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PROTOCOL NOTE

Applications in Plant Sciences

Use of electrolyte leakage to assess floral damage after freezing

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Abstract

Premise: With growing interest in the impact of false springs on plant reproduction, there is the need to develop reliable, high-throughput methods for assessing floral freezing damage. Here we present a method for use with floral tissue that will facilitate more comparative work on floral freezing tolerance in the future.

Methods and Results: We examined the effectiveness of a modified electrolyte leakage protocol to assess floral freezing damage. By comparing data from temperature response curves to an estimate of visual tissue damage, we optimized the protocol for different floral types and improved the signal-to-noise ratio for floral data.

Conclusions: Our modified protocol provides a quick and straightforward method for quantifying floral freezing damage that can be standardized across floral types. This method allows for cross-species comparisons and can be a powerful tool for studying broad patterns in floral freezing tolerance.

KEYWORDS

cold hardiness, conductivity, electrolyte leakage, flower, freezing tolerance, temperature response curve

Methods for assessing freezing damage and cold hardiness in plants have been around for over a hundred years. The most common technique—electrolyte leakage—was first developed in 1932 by Dexter and colleagues (Dexter et al., 1932). This method allows for rapid and potentially high-throughput assessment of tissue damage. It requires a small amount of tissue and has been shown to accurately predict tissue death (Zhang and Willison, 1987; Murray et al., 1989; Lim et al., 1998; CaraDonna and Bain, 2016). Over the years, many improvements have been made to this method to increase its accuracy and ease of application, but most of this work has been limited in scope to vegetative tissue (Flint et al., 1967; Burr et al., 1990; Lim et al., 1998; Kovaleski and Grossman, 2021). However, the increased prevalence of false springs in some regions has led to discussion about how freezing temperatures might impact plant survival and reproduction in the future (Augspurger, 2013; Allstadt et al., 2015; Park et al., 2023). Because many plants flower early in the growing season, the reproductive success of species under continued climate change might be influenced by the sensitivity of reproductive tissue to frost and freezing temperatures. Therefore, research on floral freezing tolerance could inform future land management decisions in seasonally cold climates.

Currently, freezing tolerance research conducted on reproductive tissue relies heavily on visual assessment of tissue color (Neuner et al., 2013; Salazar-Gutiérrez et al., 2014; CaraDonna and Bain, 2016), with two notable exceptions seen in Carter et al. (1999) and Morales et al. (2020). While tissue color can be an effective measure of damage in some species, it is challenging to standardize visual assessment across species because of morphological variation in reproductive organs. It is especially difficult to assess quantitative differences in tissue damage in dark-colored flowers and/or inflorescences without petals like catkins. Here, we outline how electrolyte leakage can be effectively used to estimate floral damage in response to freezing air temperatures with an approach that could facilitate higher-throughput analysis of floral freezing tolerance. We highlight important factors that should be controlled when comparing freezing injury across species and floral types, including modifications to sample preparation that are specific to floral tissue.

METHODS AND RESULTS

Basics of electrolyte leakage

We have developed a protocol for using electrolyte leakage to assess floral freezing damage that draws on a century of previous work on the technique, and we provide context on its specific application to flowers. For clarity, we outline all of the steps of the protocol, including the steps that do not deviate from standard protocols used on vegetative tissue, and draw attention to applicable work done on other tissue types. Electrolyte leakage methods involve exposing tissue to freezing temperatures, incubating the samples in water under controlled conditions, and measuring water conductivity to estimate changes in membrane integrity (Dexter et al., 1932; Flint et al., 1967). Higher conductivity indicates greater tissue damage (Palta et al., 1982). To interpret our data, we use a common metric of tissue damage, the index of injury, which was developed by Flint et al. (1967). This metric standardizes conductivity measurements between 0 (no damage) and 1 (complete tissue death). It requires both a control treatment to determine the conductivity of a sample with no damage and a standardized measure of tissue death. It is calculated with the following equation:

$$I_t = \left(\frac{L_t}{L_k} - \frac{L_0}{L_d}\right) \Big/ \left(1 - \frac{L_0}{L_d}\right)$$

where L_t and L_0 are the conductivities of the frozen and control samples, respectively, and L_k and L_d are the maximum conductivities measured after complete tissue death of these samples. Below, we outline our protocol for using electrolyte leakage to measure freezing damage in flowers (Figure 1, Appendix 1) and highlight the modifications and special considerations needed when working on different types of floral tissue.

