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Estimation of Cold Hardiness in Trunk Bark and Wood Tissues of Peach Trees (*Prunus persica*)

복숭아나무 조직별 원줄기의 내한성 측정

BY

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THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Estimation of Cold Hardiness in Trunk Bark and Wood Tissues of Peach Trees (*Prunus persica*)

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ABSTRACT

To establish the reliable methods for estimating cold hardiness for trunk tissues of peach trees, tissue browning, exotherm, electrolyte leakage (EL), and triphenyl tetrazolium chloride (TTC) analyses were performed on shoots, and bark and wood tissues of 5-year-old branches in ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees (*Prunus persica*) during cold acclimation. Visual estimation based on tissue browning was relatively simple and the discoloration caused by freezing temperatures was observed in both bark and wood tissues. However, its scoring was difficult to quantify the level of cold hardiness. Exotherm analysis was conducted to characterize freezing in shoots, and bark and wood tissues. High temperature

exotherms, indicating extracellular ice formation, were exhibited consistently between −5 and −10°C. However, low temperature exotherms, indicating intracellular ice formation associated with freezing injury, were not detected. Therefore, exotherm analysis was not suitable for estimating cold hardiness in all tissues of peach trees. EL and TTC analyses provided reproducible results. These two analyses were also possible to express the level of cold hardiness as temperature representing 50% injury occurred (LT_{50}). EL analysis required the additional processes for separating bark from wood tissues and for cutting wood tissues into small pieces prior to being subjected to freezing treatments, while TTC analysis did not require such processes. TTC analysis estimated cold hardiness lower than EL analysis, showing a tendency to overestimate cold hardiness in plant tissues. Thus, EL and TTC analyses could be recommended for estimating cold hardiness in thick branches or trunk in peach trees.

Key words**:** cold hardiness, differential thermal analysis, electrolyte leakage, exotherm analysis, LT_{50}, peach tree, triphenyl tetrazolium chloride analysis

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Deciduous woody plants like peach tree (*Prunus persica*) frequently suffer from extreme winter temperatures. Most tissues or parts of *Prunus* species are injured by temperatures that are closely related to the average minimum temperatures. In the northern area, most plant tissues and organs are damaged at the temperatures below −20°C, while in the southern area, temperatures below −15°C can injure plants. Lack of cold hardiness in winter and susceptibility to spring frosts are two critical problems of losses of crop yields (Arora and Rowland, 2011).

Freezing causes stress or injury to plants (Pearce, 2001) from cells to the whole-plant level. Mainly, floral buds and trunk tissues in peach trees may be injured every winter. Especially, damage in trunk tissues is relatively more lethal among different tissues. The most common low-temperature injury to trunk in *Prunus* species is oxidative browning and splitting of the wood tissues. Subsequently, secondary injuries can occur by allowing pathogens and diseases to enter through a crack on a trunk. After all, whole tree survival can be influenced, following trunk tissues damage from freezing. However, little research has been reported to assess freezing injury in trunk tissues of woody plants including peach trees.

Assessing injury following freezing treatments is an important part of laboratory freezing studies for determining cold hardiness levels of woody plants (Lindén, 2002; Väinölä et al., 1997). Many methods have been used to quantify injury of shoots of woody plants (Lee et al., 2012; Lee et al., 2013; Pagter et al., 2011;

Pramsohler et al., 2012). Discoloration or tissue browning of injured tissues is generally used to assess injury (Luoranen et al., 2004; Väinölä et al., 1997). Although visual estimation is relatively simple to perform, it requires tissues to incubate for overnight following freezing treatment to allow time for oxidative browning of injured tissues (Luoranen et al., 2004; Väinölä et al., 1997). Then, the frozen tissues are rated by naked eyes or under a microscope.

Exotherm analysis is relatively rapid and is possible to identify supercooling ability in some tissues such as floral buds and xylem ray parenchyma by occurrence of low-temperature freezing events or exotherms (LTEs) during a controlled freezing test (Flinn and Ashworth, 1995; Gao et al., 2014). These LTEs are associated with the temperature at which lethal injury occurred within various tissues or organs of plants. Therefore, it has also been used as an estimator of cold hardiness.

Electrolyte leakage (EL) analysis is another common method for evaluating tissue injury, after a freeze-thaw cycle. The reliability of the test has been proved, depending on tissue type (Calkins and Swanson, 1990; Lim et al., 1998). After thawing, electrolytes diffuse into solution causing an increase in electrical conductivity (EC). This method measures electrolytes release from all injured cells or tissues, and usually shows a range of increasing injury at decreasing temperatures rather than an identified killing point (Ebel et al., 2004; Ketchie et al., 1972). In addition, EL analysis is suitable for grape (*Vitis vinifera*) cane and bud (Jones et al., 1999), silver birch (*Betula pendula*) leaf and stem (Li et al., 2002), and blueberry

(*Vaccinium corymbosum*) shoots (Lee et al., 2012; Lee et al., 2013).

Triphenyl tetrazolium chloride (TTC) analysis has been used to measure viability of plant tissues after freezing and other environmental stresses (Ketchie and Kammereck, 1987; Parker, 1953; Prive and Zhang, 1996; Zhu et al., 2002). The colorless TTC is reduced to red triphenyl formazan by dehydrogenase activity in living cells and/or tissues of plants (Richter et al., 2007). Through this staining, viability of plant tissues to assess freezing injury is estimated (Bennett and Loomis, 1949; Kang et al., 1997; Nesbitt et al., 2002; Zhao et al., 2010; Zhu et al., 2002). Although validity of the analysis has been questioned, since TTC extraction was shown in one study to be a poor indicator rather than EL (Chalker-Scott et al., 1989), it was useful in apple roots (Prive and Zhang, 1996).

In this study, cold hardiness levels in bark and wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees were evaluated using four common methods for shoots of woody plants. This study will provide practical information to find out reliable methods for estimating cold hardiness of trunk tissues in peach trees or other woody plants.

LITERATURE REVIEW

Cold hardiness of woody plants during cold acclimation

For successful overwintering, woody plants native to the temperate zone adapt to seasonal changes in temperature. In autumn, plants develop cold hardiness during cold acclimation, then they become tolerant to mid-winter climate (Pagter and Arora, 2013; Weiser, 1970). The annual process of cold acclimation includes changes of metabolites and membrane structure to develop cold hardiness for survival in mid-winter (Gusta and Wisniewski, 2013). The survival for plants exposed to subfreezing temperatures depends on various interacting factors; timing and/or rate of acclimation, the level of cold hardiness during mid-winter, and the timing and rate of deacclimation in the early spring (Arora and Rowland, 2011; Ehlenfeldt and Arora, 2008).

Cold hardiness varies greatly among species, genotypes, provenances, and even different parts of the same plant. Northern blueberry species (e.g., *V. constablaei* and *V. corymbosum*) are more cold hardy than southern species such as rabbiteye blueberry (*V. ashei*) (Ehlenfeldt et al., 2007; Lee et al., 2012). Various levels of cold hardiness in twenty-four peach and fourteen nectarine cultivars were shown after a temperature drop from 5 to −11^{o}C within 12 h in November in the United States (Smith et al., 1994). Furthermore, the level of cold hardiness in twigs of pecan cultivars has varied, based on the region of origin (Volk et al., 2009). European oak from the northern, central, and southern portions of the Europe has different levels

of cold hardiness (Morin et al., 2007). Generally, reproductive organs, roots, and young leaves are particularly sensitive to cold (Kozlowski and Pallardy, 2002). The shoots of pine tree (*Pinus sylvestris*) could withstand further freezing temperatures than roots, with the maximum tolerance at −40 and −15°C, respectively (Smit-Spinks et al., 1985).

