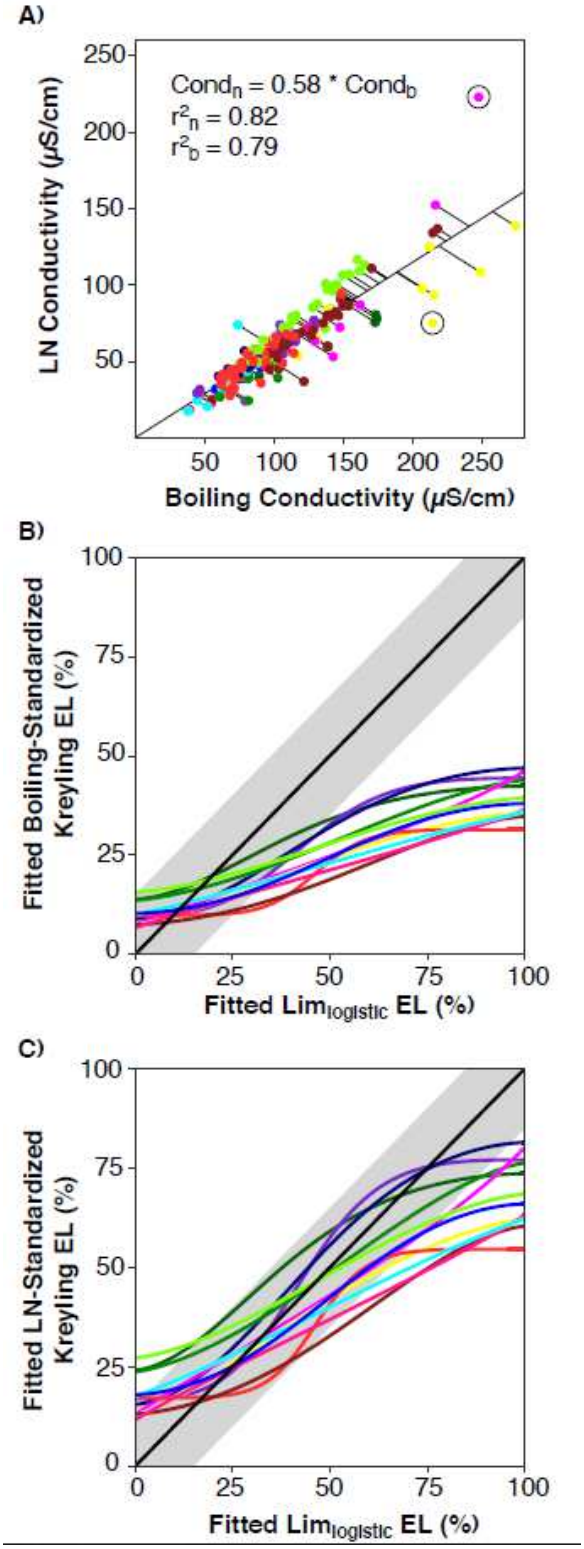

Figure 9

A) Sample conductivity following boiling predicts conductivity following immersion in liquid nitrogen across a range of values and for diverse species (color-coding indicates species as in Fig. 2.). Circled points are statistical outliers and lines indicate Deming regression error. r2 were calculated based on residuals in each direction. B) When a boiling standard is used, electrolyte leakage values derived using different curve-fitting procedures (e.g. Kreyling vs. Limlogistic) are not comparable above ~25% leakage.