Preparing tissue for temperature treatments

Freezing tests are usually conducted on tissue samples cut from an intact plant, but there is considerable variation across studies in how the tissue is sampled and treated during the freezing cycle. For example, in some cases, entire organs are cut from a plant (Sutinen et al., 1992; Peters and Keller, 2009), while in other studies a small piece of tissue (e.g., a leaf disk) is sampled (Lim et al., 1998). There is also variability in whether samples are frozen while they are submerged or partially submerged in water (Boorse et al., 1998; Kovaleski and

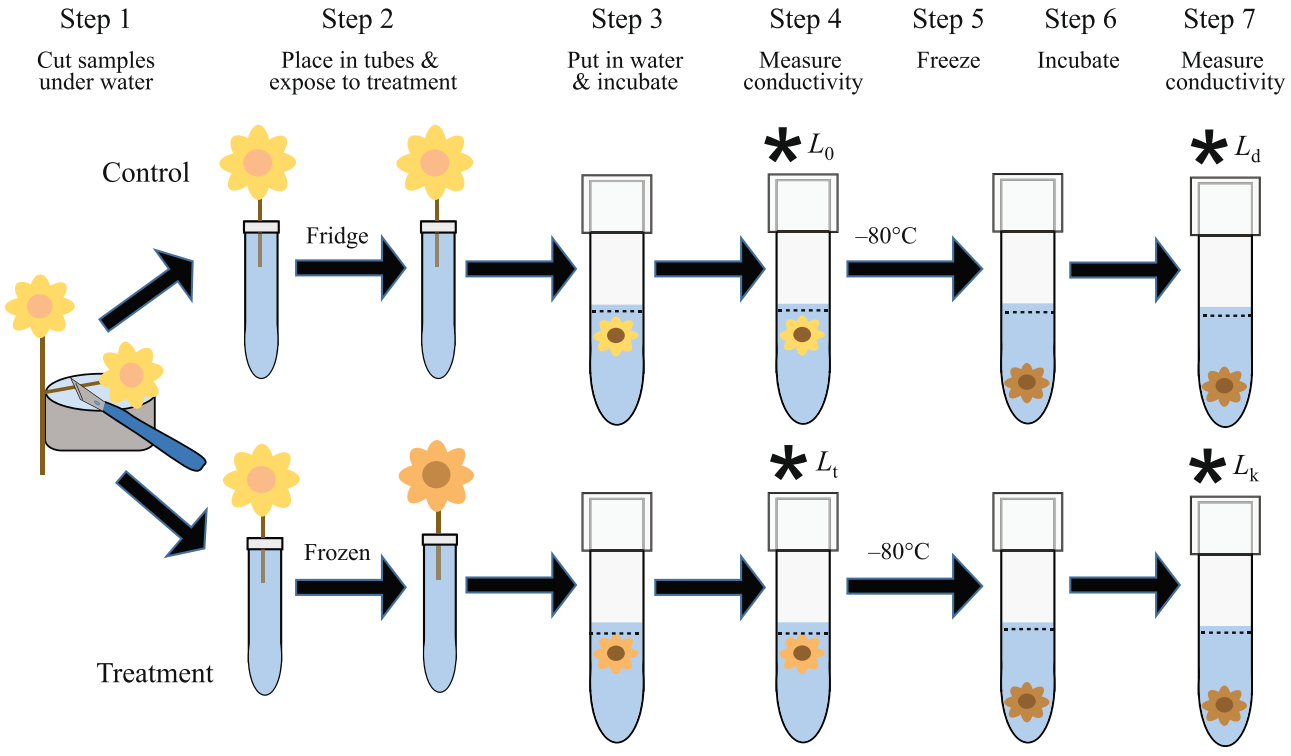

FIGURE 1 Outline of the electrolyte leakage protocol for flowers. After flowers are cut (step 1), they are placed in floral tubes and subjected to a control and freezing temperature treatment (step 2). The flowers are then placed in test tubes with water and incubated for 23–25 h at 23°C (step 3). Afterwards, the first conductivity measurements are taken (L_0 and L_t, step 4). To determine the maximum conductivity of each sample, the samples are subjected to a −80°C treatment and incubated for an additional 23–25 h in the water bath (steps 5 and 6), after which the final conductivity measurements are taken (L_d and L_k, step 7). Time points when conductivity is measured are marked with an asterisk. The control and treatment samples are on the top and bottom rows, respectively. The full protocol is provided in Appendix 1.