The strategies of woody plants to withstand subfreezing temperatures are classified into freezing avoidance and freezing tolerance (Levitt, 1980). Freezing avoidance is especially associated with supercooling, in which water remains liquid below its equilibrium freezing point because nucleating substances necessary for ice initiation is not sufficient (Burke et al., 1976; Jones et al., 1999). In general, supercooling occurs in xylem parenchyma cells, flower buds, and bark tissues in woody plants (Tanino, 2012). Some tissues survive extracellular freezing, by increasing cytoplasmic viscosity, then changing into the glassy state (Wisniewski et al., 2003). Glass is extremely viscous and metastable conditions for survival, brought by a high solute concentration at a sufficiently low temperature (Welling and Palva, 2006; Wisniewski et al., 2003). Their high viscosity may stop almost all chemical reactions that require molecular diffusion (Crowe et al., 1998; Koster and Bryant, 2006). On the other hand, freezing tolerance of extracellular ice formation and sequential cellular dehydration are another mechanisms to withstand subfreezing temperatures (Levitt, 1980). In peach stems, ice is nucleated in the cortex and grows from there to the xylem (Wisniewski et al., 2003). To reduce ice formation and growth, various reactions such as compositional changes in

membranes, compatible solute concentration, osmotic adjustments, plant hormone regulation, and antioxidant defense system occurs (Gusta and Wisniewski, 2013). Thus, plant tissues and organs have developed strategies to adapt subfreezing temperatures to protect them during cold acclimation (Weiser, 1970). Cold hardiness of bark and xylem tissues from deciduous peach trees increased from −5 and −11°C, respectively, in August to a maximum of about −50 and −36°C in February and then decreased to −4 and −3°C by May (Arora et al., 1992).

Environmental regulation of cold acclimation

Woody plants achieve cold hardiness during sequential stages of cold acclimation. The first change is initiated by short photoperiod and non-freezing temperatures and the second are influenced by subfreezing temperatures. Consequently, woody plants undergo maximum level of cold hardiness during mid-winter (Weiser, 1970).

Cold acclimation is triggered by shortening photoperiod and low temperature (Gusta and Wisniewski, 2013; Li et al., 2004). Shortening photoperiod has been known as an important signal for growth cessation, formation of terminal buds and cell wall, tissue water content, and cold acclimation (Li et al., 2003, 2004). These responses are mediated through the phytochrome protein pigment which perceives the length of the photoperiod (Kalcsits et al., 2009). Among the northern, central, and southern ecotypes of silver birch (*Betula pendula*), the northern ecotype had an earlier and quicker cold acclimation than the others; however, there were no

significant differences in the maximum levels of cold hardiness between the ecotypes after the treatment of photoperiodic control (Li et al., 2003).

When cold acclimation subsequently continues, woody plants during the mid-winter undergo numerous physiological and metabolic changes to develop cold hardiness (Li et al., 2004; Xin and Browse, 2000). They include reduction of growth, tissue water content, compatible solutes, and transient abscisic acid level, and change of membrane lipid composition (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Welling and Palva, 2006).

Compatible solutes like soluble sugars, polyols, proline, betaine, and polyamines are accumulated during cold acclimation in different tissues of woody plants (Koster and Lynch, 1992; Sasaki et al., 1996; Tao et al., 1998). In European oak species, cold hardiness are positively correlated with total soluble sugars, and most soluble sugars increase while starch contents stored at stems and buds decrease (Morin et al., 2007). Like this, one of the most prominent biochemical changes during cold acclimation is the accumulation of soluble sugars in cells and tissues of woody plants. Soluble sugars act as compatible solutes, signaling molecules, carbon skeletons, and energy reserves in overwintering plant tissues (Ma et al., 2009; Xin and Browse, 2000). The major soluble sugars associated with cold hardiness are different in species, tissues, and organs of woody plants. An inter-conversion between starch and sucrose during the autumn in stem occurs earlier than floral buds of peach tree (Bonhomme et al., 2009). In addition, accumulation of dehydrins as a group of late embryogenesis abundant II proteins is also considered as

protection of membranes and proteins after exposure of subfreezing temperatures (Close, 1997; Kosová et al., 2007). The increased amount of dehydrins in bark tissues of deciduous peach trees has shown to be associated with the increased level of cold hardiness during cold acclimation (Arora et al., 1992).

Measurements of cold hardiness according to tissue injury

Freezing temperature causes the ice formation in the inter- and intra-cellular spaces and cell walls of plant tissues. Ice formation initiates at sites that the initial spread of freezing has reached (Pearce and Fuller, 2001). The initial growth of ice is as rapid as 40 mm s^{-1} (Pearce and Fuller, 2001; Zámećník et al., 1994). The pattern of ice is differentially propagated in various organs and tissues of woody plants.

Ice formation occurs in two markedly locations within the tissues of most plants: extracellular spaces and the cells and intracellular spaces. Extracellular ice formation is not lethal, but drops water potential outside the cell (Xin and Browse, 2000). Subsequently, unfrozen water from the cytoplasm moves through the plasma membrane by osmosis, followed by cellular dehydration. The freeze-induced dehydration causes damage to membrane structure and function. Consequently, structural membrane injury resulting from progressive water loss is thought to be a close approach of membranes and fusion (Koster and Bryant, 2006). With this phase change in a fraction of the membrane lipids from a bilayer to a non-bilayer Hex II phase, the plasma membrane destabilized and loses osmotic responsiveness (Koster and Bryant, 2006; Xin and Browse, 2000). The symptoms of membrane injury at

the cellular level are leakage of electrolytes and other solutes and also inability to regain turgor after the stress (Väinölä, 2000). Generally, bark cells in apple twigs are dependent on the ability to tolerate the freezing-induced dehydration (Ashworth et al., 1988). Measuring the change in electrical conductivity of a tissue solution caused by diffusion of electrolytes is one of the most widely used method to assess freezing injury (Nesbitt et al., 2002).

When temperature drops dramatically, tissues are easily injured by intracellular ice formation. To avoid freezing injury, intercellular solution supercools (Ashworth et al., 1988). For example, bud and xylem parenchyma tissues of woody plants avoid freezing injury by deep supercooling (reviewed by Wisniewski, 2009). Water in these tissues exists in the liquid phase to temperatures as −50°C by being isolated from internal and heterogeneous ice nucleators including extracellular ice (Burke and Stushnoff, 1979). Upon nucleation freezing occurs intracellularly, which is lethal event. There is strong correlation between the temperature range of deep supercooling and tissue injury (reviewed by Wisniewski et al., 2009). Deep supercooling in plant tissues can be monitored using differential thermal analysis (DTA). This depends on the use of thermocouples in tissues to detect the latent heat released by water in tissues as it undergoes a liquid to solid phase change (Wisniewski et al., 2009).

Freezing injury is mainly known to result from membrane damage caused by cellular dehydration. However, expansion-shinkage following freezing also induces physical destruction such as frost crack and allows subsequent pathogen to enter

through frost cracks. Furthermore, freeze-induced reactive oxygen species, protein denaturation, and oxidative browning may cause cellular damage (Pearce, 2001; Thomashow, 1999). Oxidative browning is used to assess freezing injury because of simplicity and rapidity.

Finally, tissues are injured as proteins such as enzymes denatured. In living plant tissues, TTC changes to red-colored triphenyl formazan, as dehydrogenase activity increases. On the other hand, dead tissues do not show this reaction. Through this biochemical reaction, measuring viability of plant tissues after freezing or other environmental stresses have been used (Ebel et al., 2004). Extracting the formazan, visual assessment of TTC reduction and/or quantifying with a spectrophotometer could be used to assess freezing injury (Ruf and Brunner, 2003; Towill and Mazur, 1975).

MATERIALS AND METHODS

Plant materials

Two 9-year-old peach tree cultivars, ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’, were from the orchard of National Institute of Horticultural and Herbal Science, Suwon, Korea. Shoots and 5-year-old branches of these cultivars were collected from September to December, 2014. They were used for examining the changes of cold hardiness level and carbohydrate contents.

Meteorological condition

Mean temperature and minimum air temperatures at the experimental site during winter from 2010 to 2014 (Fig. 1) were provided from Korea Meteorological Administration.