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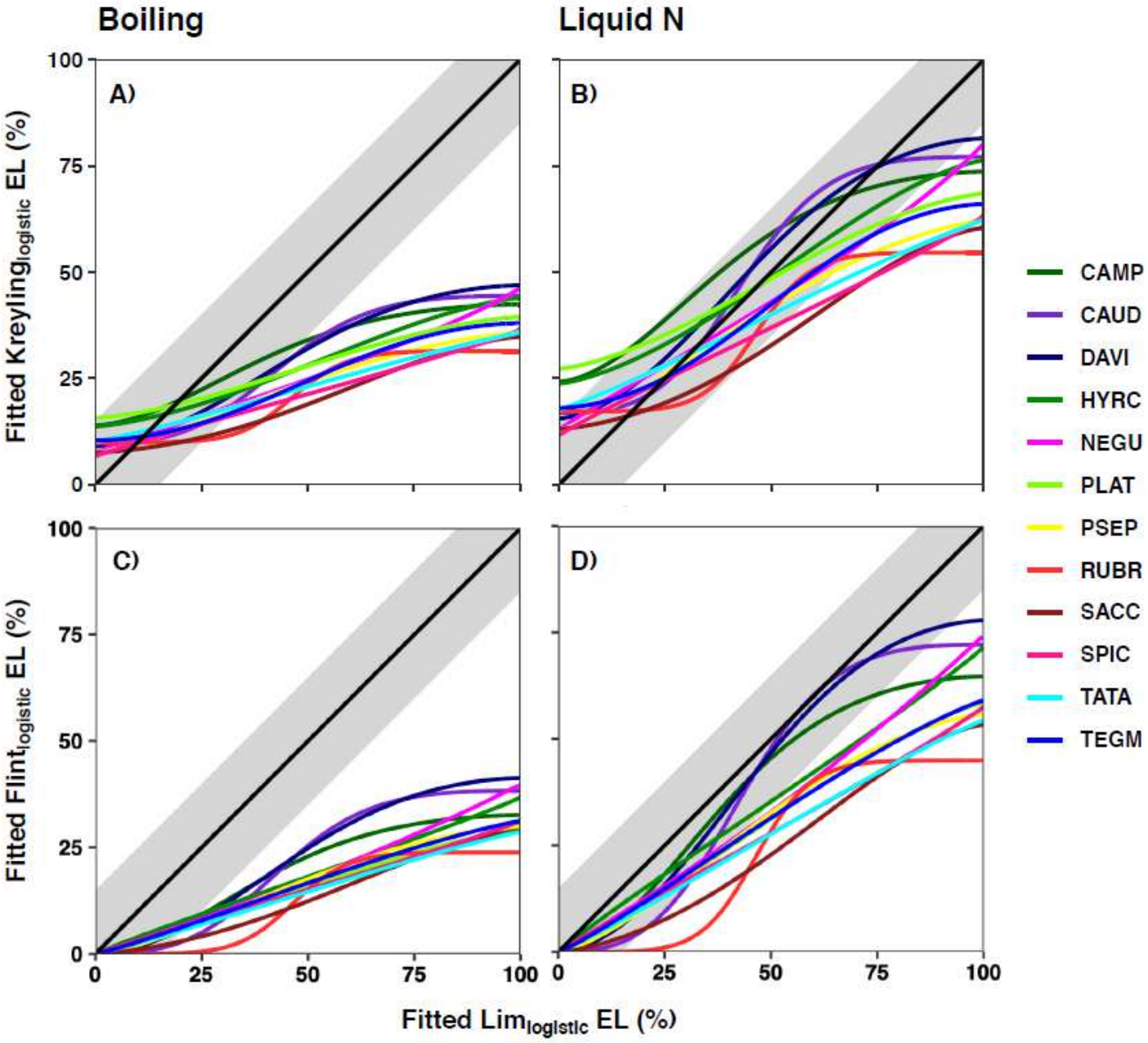

Figure 10

When a boiling standard is used, electrolyte leakage values derived using different curve-fitting procedures (e.g. Kreyling vs. Limlogistic vs. Flint approaches) are not comparable above ~25% leakage (A vs. B). However, use of a liquid nitrogen standard makes outputs of these two routines more comparable (C vs. D). Grey bar indicates a range of values within 15% of the 1:1 line.