Grossman, 2021), in a plastic bag with a wet paper towel (Sutinen et al., 1992; Whitlow et al., 1992; Lindén et al., 2000), or exposed to air (Murray et al., 1989). We found that the treatment of floral samples prior to and during the freeze and control treatment can have a significant impact on the effectiveness of the electrolyte leakage technique.

In general, any method that involved exposing a cut end of a flower to air, even in a plastic bag, often led to petal wilting. If wilting occurred in the control treatment, the wilted control samples (L_0) can have a conductivity close to that of the dead samples (L_d). In Figure 2A, we show an example where the conductivity of dead tissue of *Petunia ×atkinsiana* (Sweet) D. Don is not significantly different than wilted control flowers, but is significantly different than fresh, non-wilted flowers (ANOVA, $F_{2,14} = 20.9$, $P < 0.0001$). Note that in this example, wilted flowers also have a higher standard deviation of conductivity than non-wilted flowers.

To demonstrate how wilting in the control treatment can impact a temperature response curve, we randomly sampled the data from Figure 2A (100 times with replacement) and estimated the conductivity of fresh and wilted samples at different levels of injury. We assumed that L_d and L_k were both equal to the conductivity of the dead samples and that L_0 was determined based on either the fresh or wilted flowers. This simple exercise allowed us to model a temperature response curve and visualize differences in the standard deviation of each point along the curve based on the control treatment (Figure 2B). The results show that the standard deviation of a curve made from wilted flowers is amplified because of how close L_0 and L_d are numerically.

Through various trials, we found the most effective way to collect and process flowers was to cut the flowers off the plant while underwater and place the samples in floral tubes with water during our freezing and control treatments (Figure 1, steps 1 and 2). The goal was to only have the pedicel or stem of the plant in contact with the water during the treatments, leaving the floral tissue dry. When possible, we used larger samples containing more than just the flowers, which helped stabilize the tissue in the tubes while freezing. Any samples that lost contact with water during temperature treatments were discarded. After the freezing tests, we subsampled the tissue of interest and put it in water for incubation (Figure 1, step 3).

In our work, we froze samples in a controlled temperature freezer (Tenney Junior Upright Chamber [TUJR]; Tenney Environmental, New Columbia, Pennsylvania, USA). To simulate an overnight freeze event, we cooled and warmed the samples at a rate of 4°C per hour and held the minimum temperature for 3 h. During the freezing trials, floral temperatures were monitored using beaded thermocouples (Type T; Onset, Bourne, Massachusetts, USA). Low-temperature exotherms were assumed to indicate ice nucleation in the samples. It is also important to note that although we did our freezing tests on cut samples, previous work has established that freezing tolerance estimates in the lab on cut tissue are often consistent with measurements in the field (Marshall, 1965; Sakai, 1970).

Incubating samples in water

Prior to measuring conductivity, samples were incubated in 15 mL of water (Figure 1, steps 3 and 6). Recent evidence suggests that how long samples are incubated does not impact the index of injury (Kovaleski and Grossman, 2021), but it is important that the control and treatment samples are allowed to soak in water for the same amount of time because conductivity increases over time (Murray et al., 1989). In our protocol, we placed our samples in a shaking water bath at 23°C for 23–25 h before each measurement. We chose this timing because it was convenient and consistent with previous studies on other types of tissue (Friedman et al., 2008; Thalhammer

