Evaluation of freezing injury in temperate fruit trees

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Abstract

Freezing is a major environmental stress limiting the geographical distribution, growth, and productivity of temperate fruit trees. The extent of freezing injury in the trees depends on the rate at which the temperature decreases, the minimum temperature reached, and the duration of the freezing conditions. The ability to tolerate freezing temperatures under natural conditions varies greatly among fruit tree species, cultivars, and tissues. Freezing injury must be precisely evaluated to reliably predict the winter survival and productivity of the trees in specific regions, to screen for tolerant species and cultivars, and to develop cultural strategies that reduce freezing stress. Various methods are used to evaluate freezing injury in temperate fruit trees under field and artificial conditions, including visual evaluation of tissue discoloration, thermal analysis, determination of electrolyte leakage, and triphenyl tetrazolium chloride reduction analysis. In this review, we describe the most frequently used experimental procedures for evaluating freezing injury.

Keywords Dehydration · Electrolyte leakage · Exotherm · Freezing injury · Fruit tree · Tetrazolium

1 Introduction

Freezing injury in plants occurs when temperatures drop below the freezing point of water. When plant cells are exposed to subfreezing temperatures, ice first forms in the extracellular spaces that have the highest osmotic potential and the highest levels of ice nucleators and continues to form toward the decreasing water potential (Pearce 2001; Wisniewski et al. 2014). As the temperature decreases, the extracellular ice crystals grow with the withdrawal of liquid water from the unfrozen protoplasts and ultimately the intracellular ice crystals form, which is lethal to the cells (Pearce 2001; Guy 2003; Arora 2018). Freezing injury appears to depend on a common mechanism involving cell dehydration and membrane disintegration via ice formation during freezing (Steponkus 1984; Yamazaki et al. 2009; Moran et al. 2011; Arora 2018).

Freezing is a major environmental stress that limits the geographical distribution, growth, and productivity of temperate fruit trees. Freezing injury in the trees includes winter sunscald of thin-barked species, frost splitting of trunks, blackheart of stems, freezing of roots, midwinter kill of dormant flower buds, death of cambium in twigs, branches, and trunks, and frost damage to flowers and fruit during spring and fall (Weiser 1970; Pearce 2001).

The ability to tolerate freezing temperatures under natural conditions varies greatly among fruit tree species, cultivars, and tissues (Rodrigo 2000; Neuner et al. 2010; Aslamarz et al. 2011; Moran et al. 2011; Lee et al. 2013; Yu et al. 2017). Evaluation of freezing injury is important for predicting winter survival and regrowth the following spring, and productivity of temperate fruit trees in specific regions. Such evaluation is also needed to identify freezing-tolerant species and cultivars and to establish cultural strategies that reduce freezing stress. In this review, we explain how freezing injury can be evaluated in temperate fruit trees and describe the most frequently used experimental procedures to evaluate freezing injury.

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2 Conditions for evaluating freezing injury

2.1 Field conditions

Freezing injury in temperate fruit trees can be evaluated in the field by determining their survival and regrowth capacity during or following a test winter (Proebsting and Mills 1972; Clore et al. 1974; Layne and Ward 1978; Rajashekar et al. 1982). However, evaluation of freezing injury in the field is complex and often unreliable, because the temperatures of test winters fluctuate, sometimes greatly, from year to year. The winter survival of the trees is also influenced by a variety of factors, including the timing, duration, and rate of temperature decline and rise, the minimum temperature reached, the nucleation temperature, the number of freeze-thaw cycles, and snow cover (Proebsting and Mills 1972; Guy 2003).

2.2 Artificial conditions

Various methods have been employed to evaluate injury in temperate fruit trees under artificial freezing conditions (Steponkus and Lanphear 1967; Ristic and Ashworth 1995; Nesbitt et al. 2002; Neuner et al. 2010; Aslamarz et al. 2011; Moran et al. 2011; Lee et al. 2012, 2013; Pramsohler et al. 2012; Szymajda et al. 2013; Yu et al. 2017). Samples can be subjected to precisely controlled freezing temperatures of various durations and rates of cooling in a growth chamber or bath circulator and then evaluated for freezing injury. Containerized whole trees or tree parts can be tested, with most studies focusing on flower buds and shoots. In this way, large numbers of samples can be handled within a short period of time and reproducible values can be obtained for any given set of controlled freezing conditions. However, the amount of freezing injury can change depending on the rate and duration of the artificial freezing. Furthermore, the values obtained under artificial conditions do not always reflect the true cold hardiness of a species or cultivar, or predict its survival under natural conditions. Nevertheless, such values can be used as valid parameters for predicting the relative

amount of freezing injury during winter and the severity of spring frost damage (Barranco et al. 2005; Ehlenfeldt et al. 2006; Imani et al. 2011; Lee et al. 2013; Moghadam et al. 2015).