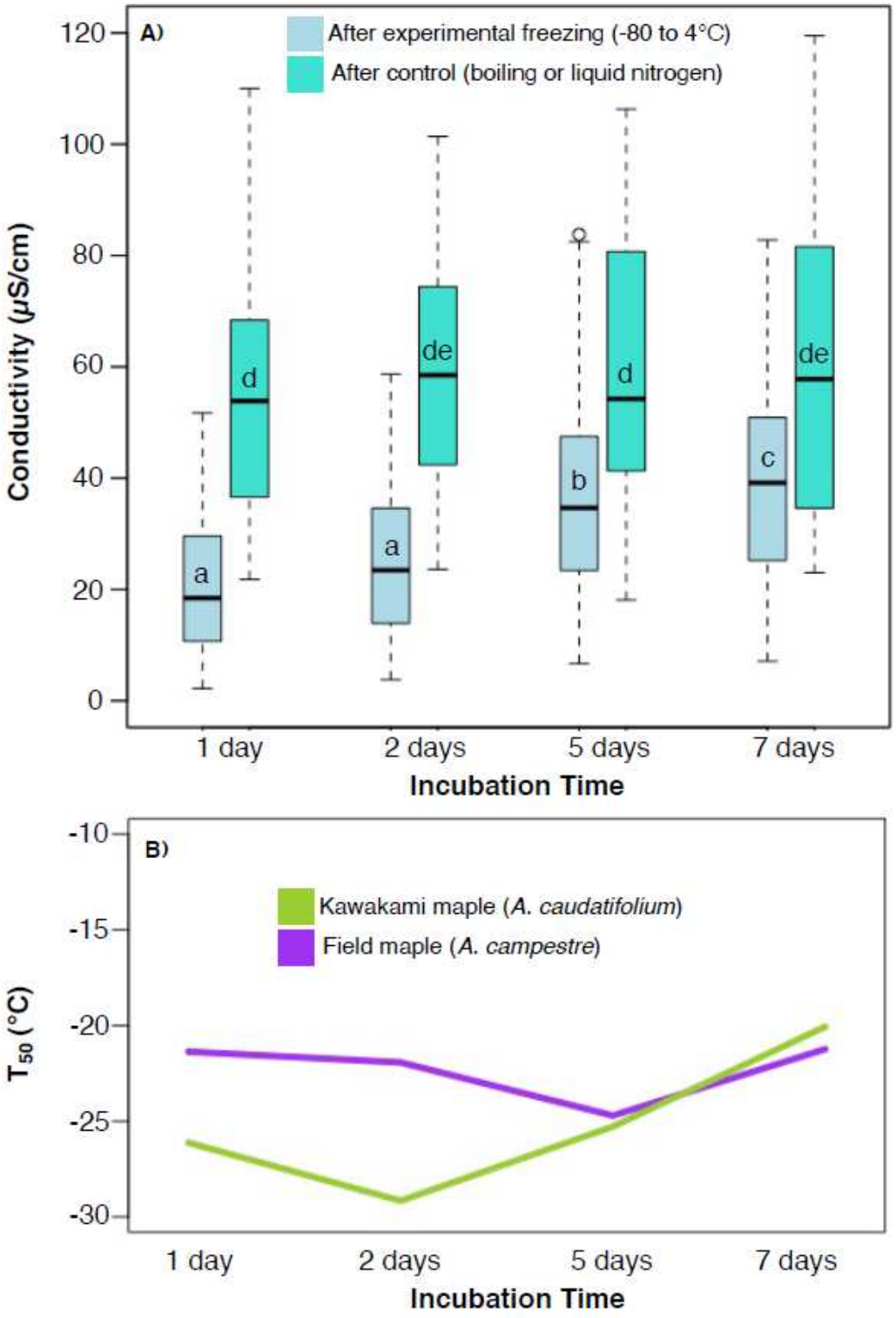

Figure 11

A) Electrolyte leakage increased gradually over seven days following experimental freezing (light blue), but not exposure to a boiling or liquid nitrogen control (turquoise), with no evidence of a significant difference when conductivity was measured over the first 48 hours after freezing. Lowercase letters indicate significant differences in conductivity measured at different time points at the 0.05 level based on models reported in Additional file 3 panels A (light blue, a-c) and B (turquoise, d-e). B) As a result of