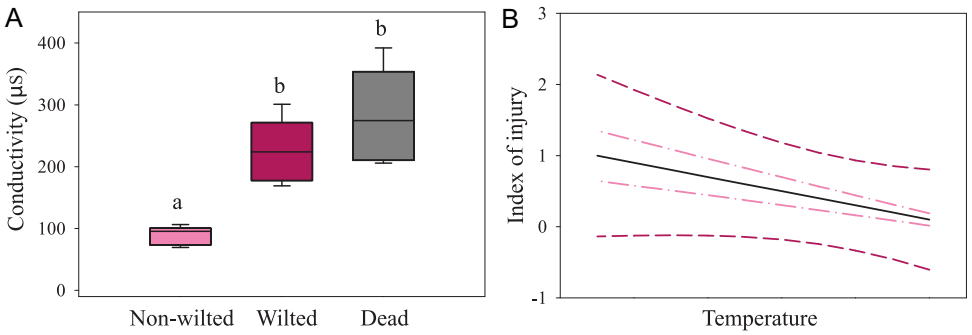

FIGURE 2 Comparison of the conductivity and modeled index of injury of wilted and non-wilted flowers. (A) Conductivity of *Petunia ×atkinsiana* flowers that were non-wilted (light pink), wilted (dark pink), and dead (gray, killed with a −80°C treatment). There were significant differences among treatments (ANOVA: $F_{2,14} = 20.9$, $P < 0.0001$), and letters indicate treatments that were different based on Tukey's honestly significant difference test ($\alpha = 0.01$). Sampling involved six replicates of single flowers incubated in 15 mL of water for 24 h. (B) Theoretical temperature response curve (black line) with modeled standard deviation lines at different levels of injury when non-wilted (light pink lines) and wilted flowers (dark pink lines) were used as a non-frozen control (L_0). Temperature units are arbitrary because the data were generated based on a set index of injury values. The model was created by randomly sampling data in (A) one hundred times with replacement.

et al., 2014; Zhang et al., 2016; O'Connell and Savage, 2020). We have only used this technique for plants with a floral lifespan longer than four days; the timing of incubation might have to be modified for shorter-lived flowers.

One challenge with incubating floral tissue is that it can be buoyant and will often float on top of water when it is not damaged. When flowers float, there is inconsistency in how much tissue is in contact with the water, which leads to visual differences in the browning of the tissue. We also found that samples that were floating had a lower conductivity after incubation than those that were kept submerged using a piece of mesh (one-sided paired *t*-test, $T_9 = 2.58$, $P = 0.01$; Figure 3A). Because floating only occurs in control flowers or in non-damaged flowers, it can inflate differences between non-damaged and damaged tissue (Figure 3B). To address this issue, we inserted a small piece of plastic mesh into each tube to keep the sample submerged. This problem also occurs with leaf tissue and can be solved by vacuum infiltrating the sample; however, we did not test the effectiveness of this method on flowers.

Standardizing damage for index of injury calculations

There are many methods used to determine the standard for complete tissue death (L_k and L_d), including boiling (Friedman et al., 2008; Zhang et al., 2016) and autoclaving samples (Koehler et al., 2012; Lenz et al., 2013; Savage and Cavender-Bares, 2013) or freezing samples at very low temperatures (Kovaleski and Grossman, 2021). When measurements of complete tissue death do not correspond with maximum freezing injury (either because they are too high or too low), temperature response curves do not plateau at 1. For example, protocols that rely on heat, such as boiling and autoclaving, often have a maximum index of injury that is lower than what is estimated for death by freezing (for further discussion of this pattern, see Lim et al., 1998; Kovaleski and Grossman, 2021). This would not be a problem if all species showed a similar maximum value, but differences among species in their temperature response curves complicate making species comparisons without normalizing the data (Sutinen et al., 1992; Lim et al., 1998; Kovaleski and Grossman, 2021). We found a similar problem in flowers when we used an autoclave treatment as our standard for complete tissue death (Table 1); the temperature response curves reached a maximum at different points for each species (ANOVA, $F_{5,54} = 4.06$, $P = 0.003$) and always at a point less than 1.

One way to correct maximum values in a temperature response curve is to calibrate it based on the maximum damage measured at −80°C when fitting a response curve (Lim et al., 1998; O'Connell and Savage, 2020). We chose an alternative approach that bypasses the need for this correction. Instead of using the maximum damage measurement at −80°C to fit our curve, we used this treatment to determine the point of complete tissue death (L_k and L_d). We selected this temperature because it is colder than any experienced on Earth and is well below temperatures that flowers are known to survive (CaraDonna and Bain, 2016; Ishikawa et al., 2016; Wagner et al., 2021). Consistent with

TABLE 1 Maximum freezing injury of flowers (average ± standard deviation) when using an autoclave to assess complete tissue damage.