Exotherm analysis

Thermal analysis to obtain freezing profiles for bark and wood tissues of 5-year-old branches or trunk were utilized as described by Arora et al. (1992). Samples of bark and wood tissues were utilized to detect exotherms. Oven-dried 3-year-old branches were used as a reference. Bark and wood tissues of branches were taped to a copper-constantan thermocouple and placed in a 50 mL conical

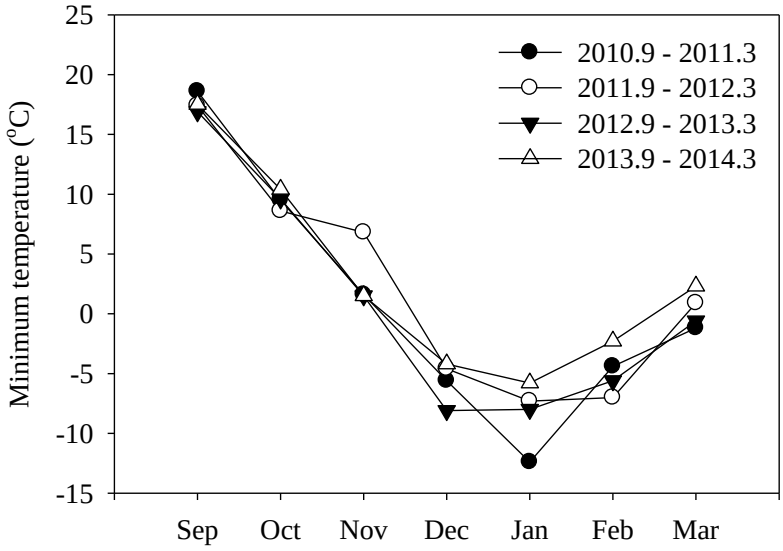

Fig. 1. Minimum temperatures at the National Institute of Horticultural and Herbal Science, Suwon, Korea (37°15' N, 126°98' E) during cold acclimation and deacclimation of 2010-2014.

tube. The tubes were cooled at a rate of 2°C/h, in a bath circulator (RW-1040G and RW-2040G, Jeio Tech, Seoul, Korea) equipped with a temperature controller (UP351E, Yokogawa Electric Korea Co., Seoul, Korea). No significant differences were observed between the exothermic patterns of 2 and 20°C/h, thus cooled at a rate of 2°C/h. Stem temperature was monitored by a copper-constantan thermocouple, and recorded every two seconds with a data logger (CR1000M, Campbell Scientific, Inc., Logan, UT, USA).

Visual estimation based on tissue browning

After freeze-thaw treatment, detached wood and bark tissues were separated to observe tissue browning on naked eyes.

EL analysis

Cold hardiness of the bark and wood tissues was estimated by the method of Lee et al. (2012) with slight modifications. Shoots, 3-year-old and 5-year-old branches, and trunk tissues were randomly collected from the peach trees. The samples were rinsed under running cold distilled water. After rinsing, the samples were placed in a 50 mL conical tube containing 2 mL of distilled water. The tubes were incubated in a bath circulator equipped with a temperature controller and cooled at a rate of 2°C/h to the target temperature, and then maintained for 2 h at each target temperature. The tubes were removed and thawed at 4°C. Five target temperatures were selected in ranges of −2 to −24°C in September, −4 to −30°C in October and

November, and −8 to −40°C in December, 2014. The temperatures were monitored every two seconds with a data logger (CR1000M, Campbell Scientific, Inc.) using a copper-constantan thermocouple.

After the freezing-thawing treatment, shoots were cut into 1-cm-long, and 5-year-old branches were separated into bark and wood tissues, placed in a 50 mL conical tube containing 20 mL of distilled water, and then vacuum-infiltrated for 3 min. The tubes were shaken at 125 rpm on an orbital shaker (SupertechTM Orbital Shaker, SeouLin Bioscience, Seoul, Korea) at room temperature for 20 h and EC of the aliquots were measured using an EC meter (Orion Star A215, Thermo Scientific, Waltham, MA, USA). After autoclaving at 120°C for 30 min, EC was measured again. Then, percent injury was calculated according to the method of Lee et al. (2012);

$$\% \text{ Injury} = (\% \text{ EL}_{(t)} - \% \text{ EL}_{(4°C)})/(100 - \% \text{ EL}_{(4°C)}) \times 100$$

Where % $EL_{(t)}$ and % $EL_{(4°C)}$ are percentages of EL for initially each freeze-treatment temperature and unfrozen control, respectively. The percent injury data were adjusted using the method of Lim et al. (1998). Samples treated at −80°C were representing 100% freeze-injured samples by extreme freezing, and the percent injury was then transformed by following equation;

$$\% \text{ Adjusted injury} = (\% \text{ injury}_{(t)}/\% \text{ injury}_{(-80°C)}) \times 100$$

With these percent adjusted injury data, a quantitative estimate of cold hardiness, temperature at which 50% injury occurred (LT_{50}), were calculated using the Gompertz function; an asymmetric sigmoid curve which is appropriate to be used in data fitting of plant response to temperature stress (Lim et al., 1998). To obtain more practical and efficient LT_{50} estimates without the repeat of whole experiment, the five adjusted injury values at each treatment temperature were resampled 30 times according to the method by Arora et al. (2004). Using the 30 sets of resampled data and 30 resulting values of LT_{50}, standard errors (SE) of LT_{50} were predicted.

TTC analysis

Cold hardiness of each individual tissue was determined by measuring viability using TTC staining, according to the method by Ruf and Brunner (2003) and Nesbitt et al. (2002) with slight modifications. Shoots and 5-year-old branches were randomly collected from peach trees and then rinsed under cold tap water for 5 s. The samples were placed in 50 mL conical tubes, and then incubated in a bath circulator equipped with a temperature controller. The tubes cooled at a rate of $2^{o}C$/h until the target temperature was reached and maintained for 2 h, thereafter thawed at $5^{o}C$, as for EL analysis.

After the freezing-thawing treatment, buds were removed from shoots and cut into 3-cm-long sections. Branches were cut into 1-cm-long sections. Then, they were placed in 50 mL conical tubes containing 10 mL of 0.8% TTC in a 0.05M Na_2HPO_4/KH_2PO_4 buffer (pH 7.4) for stem pieces and 5 mL for shoots. The

samples were placed under vacuum for 15 min to remove air from the intercellular spaces, thereby allowing tissue infiltration by the TTC solution. The tubes were incubated for 20 h in a growth chamber (HB-301S-3, Hanbaek Scientific Co., Seoul, Korea) set in the dark at 28ºC. After incubation, TTC solution was decanted and the samples were rinsed three times with distilled water. Bark and wood tissues were dissected from branches. Then, to extract the water-insoluble formazan, the sample tissues were boiled for 10 min following the addition of 7 mL of 95% (v/v) ethanol (Fig. 2). TTC reduction was determined spectrophotometrically by measuring absorbance at 490 nm (Towill and Mazur, 1975).

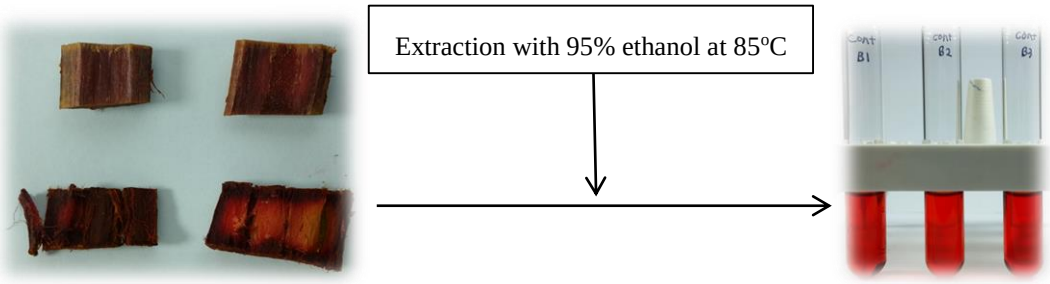

Fig. 2. Experimental procedure of triphenyl tetrazolium chloride analysis in different tissues of 5-year-old branches of ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees, sampled in September, 2014.