3 Methods for evaluating freezing injury

3.1 Visual evaluation

Membrane disruption caused by freezing allows cytosolic enzymes to act on substrates that are normally sequestered (Rouet-Mayer et al. 1990). The injured tissues can discolor as cytosolic enzymes act on phenolic substrates. Polyphenol oxidase is one enzyme known to be responsible for discoloration in mechanically injured tissues (Vámos-Vigyázó 1981; Van Lelyveld and Bower 1984; Lee et al. 2005). Polyphenol oxidase catalyzes the oxidation of phenolic compounds to *o*-quinones, which subsequently polymerize to form dark-colored pigments (Rouet-Mayer et al. 1990). The discoloration of freeze-injured tissues can be evaluated visually under field or artificial freezing conditions (Rodrigo 2000; Ehlenfeldt et al. 2006; Mills et al. 2006; Lenz et al. 2013, 2016; Moghadam et al. 2015).

Artificial freezing experiments usually employ three to five appropriate target freezing temperatures. Freezing and thawing are performed in a growth chamber or bath circulator equipped with a temperature controller, with temperature changes applied at a rate of 2 °C/h. Flower buds or shoots are kept at the target freezing temperature for 4–6 h. After freezing and thawing, samples are kept at room temperature for some time to allow symptoms to develop. This incubation can last from several days to one week depending on the tissue type and growing season. Samples are cut longitudinally and their injury is evaluated based on discoloration, sometimes with an aid of a digital microscope (Fig. 1).

Percent injury is plotted against freeze temperature to generate a sigmoid curve. The sigmoid curve associated with the temperature at which 50% injury occurred (LT_{50}) is generally used to characterize freezing injury by regression analysis. LT_{50} values can also be calculated using a linear

Fig. 1 Discoloration of blueberry flower buds caused by various freezing temperatures. The tissues were subjected to various freezing temperatures in a circulating water bath for 2 h and their discoloration was visually evaluated by using a digital microscope following the incubation at room temperature for 24 h

regression model, assuming that the section of the sigmoid curve between 20 and 80% can be regarded as linear (Gu 1999; Szalay et al. 2016).

Visual evaluation requires less instrumentation than other methods and is considered reliable. However, it is qualitative and subjective in nature as well as being more time consuming and requiring larger amounts of sample material than other methods. Nevertheless, visual evaluation has widely been used to determine freezing injury in many temperate fruit trees, including apple (Embree and McRae 1991; Kang et al. 1998; Moran et al. 2011), pear (Kang et al. 1998), peach (Salazar-Gutiérrez et al. 2014; Liu et al. 2019), grape (Jones et al. 1999; Mills et al. 2006), and blueberry (Ehlenfeldt et al. 2006; Kishimoto et al. 2014).

Visual evaluation of tissue discoloration is sometimes combined with observations of regrowth capacity, especially when testing whole trees (Embree and McRae 1991). Callus growth on injured woody stems has also been suggested as a measure of freezing injury (Pellett and Heleba 1998; Nesbitt et al. 2002). Regrowth tests require lengthy incubation under favorable growing conditions and the results may be hampered by dormancy. Nevertheless, normal regrowth of a tree is the ultimate measure of its survival and the most reliable parameter for evaluating freezing injury. Visual evaluation of tissue discoloration and regrowth are often used as controls for more quantitative analyses.

3.2 Thermal analysis

When temperatures drop below the freezing point, there are abrupt increases in tissue temperatures due to the latent heat release of fusion during ice formation (Quamme et al. 1972; Burke et al. 1976; Kaya and Köse 2017). These temperature increases are called exotherms. Freezing injury can be monitored based on exotherm appearance as ice forms (Mills et al. 2006; Salazar-Gutiérrez et al. 2016; Kaya and Köse 2017; London and Kovaleski 2017; Liu et al. 2019).

Thermal analysis for detecting exotherms is carried out by embedding a small probe of copper-constantan

thermocouple under the bark of a moist woody twig or on the surface of buds (Fig. 2). A second thermocouple is commonly embedded in a dry sample of the tissue as a reference. Tissues are usually cooled to $-40\text{ }^{\circ}\text{C}$ at a rate of -2 or $-20\text{ }^{\circ}\text{C/h}$ in a bath circulator equipped with a temperature controller. Temperatures of the sample tissues are then recorded at 2 s intervals using a data logger as the surrounding air temperature is steadily lowered.