Species[a]	Index injury[b]	Visual damage[c]
Acer rubrum L.	0.56 ± 0.14	1 ± 0
Alnus incana (L.) Moench	0.48 ± 0.17	1 ± 0
Campanula carpatica Jacq.	0.54 ± 0.12	1 ± 0
Populus balsamifera L.	0.64 ± 0.10	1 ± 0
Rhododendron L. 'Rosy Lights'	0.55 ± 0.15	1 ± 0
Salix discolor Muhl.	0.38 ± 0.07	1 ± 0

[a]Ten samples per species were used except for *Rhododendron*, which only had $n = 9$.
[b]Average index of injury was significantly different among species (ANOVA, $F_{5,54} = 4.06$, $P = 0.003$).
[c]To assess visual damage, flowers were cut in half and the amount of browning was determined based on a scale of 0 to 1, with 0 being no damage and 1 being completely brown.

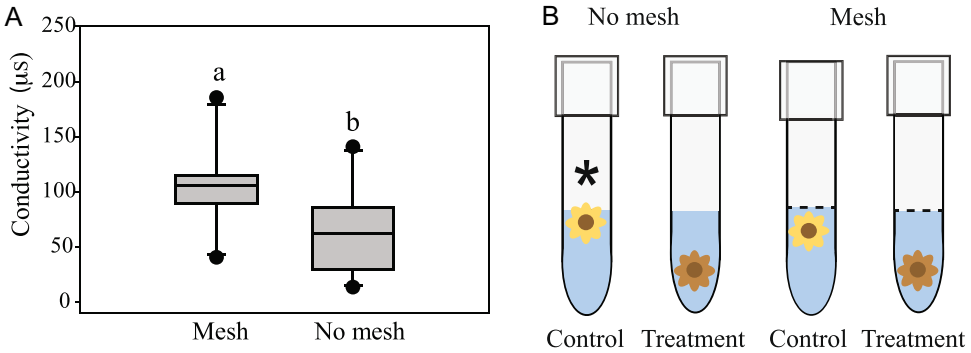

FIGURE 3 Conductivity of *Petunia ×atkinsiana* flowers that were placed in test tubes with and without mesh. Samples with no mesh had significantly lower conductivity than those with mesh (paired *t*-test: $t = 2.58$, $df = 9$, $P = 0.01$). Sampling involved six replicates of single flowers incubated in 15 mL of water for 24 h. (B) Image showing how mesh can be used to standardize treatments by keeping tissue submerged in water. If mesh is not used, non-damaged tissue tends to float (asterisk), leading to differences in the control and freezing treatments.

previous work on vegetative tissue (Lim et al., 1998; Melcher et al., 2000; O'Connell and Savage, 2020; Kovaleski and Grossman, 2021), we found this treatment was effective in determining the maximum freezing injury in all the species we studied (see subset of response curves in Figure 4). Note that for all the curves, the patterns of visual damage matched those quantified using electrolyte leakage, with damage first appearing when the index of injury reached around 0.4, and differences among species matched the known differences in the cold hardiness of the plants. One advantage of using −80°C as an estimate of complete tissue death is that it avoids the need to have a complete temperature response curve to assess differences in species' freezing tolerance. As a result, this method could be used to compare the injury of species to a subset of ecologically relevant temperatures like those experienced during a false spring.

Optimizing the signal-to-noise ratio of the data

One of the main challenges with using the electrolyte leakage method is the presence of variation between flowers of the same species (even on the same plant). If there is high variability between samples, it can lead to problems with comparing the treatment and control samples and result in a low signal-to-noise ratio. This variation reflects natural biological variation in flowers but can make it difficult to discern differences between species if tissue preparation is not standardized.

One of the largest contributors to within-species variability in conductivity is pollen, because the amount of pollen in a sample is directly proportional to its conductivity (see data from *Alnus incana* (L.) Moench, *Betula papyrifera* Marshall, *Populus tremuloides* Michx.; Figure 5). This variability is a problem if there are differences in the amount of pollen released in the control and freezing treatments. In some species, this problem can be avoided by collecting samples at a similar phenological stage. However, this approach does not always work. For example, if flowers are collected before complete pollen release, the flowers in the control treatment may continue to mature and release pollen, while the freezing treatment is happening. As a result, the control flowers may be more advanced in their phenology after the freezing treatment, even if the flowers were collected at the same phenological stage initially. In general, careful attention should be paid to differences in pollen between the control and freezing treatments after the temperature treatment is complete, and modifications should be made as necessary. In some species, it is possible to prevent pollen from inflating conductivity measurements by removing pollen from flowers before running the tests. However, species with large variation in the amount of pollen released per inflorescence or flower (like male catkins in *B. papyrifera*) or plants that are collected at different phenological stages may require higher replication to get an accurate temperature response curve.