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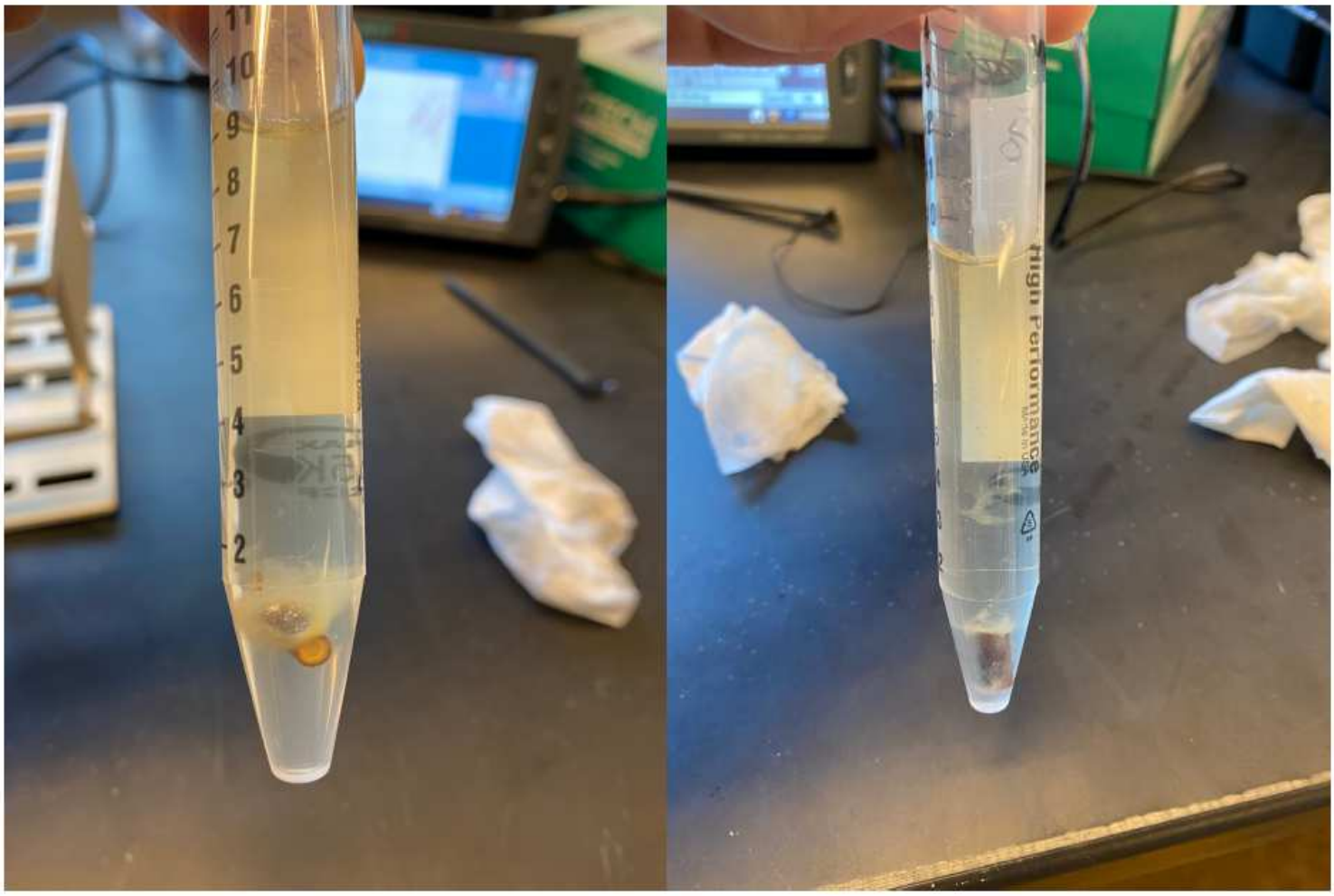

Figure 12

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- renamed495b1.xlsx