During thermal analysis, the sample tissues initially cool well below the freezing point of water with no apparent ice formation (Fig. 3). The failure to form ice at temperatures below the freezing point is called supercooling (Burke et al. 1976). At approximately $-5\text{ }^{\circ}\text{C}$, the first exotherm appears, which represents ice formation in the apoplastic space of xylem tracheary elements and in the intercellular spaces in the cortex, bark, phloem, and so forth (Burke et al. 1976; Neuner et al. 2010). The ice crystals continue to grow, fed by the gradual withdrawal of liquid water from unfrozen protoplasts (Arora 2018). The second exotherm is seen as a shoulder rather than a distinct peak and represents the

Fig. 3 Changes in tissue temperature during controlled freezing of woody stems. Exotherms are the abrupt tissue temperature increases significantly above ambient due to the latent heat release of fusion during the freezing of water in the excised stem. (modified from Weiser 1970)

Fig. 2 A copper-constantan thermocouple probe embedded in a grapevine bud

enlargement of extracellular ice crystals. The first and second extracellular exotherms are called high-temperature exotherms. With further cooling, a third, low-temperature exotherm is seen in the thermal analysis curve. The third exotherm represents intracellular ice formation, which is invariably lethal to the cells in which it occurs (George et al. 1974; Mills et al. 2006; Tanino 2012; Charrier et al. 2015; Grant and Dami 2015; Rubio et al. 2016).

The ability to supercool varies among fruit tree species (Quamme 1991), and this species-specific threshold temperature limits their geographical distribution (George et al. 1974; Burke et al. 1976; Wisniewski et al. 2003; London and Kovalski 2017). In many temperate fruit trees, acclimation to freezing involves the suppression of ice formation at temperatures far below the freezing point (Pramsohler et al. 2012; Salazar-Gutiérrez et al. 2014; Kaya and Köse 2017; Liu et al. 2019). Deep supercooling to as low as -40°C can take place in flower buds and stems, despite ice formation in bud scales and adjacent tissues (George and Burke 1977; Ashworth et al. 1988; Kang et al. 1998). The flower bud primordium is surrounded by an impenetrable cell layer containing waxy deposits in the cell walls (Ashworth 1982; Ashworth et al. 1989). This cell layer isolates the meristematic cells of the primordium from the scales and bud axis and excludes ice crystal penetration. Inside the primordium, there are no strong ice nucleation sites. Thus, as the temperature is lowered, the water in the primordium supercools until the temperature reaches the critical threshold temperature for ice nucleation.

Since low-temperature exotherms are produced by freezing of flower buds and the xylem parenchyma cells in stems (Ashworth et al. 1989; Arora et al. 1992; Volk et al. 2009; Pramsohler et al. 2012), thermal analysis has sometimes been employed to rapidly identify the ability to undergo supercooling during a controlled freezing test in many temperate fruit trees, including apple (Salazar-Gutiérrez et al. 2016), pear (Rajashekar et al. 1982), peach (Ashworth 1982; Ashworth et al. 1989; Kang et al. 1998; Liu et al. 2019), sweet cherry (Salazar-Gutiérrez et al. 2014), grape (Kang et al. 1998; Jones et al. 1999; Mills et al. 2006; Ferguson et al. 2011; Grant and Dami 2015; Rubio et al. 2016; London and Kovalski 2017; Kaya and Köse 2017), and blueberry (Kishimoto et al. 2014). In some cases, however, the low-temperature exotherms are undetectable or detected over a wide temperature range, because the xylem parenchyma cells do not freeze simultaneously but rather small groups of the cells appeared to freeze at random points (Neuner et al. 2010; Tanino 2012; Yu et al. 2017).

3.3 Electrolyte leakage analysis

Any process that damages the plant membrane will cause the contents of the cell to leak out (Steponkus and Lynch

1989; Duke and Kenyon 1993; Lee et al. 1995; Lee and Cho 1996; Lindén et al. 2000; Nesbitt et al. 2002; Demidchik et al. 2014; Arora 2018). Cellular leakage is a symptom of membrane damage caused by freezing stress (Lindén et al. 2000; Aslamarz et al. 2011; Arora 2018). Leakage can occur without membrane rupture, but is more pronounced when the membrane ruptures and the entire cell contents are released (Duke and Kenyon 1993; Améglío et al. 2005; Yamazaki et al. 2009). Thus, recording the amount of cellular leakage provides an estimate of freezing injury in many temperate fruit trees (Lee et al. 2012, 2013; Moghadam et al. 2015; Yu et al. 2017).