RESULTS AND DISCUSSION

Exotherm analysis

High temperature exotherm (HTE) occurred between −4 and −8°C in bark and wood tissues of 5-year-old branches (Fig. 3). However, LTE was not detected in both tissues at a cooling rate of 2°C/h. Thermal analysis in the wood tissues of branches showed the large initial peak, HTE between 0 and −4°C (Fig. 4). However, LTE was not detected in the same tissues of branches. DTA with the wood tissues of branches showed only HTE, but no LTEs were identified (Fig. 4). The HTE temperature from each wood tissue was −2.2 and −3.9°C, at a cooling rate of 2°C/h. HTEs still occurred at cooling rates of 2 and 20°C/h (Fig. 5). LTEs were not detected even at rapid cooling rate (−20°C/h) in thermal analysis (Fig. 5) and DTA in wood tissues of branches (Fig. 6). Therefore, cooling rates may not be associated with the existence of LTEs. With only one exotherm, it was actually difficult to distinguish between non-lethal and lethal temperatures. Similar result was shown in some subtropical species (Nesbitt et al., 2002).

Visual estimation based on tissue browning

As tissues were frozen at a rate of −2, −10, and −20°C/h, at a cooling rate of 2°C/h, both bark and wood tissues of branches were slightly damaged, while both tissues were shown severe oxidative browning at a rate of −20°C/h (Fig. 7).

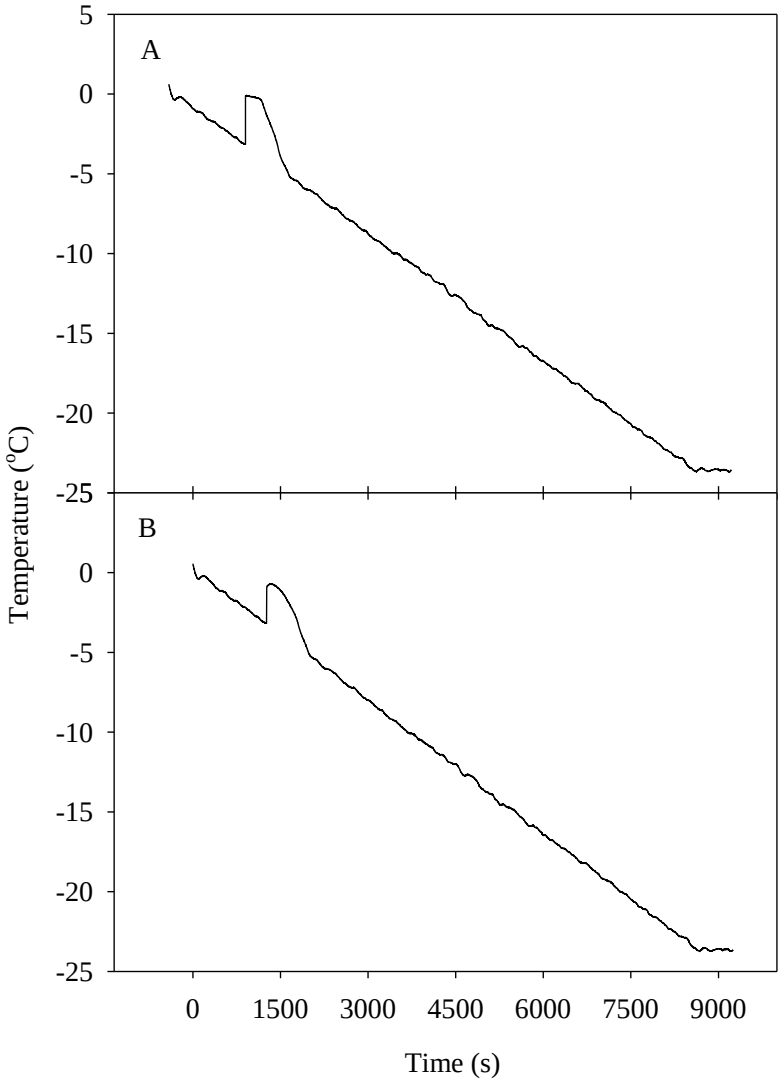

Fig. 3. Thermal analysis with bark (A) and wood tissues (B) of 5-year-old branches of ‘Janghowonhwangdo’ peach trees.

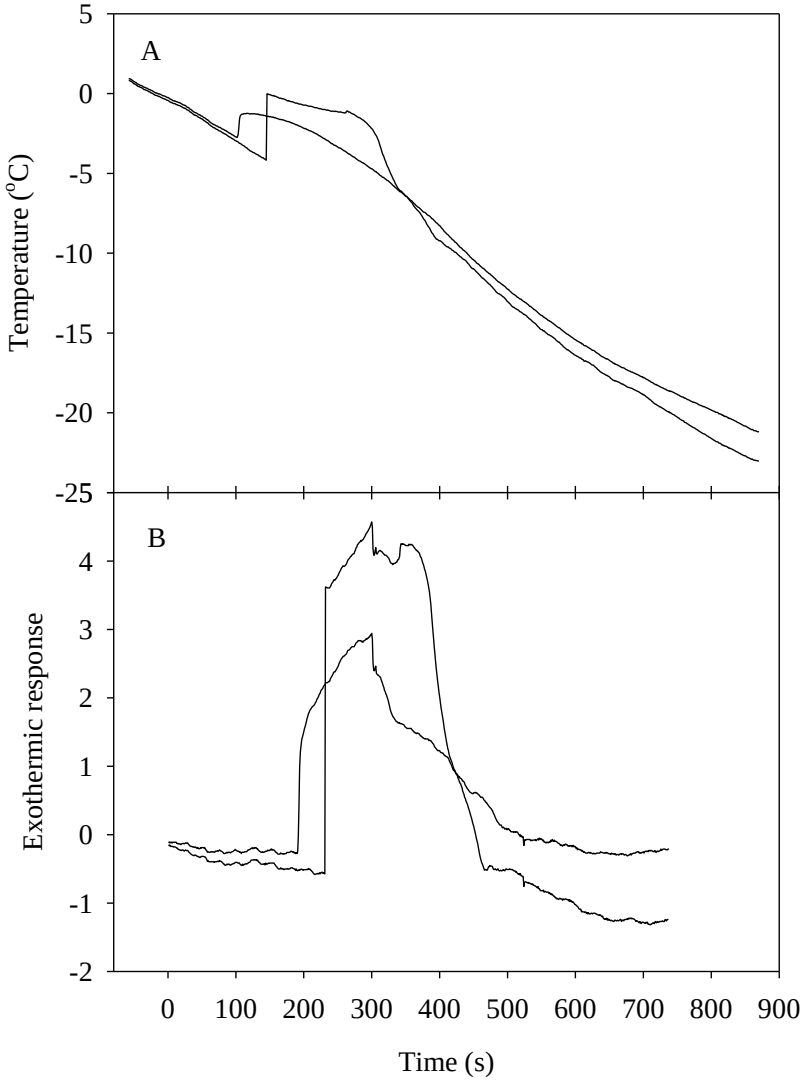

Fig. 4. Thermal analysis (A) and differential thermal analysis profile (B) with wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ peach trees at a cooling rate of 2°C/h.