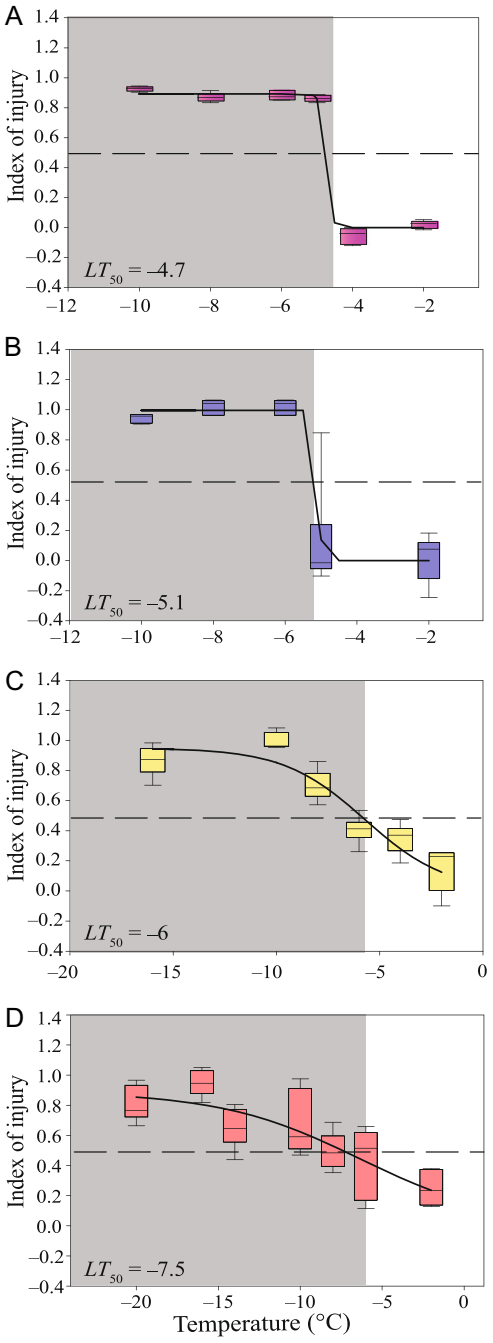

FIGURE 4 Temperature response curves based on electrolyte leakage data from flowers of (A) *Dendrobium nobile* Lindl., (B) *Dendrobium* Sw. 'Micro chip', (C) *Viola* ×*wittrockiana* Gams, and (D) *Pelargonium zonale* (L.) L'Hér. A three-parameter sigmoidal curve was fit to the data for each species using JMP Pro (version 13.0.0; SAS Institute, Cary, North Carolina, USA). Dashed lines indicate the point of 50% damage (LT_{50}). The panels are ordered based on the cold hardiness of each species according to the United States Department of Agriculture (USDA) hardiness zones (*D. nobile* and *Dendrobium* 'Micro chip' – USDA Zone 11, *Viola* – USDA Zone 6, *Pelargonium* – USDA Zone 5). Shading indicates samples with visible browning. Each temperature treatment had a $n = 5–6$.

Another factor that can cause problems with the comparability of the control and freezing treatments is

inconsistency in the amount of tissue used in each sample. This is especially important when considering plants with variation in the number of flowers per inflorescence. For example, in *Acer rubrum* L., we observed female inflorescences had between three and 10 flowers per inflorescence. In this situation, it is important to control both the number of flowers and the number of inflorescences in the samples. When we compared the conductivity of different inflorescences in this species, we found a significant relationship between conductivity and flower number ($F_{1,42} = 426$, $P < 0.0001$; Figure 6A). However, not all species have inflorescences with multiple flowers. Therefore, we also examined whether it is important to control floral size when working with species that exhibit variation in the surface area and mass of individual flowers. We tested whether conductivity was related to floral size in *Forsythia* Vahl 'Meadowlark' but did not find evidence that differences in surface area and mass among flowers had a large impact on conductivity in this species (α = 0.01; Figure 6B, C). It is possible that this may not be true for all floral types and effort should be made to keep samples as similar as possible when using electrolyte leakage to assess freezing damage.