Many different types of cellular contents can be sampled to determine cellular leakage. However, electrolyte leakage is the most frequently measured parameter for evaluating tissue injury, because electrolyte leakage can be determined continuously with an electrical conductivity probe and does not require destructive sampling (Duke and Kenyon 1993; Lindén et al. 2000; Aslamarz et al. 2011; Lee et al. 2012, 2013). Furthermore, electrolyte leakage analysis is simple, rapid, and inexpensive, requires small amounts of sample, and provides quantitative data.

Electrolyte leakage analysis often begins with rinsing the samples with deionized water to remove surface contaminants. Shoot or stem tissues are cut into small pieces and placed into 50-mL conical tubes containing 1 mL of distilled water. The tubes are incubated in a circulating water bath equipped with a temperature controller. The tubes are cooled at a rate of $2^{\circ}\text{C}/\text{h}$ until the target temperature (as low as -40°C) is reached, maintained at the target temperature for 2 h, and then thawed at a rate of $2^{\circ}\text{C}/\text{h}$ to 4°C . The temperatures are monitored with a copper-constantan thermocouple and recorded with a data logger. After the freeze-thaw treatment, the tissues are cut into 1-cm long pieces, then placed in a 15-mL conical tube containing 8 mL of deionized water, and vacuum-infiltrated for 5 min. The tubes are incubated at room temperature for 20 h on an orbital shaker at 120 rpm. Initial electrical conductivity of the aliquots is measured using an electrical conductivity meter. After autoclaving at 120°C for 30 min, followed by incubation at room temperature for 20 h, final electrical conductivity is measured.

The simplest method of expressing the data is to plot the change in electrical conductivity of treated tissue minus the change in electrical conductivity of the control at each temperature, assuming that the differential increase in electrolyte leakage is due to the freezing temperature. Alternatively, the data can be normalized based on percent of total capacity to leak (Lee et al. 2013; Yu et al. 2017). The total capacity to leak can be determined by autoclaving the tissues and measuring the electrical conductivity of the aliquot.

Percent injury is calculated as follows (Arora et al. 1992):

$$\% \text{ Injury} = (\% \text{EL}_{(T^{\circ}\text{C})} - \% \text{EL}_{(4^{\circ}\text{C})}) / (100 - \% \text{EL}_{(4^{\circ}\text{C})}) \times 100,$$

where $\% \text{EL}_{(T^{\circ}\text{C})}$ and $\% \text{EL}_{(4^{\circ}\text{C})}$ are percent electrolyte leakage (EL) values based on initial electrical conductivity over total electrical conductivity for each freeze target temperature ($T^{\circ}\text{C}$) and unfrozen control (4°C), respectively. The percent injury data are transformed using the method of Lim et al. (1998), with the percent electrolyte leakage for the -80°C -treated sample set at 100%, and adjusted using the following equation (Lee et al. 2013; Yu et al. 2017):

$$\% \text{ Adjusted injury} = (\% \text{ injury}_{(T^{\circ}\text{C})} / \% \text{ injury}_{(-80^{\circ}\text{C})}) \times 100$$

Using the percent-adjusted injury data, quantitative estimates of freezing injury or cold hardiness, that is, LT_{50} and the temperature at which the rate of injury is maximal (T_{max}), are calculated using the Gompertz function, an asymmetric sigmoid curve appropriate for use in data fitting of a plant's response to temperature stress, as described by Lim et al. (1998) and Lindén et al. (2000).

Electrolyte leakage analysis has been used to evaluate freezing injury in grape canes and buds (Jones et al. 1999), red raspberry canes (Lindén et al. 2000), olive leaves and shoots (Barranco et al. 2005), blueberry shoots (Lee et al. 2012, 2013), and peach trunk bark and wood tissues (Yu et al. 2017). Although electrolyte leakage is not a direct measurement of cellular injury caused by freezing, the values from electrolyte leakage analysis have correlated well with cold hardiness in many temperate fruit trees (Lindén et al. 2000; Nesbitt et al. 2002; Lee et al. 2013; Yu et al. 2017).