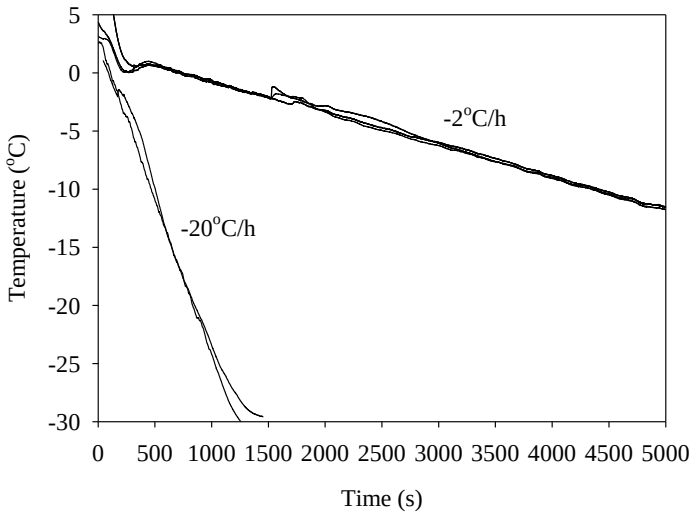

Fig. 5. Thermal analysis with wood tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees with a cooling rate of 2 or 20°C/h.

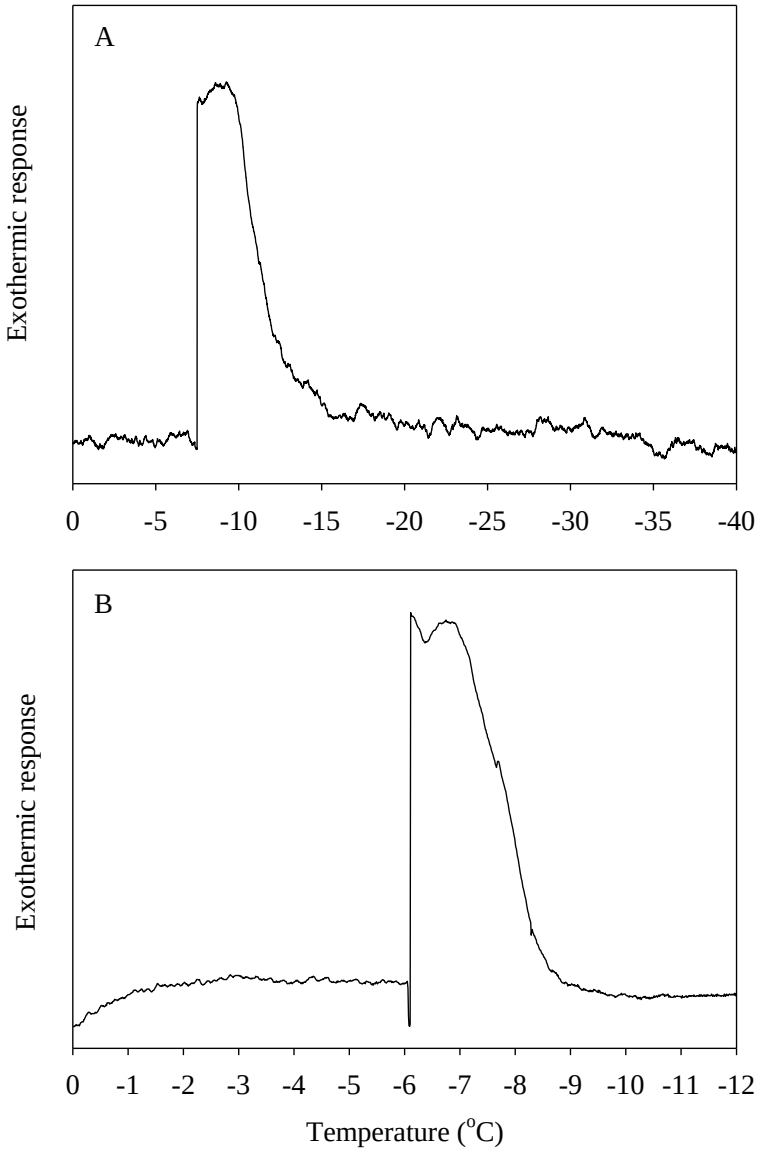

Fig. 6. Differential thermal analysis with wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ peach trees with a cooling rate of 2 (A) or 20°C/h (B).

−2ºC/h −10ºC/h −20ºC/h

Fig. 7. Freezing injury in 5-year-old branches of ‘Janghowonhwangdo’ peach trees according to different cooling rates.

According to data provided by Korea Meteorological Administration, naturally air temperatures usually freeze at a rate of approximately −2°C/h. As temperature dropped at a rate of −2 to −14 and −24°C, both bark and wood tissues were more severely damaged at −24°C than at −14°C (Fig. 8).

The low temperature-caused injury could be observed relatively easier than by other analyses; nevertheless, quantifying the extent of freezing injury was difficult. When the same subzero temperatures were treated, the same response in both tissues was not always shown. Therefore, visual observation to estimate cold hardiness levels on both tissues of branches and trunk tissues was considered not to be suitable due to its low reproducibility.

Evaluation of EL analysis in different tissues at various subzero temperatures

With decreasing temperature, EL increased in shoots, and bark and wood tissues of 5-year-old branches in ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees in September, 2014. Most tissues in the two cultivars were damaged with different patterns.

In both bark and wood tissues in ‘Janghowonhwangdo’and ‘Hikawa Hakuho’ peach trees, EL dramatically varied between −3.8 and −26°C (Fig. 9). When treating at −12, −18, and −22°C for 2, 6, and 12 h, tissues showed different patterns (Fig. 10). Commonly, EL increased at lower temperatures maintained longer. For example, in bark tissues of 5-year-old branches of ‘Janghowonhwang-

5ºC/h −14ºC/h −24ºC/h

Fig. 8. Freezing injury in 5-year-old branches of ‘Janghowonhwangdo’ peach trees at subzero temperatures.