Ultimately, the amount of noise that is acceptable in electrolyte leakage measurements is dependent on the signal strength. We found the best way to analyze the signal-to-noise ratio of a new species is to measure variability in the control treatment. In Figures 4A and 4B, the two *Dendrobium* Sw. species show minimal variation in the index of injury measured at each temperature. These plants all had a signal-to-noise ratio (average conductivity/standard deviation) that was greater than 3 (3.7 and 5.0, respectively) in the control treatment. Meanwhile, *Pelargonium zonale* (L.) L'Hér. (Figure 4D), which had the most variation at each temperature point, had a signal-to-noise ratio of 2.1. In general, we found that as the signal-to-noise ratio decreased below 2, the quality of the response curves greatly declined. If an effort has been made to reduce flower-specific noise as discussed above, the easiest solution for increasing the signal-to-noise ratio is to boost the signal by adding more plant tissue to each tube.

It is important to note that there will always be a certain amount of variability in electrolyte leakage data because there is natural variation in the timing of ice formation within samples. The only way to eliminate this variation is to directly nucleate ice by putting ice, bacteria, or another type of nucleating agent in the tubes before freezing. Although this is done in some freezing studies (Boorse et al., 1998; Friedman et al., 2008), we chose to not nucleate ice directly because we wanted to simulate how flowers respond to freezing air temperatures, while taking into account any supercooling that might occur in the tissue (for further discussion, see Neuner et al., 2010; Zhang et al., 2016). Therefore, our temperature response curves are influenced by both the probability of freezing at a certain temperature and the amount of damage incurred by that freezing. As a result, our curves have more variation at the temperatures close to where ice nucleation occurs (around −5°C; see Figure 4A).

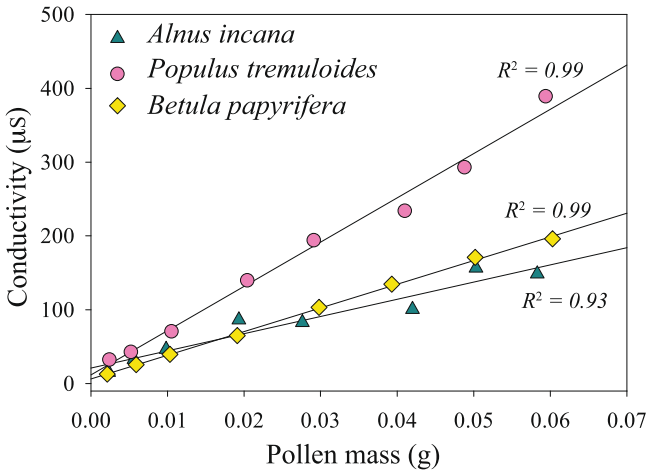

FIGURE 5 The relationship between conductivity and mass of pollen in 15 mL of water for *Alnus incana*, *Populus tremuloides*, and *Betula papyrifera* ($n = 8$ per species). Samples were incubated in a water bath for 24 h before measurement. Significant linear relationships are shown ($P \leq 0.0001$).