3.4 Triphenyl tetrazolium chloride (TTC) reduction analysis

TTC (2,3,5-triphenyl-2 H-tetrazolium chloride) is a redox indicator commonly used to measure cellular respiration. In TTC reduction analysis, the colorless TTC is reduced to red 1,3,5-triphenylformazan by the action of various dehydrogenases in living tissues (Lakon 1949). It is sometimes difficult to visually differentiate between living and non-living tissues based on the degree of TTC reduction because of wide variation in the intensity and pattern of the red coloration. Following extraction of the triphenylformazan with an organic solvent, however, the color change can be quantitatively determined spectrophotometrically (Lopez del Egado et al. 2017). Thus, the TTC reduction analysis may be useful for evaluating freezing injury in temperate fruit trees.

For TTC reduction analysis, shoot or stem tissues are subjected to various freezing temperatures as described for the electrolyte leakage analysis. After the freeze-thaw treatment, the samples are placed in 50-mL conical tubes containing 10 mL of 0.8% TTC in 0.05 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$

buffer (pH 7.4) and vacuum-infiltrated for 15 min to facilitate absorption of the TTC solution. The tubes are incubated in a growth chamber in darkness at 28°C for 20 h, followed by three rinses of the tissues with distilled water. The water-insoluble triphenylformazan is extracted from each sample with 7 mL of 95% (v/v) ethanol in a water bath at 85°C for 10 min. The amount of triphenylformazan, which equals the amount of TTC reduced, is measured at 490 nm using a spectrophotometer. Percent-adjusted injury data and LT_{50} values are calculated using the same methods employed for electrolyte leakage analysis.

TTC reduction analysis has been used to evaluate freezing injury in many tree species (Steponkus and Lanphear 1967; Nesbitt et al. 2002; Ruf and Brunner 2003), including walnut (Aslamarz et al. 2011) and peach (Yu et al. 2017). However, its usefulness is limited, since dormant tissues are sometimes erroneously considered to be dead (Busso et al. 2015).

3.5 Other methods

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride), a weak cationic dye, can be used to evaluate freezing injury, since it crosses the membranes of viable cells and accumulates intracellularly (Winckler 1974; Borenfreund and Puerner 1984; Repetto et al. 2008). As cells are injured by freezing, their ability to incorporate neutral red diminishes. Thus, loss of neutral red uptake corresponds to loss of cell viability.

Nuclear magnetic resonance (NMR), a type of spectroscopy which employs radio frequency light, has also been used to assess freezing injury (Burke et al. 1974, 1976). NMR can distinguish between ice crystals and unfrozen water in plant tissues based on their radio frequency spectra (Burke et al. 1974). Since the spectrum becomes progressively broader as freezing progresses, it is possible to measure the amount of unfrozen water in various tissues and thereby estimate freezing injury. Furthermore, localization of unfrozen water and freezing behaviors of various tissues at subzero temperatures can be visualized by using the NMR technology combined with image scanners (Liu et al. 1993; Gamble 1994; Kerr et al. 1997; Price et al. 1997). In the images, unfrozen water appears as a light area.

In addition, infrared thermography has been used to monitor ice nucleation and propagation in a variety of plant species and tissue types (Ceccardi et al. 1995; Wisniewski et al. 1997; Fuller and Wisniewski 1998; Workmaster et al. 1999; Neuner et al. 2010, 2019; Pramsohler et al. 2012). In the infrared thermography using an imaging radiometer or a digital infrared camera, ice formation during freezing is visible as a light area by the latent heat release of fusion as water undergoes phase transition from a liquid to a solid (Ceccardi et al. 1995; Wisniewski et al. 1997; Neuner et al. 2010).

4 Conclusion

To cope with freezing stress, temperate fruit trees have developed various mechanisms regulating ice formation in their tissues during freezing, which results in diverse but species- and tissue-specific freezing patterns. Various methods have been attempted to evaluate freezing injury in the trees under field and artificial conditions. To precisely evaluate the freezing injury, the methods should be chosen based on the species- and tissue-specific freezing patterns. The most frequently used methods are visual evaluation of tissue discoloration, thermal analysis, electrolyte leakage analysis, and TTC reduction analysis. Considering the diverse freezing patterns, freezing injury should not be evaluated based on a single method but rather on at least a couple of methods under both field and artificial conditions. In addition, cumulative data from multi-year evaluations at different phenological stages are needed for more accurate prediction of the survival of fruit trees during winter and their regrowth the following spring. The integrated evaluation of freezing injury will help to ensure successful cultivation of temperate fruit trees.

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Authors’ contribution DJY planned and wrote the manuscript; HJL supervised and wrote the manuscript.

Compliance with ethical standards

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

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