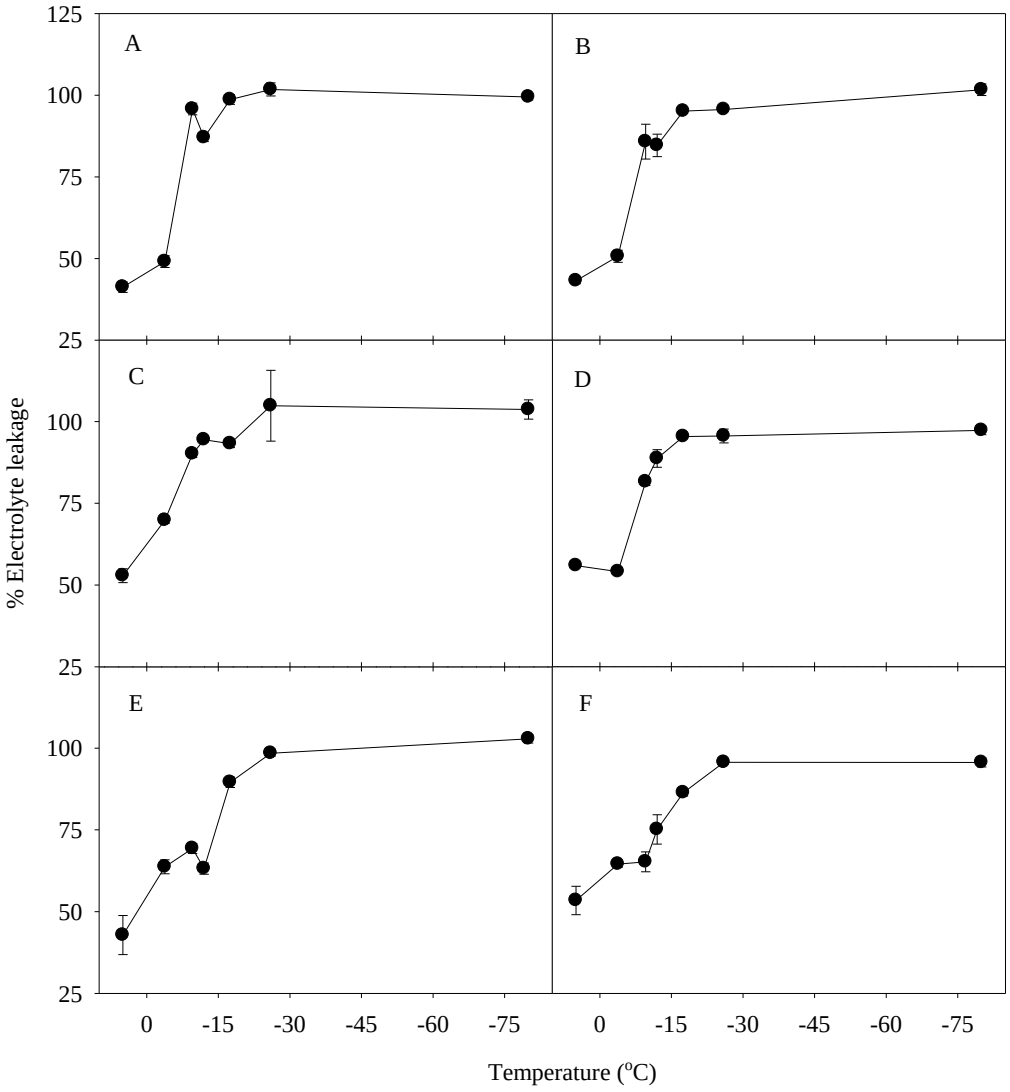

Fig. 9. Electrolytes leakage from shoots, and bark and wood tissues of 5-year-old branches of 'Janghowonhwangdo' (A, C, E) and 'Hikawa Hakuho' peach trees (B, D, F) after exposure to subfreezing temperatures, sampled in September, 2014. A, B, shoots; C, D, bark tissues; E, F, wood tissues. Data are means ± SE (n = 4).

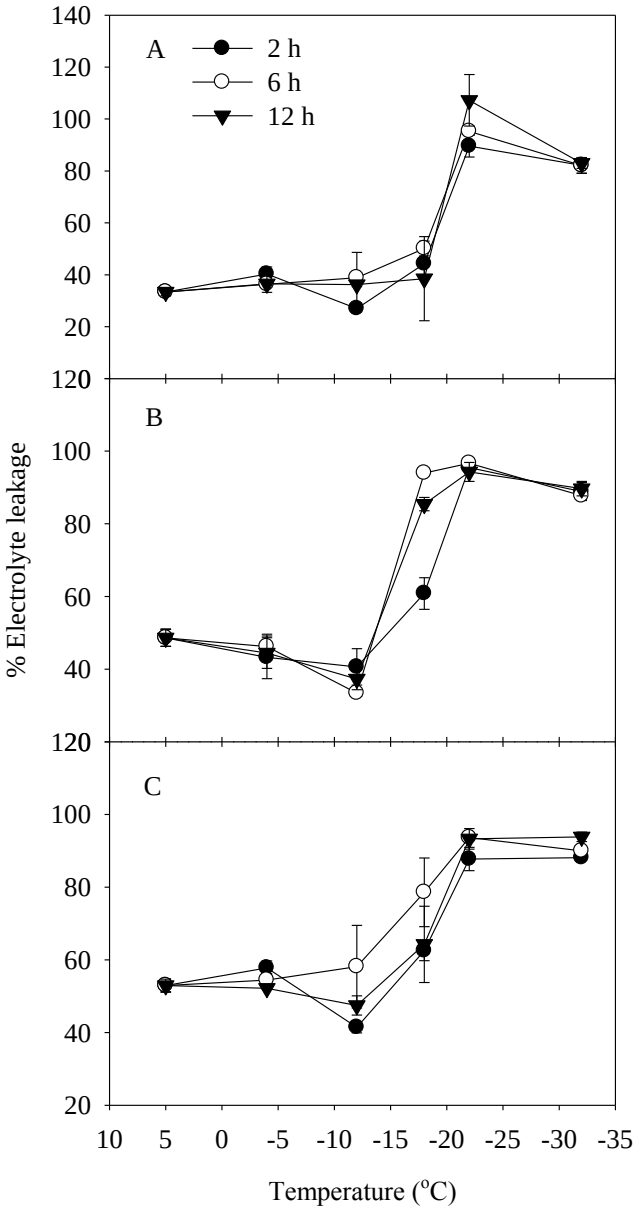

Fig. 10. Electrolyte leakage from shoots, and bark and wood tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees after exposure to subfreezing temperatures for various durations. A, shoots; B, bark tissues; C, wood tissues. Data are means ± SE (n = 4).

do' peach trees, EL dramatically increased at −12, −18, and at −22ºC for 2, 6, and 12 h, respectively (Fig. 10A). When estimating cold hardiness levels seasonally, 2 h of duration time was determined based on these results (Fig. 10). Based on this freezing injury to cell membranes, cold hardiness level in branches was possibly determined using EL analysis. This way has widely been used in various woody plants such as yellow birch (Zhu et al., 2002), satsuma mandarin (Nesbitt et al., 2002), and peach tree (Arora et al., 1992).

Data transformation and estimation of LT_{50} by EL analysis

The percent EL of unfrozen samples was 41.2, 52.8, and 42.8% for shoots, and bark and wood tissues respectively, in 'Janghowonhwangdo', and 43.2, 55.2, and 53.4% for shoots, and bark and wood tissues, respectively, in 'Hikawa Hakuho' peach trees (Table 1, data not shown for wood tissues and shoots in both cultivars). The EL values in the unfrozen tissues indicated the amount of electrolytes originally located in extracellular space. EL from the cutting plane might also occur due to the destruction of cells and/or tissues. The percent injury values of the samples treated at −80ºC did not reach at 100% (Table 1). Since the cell wall containing various ions was also damaged besides the plasma membranes and organellar membranes during the autoclave for releasing total electrolytes, this fact tended to overestimate total EC.

Gompertz function was well-fitted with the percent adjusted injury data; r^2 =

Table 1. An example for calculation of percent adjusted injury at various temperatures through electrolyte leakage (EL) analysis in 5-year-old branches of 'Janghowonhwangdo' peach trees during cold acclimation.

Treatment temp. (°C)	Initial EC[z](μS)	Total EC (μS)	% Electrolyte leakage (EL[y])	% Injury[x]	% Adjusted injury[w]
5	42.83	200.70	21.34	0	0
–3.8	95.60	176.42	54.19	41.76	44.66
–9.6	150.15	181.90	82.55	77.81	83.22
–12.0	191.38	220.18	86.92	83.37	89.17
–17.5	171.68	185.48	92.56	90.54	96.83
–26	245.40	260.85	94.08	92.47	98.90
–80	256.80	270.63	94.89	93.50	100

[z]Raw data of electrical conductivity (EC).

[y](Initial EC/total EC) ×100.

[x]$(\%EL_{(t)} - \%EL_{(5°C)})/(100 - \%EL_{(5°C)}) \times 100$.

[w]$(\%\ Injury_{(t)}/\%\ injury_{(-80°C)}) \times 100$.

0.99, $P < 0.001$ (Fig. 11) in bark tissues of 5-year-old branches of ‘Janghowonhwangdo’ peach trees. Thus, the percent adjusted injury data obtained through EL analysis at various temperatures was appropriate for estimating LT_{50} representing cold hardiness levels in each tissue of peach trees. Not only bark tissues but also other tissues were applicable to calculate the percent adjusted injury for estimating LT_{50} during September, 2014 (Fig. 12).

However, before freeze-thaw treatment, separating wood tissues from bark tissues was difficult, and thus it should be careful. In addition, when conducting the EL analysis, more samples were required than the other analyses. Compared to visual estimation based on browning, percent EL increased significantly between −12 and −18°C in most tissues, but EL was greater at −24°C than at −14°C. This fact accounted for the possibility for overestimation and/or underestimation of woody tissue survival. This could also be due to some differences of membrane composition of branches and shoots.

Evaluation of TTC analysis in different tissues at various subzero temperatures

With decreasing temperature, TTC reduction decreased in shoots, and bark and wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees in September, 2014. Most tissues in the two cultivars showed different patterns.