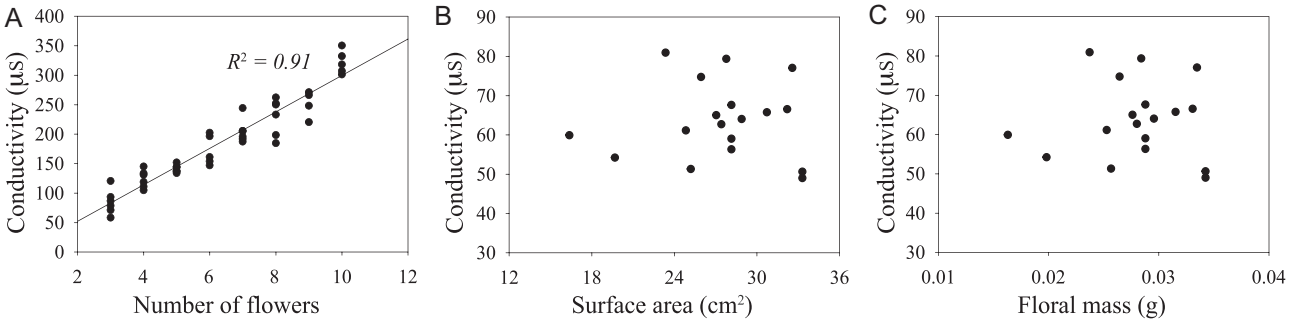

FIGURE 6 Relationship between conductivity and floral sample traits. (A) Relationship between conductivity and the number of *Acer rubrum* flowers ($n = 44$) in a sample. (B, C) Relationship between conductivity and estimated floral surface area (B) and between conductivity and floral dry mass (C) of *Forsythia* 'Meadowlark' flowers ($n = 18$). All samples were incubated in 15 mL of water for 24 h. For the *Forsythia* data, four flowers were used per tube. Only the number of flowers showed a significant linear relationship with conductivity ($F_{1,42} = 426$, $P < 0.0001$).

CONCLUSIONS

By making modifications to the standard electrolyte leakage method, we have laid out a straightforward and rapid technique to measure freezing damage in flowers that builds on previous research on vegetative tissue. Our technique improves upon more traditional approaches to study floral freezing tolerance that rely on visual assessment, because it provides a standardized approach to quantifying tissue damage regardless of floral structure. Having a reliable approach to assess freezing damage will allow for more cross-species comparisons and facilitate future work examining larger patterns of floral freezing tolerance and the potential implications of false springs on plant reproduction.

AUTHOR CONTRIBUTIONS

J.A.S. wrote the manuscript, conducted freezing experiments, and designed the protocol. S.J.H. and M.R.O. helped develop the protocol and collected data on floral freezing tolerance including temperature response curves. All authors approved the final version of the manuscript.

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DATA AVAILABILTY STATEMENT

Data are openly available in the Data Repository for University of Minnesota (DRUM) (https://doi.org/10.13020/k4xq-ab91; Savage et al., 2023).

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Appendix 1: Protocol for assessing floral freezing tolerance using electrolyte leakage.

Supplies

- Transfer tubes with lids (30 mL)
- Floral water tubes that have a cap with a hole in it
- Small pieces of plastic mesh
- Purified water (Milli-Q [MilliporeSigma, Burlington, Massachusetts, USA] or deionized water)
- Blunt-tipped tweezers
- Pruners or razor blades
- Conductivity meter (Oakton PC 700 Benchtop meter; Environmental Express, Charleston, South Carolina, USA)
- Programmable freezer (Tenney Junior Upright Chamber [TUJR]; Tenney Environmental, New Columbia, Pennsylvania, USA)
- −80°C freezer

Material collection

1. Cut flowers/inflorescences off plants underwater.
2. Place samples in labeled floral tubes filled with tap water. Make sure that the pedicel or stem is in contact with the water and is stable in the tube.
3. Allow buds/flowers/leaves to air dry before freezing.

Freezing cycle

1. Create a program that ramps down at a rate of 4°C per hour until the target temperature is reached, holds that temperature for 3 h, and ramps back up at the same rate.
2. Switch the fan on.
3. Place one set of samples in the freezing chamber and the other set of samples (the control samples) in a refrigerator.
4. Attach thermocouples to the tissue of interest.
5. Run the freezing cycle.
6. After the cycle is complete, remove the samples from the freezer and the control samples from the refrigerator.
7. Note any visual freezing damage (e.g., browning or wilting). When possible, an extra piece of floral tissue should be put in a plastic bag in the refrigerator for two days to confirm tissue damage.

Incubation and first set of conductivity measurements

1. Place the desired number of samples/flowers in a labeled tube with 15 mL of purified water.
2. Make sure that the sample is entirely submerged. You may use a circle of mesh to keep the sample from floating.
3. Place samples in the shaking water bath at room temperature for 23–25 h.
4. Rinse the probe of the conductivity meter with purified water and dry completely with a lab wipe.
5. Invert each sample 2–3 times to disperse the material in the water. Do not shake.
6. Use the conductivity meter to measure the conductivity of each sample.
7. Rinse and dry the probe and make sure no sample is stuck on the probe.
8. Repeat for each sample.

Measurement of maximum conductivity

1. Move the samples into −80°C freezer for 24 h.
2. Thaw samples for 30 min on the benchtop and then place in a shaking water bath for 23–25 h.
3. Measure the conductivity again following steps 4–8 above.