In both bark tissues in ‘Janghowonhwangdo’and ‘Hikawa Hakuho’ peach trees,

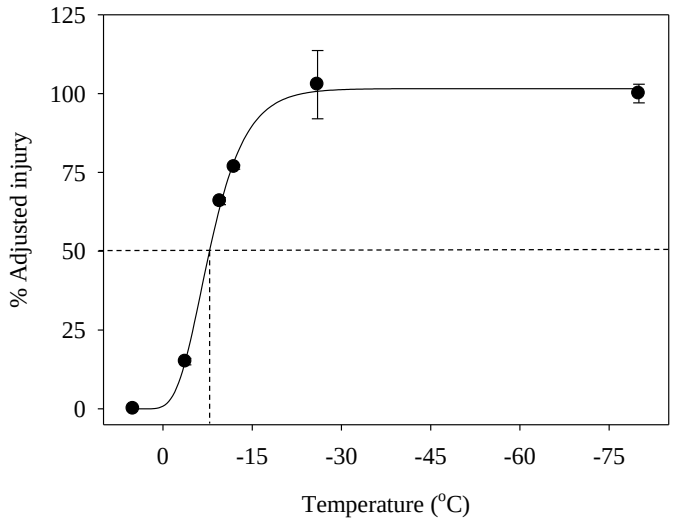

Fig. 11. Estimation of LT_{50} using electrolyte leakage analysis in wood tissues of 5-year-old branches on 'Janghowonhwangdo' peach trees using the Gompertz function fitted to means of the percent adjusted injury data per treatment temperature during cold acclimation. Data are means ± SE (n = 4).

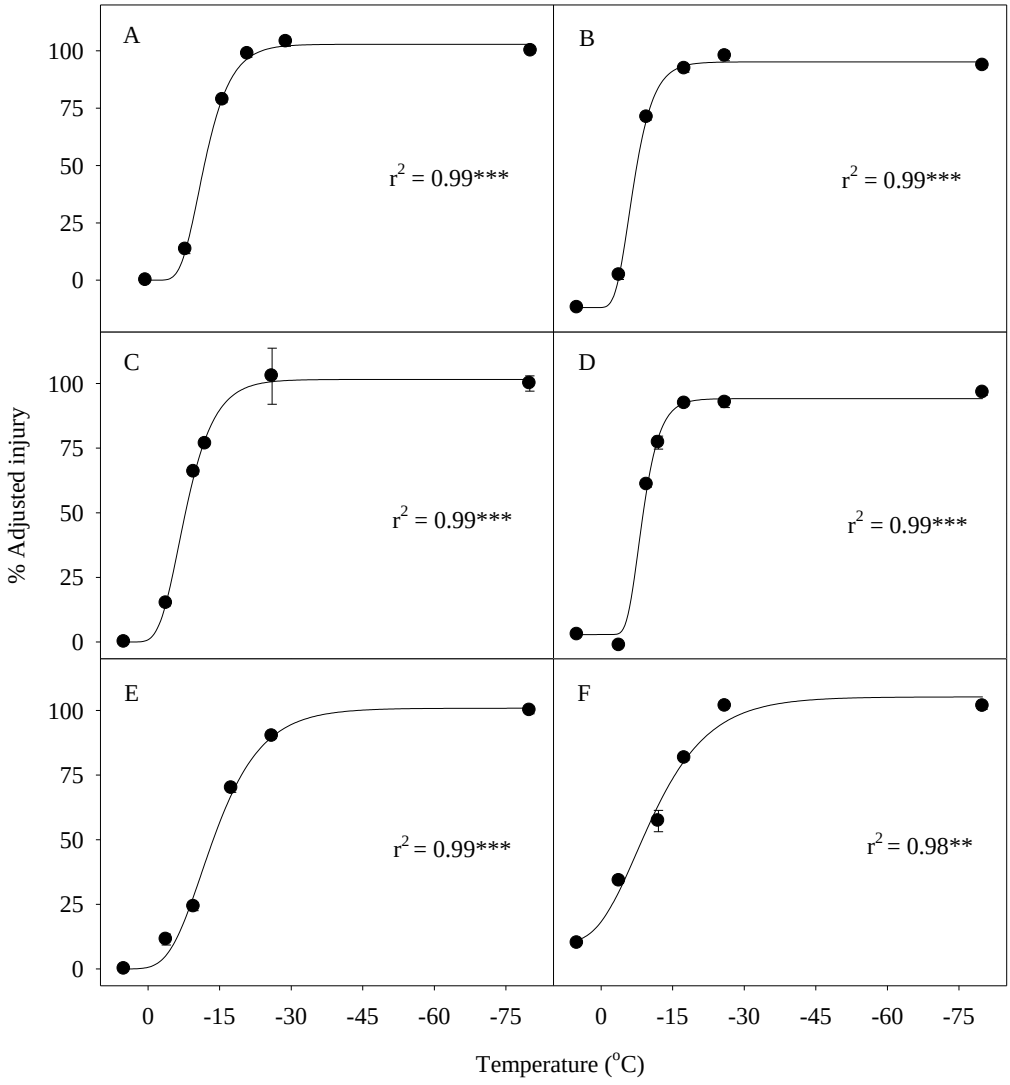

Fig. 12. Estimation of LT_{50} using electrolyte leakage analysis in shoots, and bark and wood tissues of 5-year-old branches of 'Janghowonhwangdo' (A, C, E) and 'Hikawa Hakuho' peach trees (B, D, F), sampled in September, 2014. A, B, shoots; C, D, bark tissues; E, F, wood tissues. Data are means ± SE (n = 4).

TTC reduction measurements dramatically varied between −3.8 and −17.5ºC (Fig. 13). TTC reduction measurements in other tissues showed similar trends to bark tissues (Fig. 14.). When treating at −12, −18, and −22ºC for 2, 6, and 12 h, respectively, the tissues showed different patterns (Fig. 15). Commonly, TTC reduction measurements decreased at lower temperatures maintained longer. For example, in bark tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees, TTC reduction dramatically decreased at −12 and −18ºC for 6 and 12 h, respectively, in bark and wood tissues of 5-year-old branches, and −18 and −22ºC for 6 and 12 h, respectively (Fig. 15). When estimating cold hardiness levels seasonally, 2 h of duration was determined based on these results (Fig. 15). Based on this freezing injury to metabolic activity, cold hardiness levels in branches was possibly determined using TTC analysis. This way has widely been used in various woody plants such as persimmon buds (Kang et al., 1997) and satsuma mandarin (Nesbitt et al., 2002).

Data transformation and estimation of LT_{50}

Of the values, the relative viability of the unfrozen samples (5ºC) was and 47, 595, and 82 in shoots, and bark and wood tissues of 5-year-old branches of 'Janghowonhwangdo' species, and 214, 477, and 66 in the tissues of the branches of 'Hikawa Hakuho' peach trees (Table 2, data shown in only wood tissues of the branches in 'Janghowonhwangdo'). Using the values of relative viability, the percent adjusted viability is calculated to make the samples treated at 5ºC reach at

100% (Table 2) and then changes to the percent adjusted injury, similar to the method of EL analysis (Table 2). Gompertz function also fitted with the percent adjusted injury data obtained through the TTC analysis; $r^2 = 0.99$, $P = 0.0009$ (Fig. 16). Thus, this method was also found to be suitable for determining cold hardiness levels in different tissues of 5-year-old branches in peach trees (Fig. 17). Compared to EL analysis, TTC analysis required less samples and relatively easier to separate wood tissues from bark tissues after freezing treatments.

Changes of cold hardiness for bot bark and wood tissues and shoots during cold acclimation, using EL and TTC analyses

Cold hardiness levels of branches and shoots significantly differed according to seasonal changes, but showed in different ways in the two cultivars. Substantial hardening of both bark and wood tissues of 5-year-old branches and shoots began by September and/or October. Freezing temperatures are thought to be required for induction of the second and most extensive stage of cold acclimation in woody plants. When the minimum air temperature was consistently below 0^{o}C in November, there was a sharp increase in the levels of cold hardiness in different tissues of both cultivars (Fig. 18).

Compared to the results from EL and TTC analyses, EL analysis tended to overestimate the values. That is because separating wood tissues from bark tissues in 5-year-old branches before freezing treatment could be erroneous. For example, there was similar to the *Hydrangea* shoots. In January and February, the levels of

cold hardiness were lower than −30°C, thus could not be determined precisely (Pagter et al., 2008).

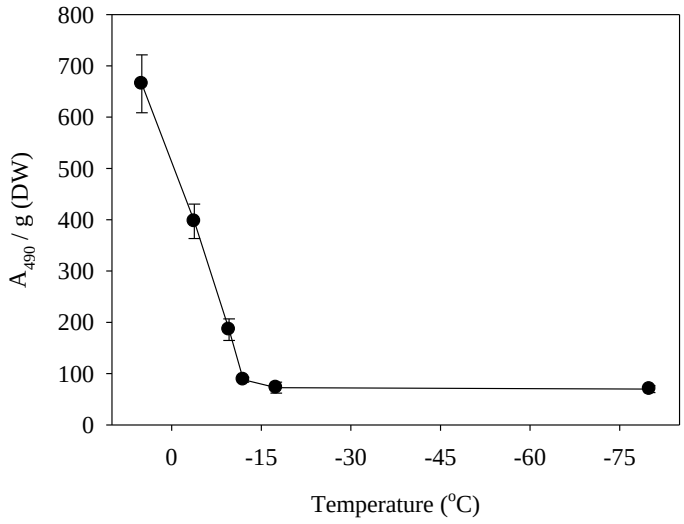

Fig. 13. Changes of absorbance at 490 nm in bark tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees at various subzero temperatures, sampled in September, 2014. Data are means ± SE (n = 4).

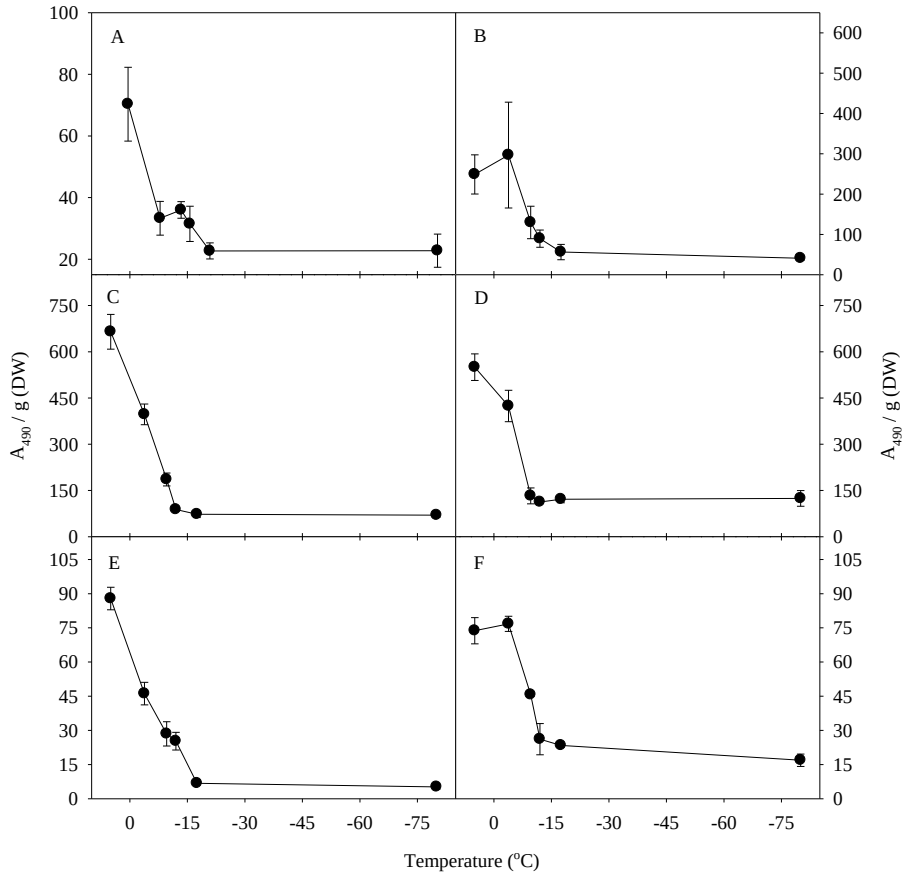

Fig. 14. Changes of absorbance at 490 m in shoots, and bark and wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ (A, C, E) and ‘Hikawa Hakuho’ (B, D, F) peach trees at various subzero temperatures, sampled in September, 2014. A, B, shoots; C, D, bark tissues; E, F, wood tissues. Data are means ± SE (n = 4).

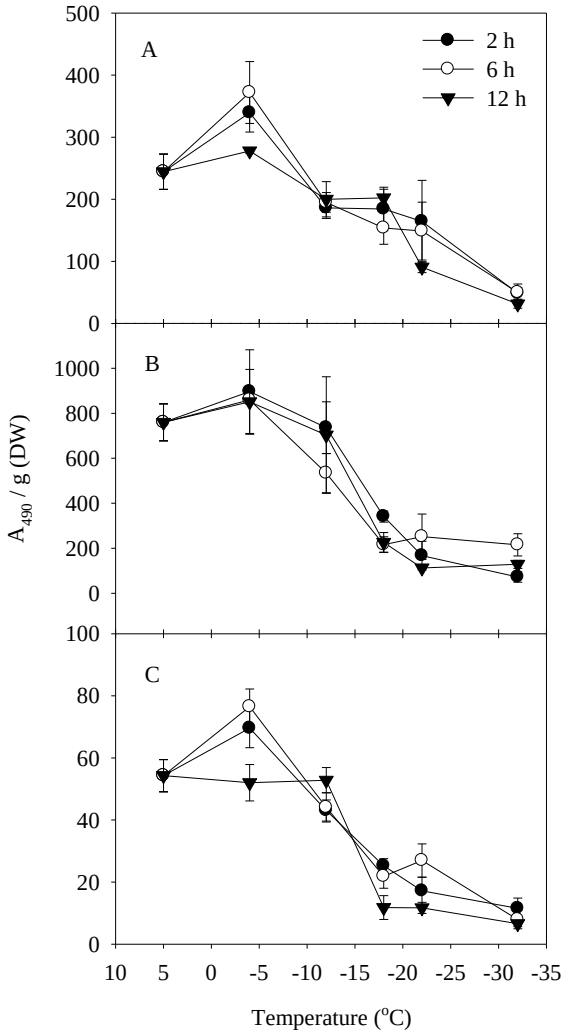

Fig. 15. Triphenyl tetrazolium chloride analysis on shoots, and bark and wood tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees after exposure to subfreezing temperatures for various durations. A, shoots; B, bark tissues; C, wood tissues. Data are means ± SE (n = 4).

Table 2. An example for calculation of percent adjusted injury at various temperatures through triphenyl tetrazolium chloride analysis in 5-year-old branches of 'Janghowonhwangdo' peach trees during cold acclimation.

Treatment Temp. (°C)	A_{490}[z]	RV[y]	% Adjusted RV[x]	% Adjusted injury[w]
5	87.86	82.67	100.00	0
–3.8	46.13	40.94	49.52	50.48
–9.6	28.45	23.26	28.14	71.86
–12	25.26	20.07	24.28	75.72
–26	8.17	2.98	3.60	96.40
–80	5.19	0	-	100.00

[z]Raw data of absorbance at 490 nm (A_{490}).

[y]Relative viability (RV) = $A_{490} - A_{490(-80°C)}$.

[x]$(RV_{(t)} / RV_{(5°C)}) \times 100$.

[w]$(100 - \%\ \text{Adjusted}\ RV_{(t)})$.

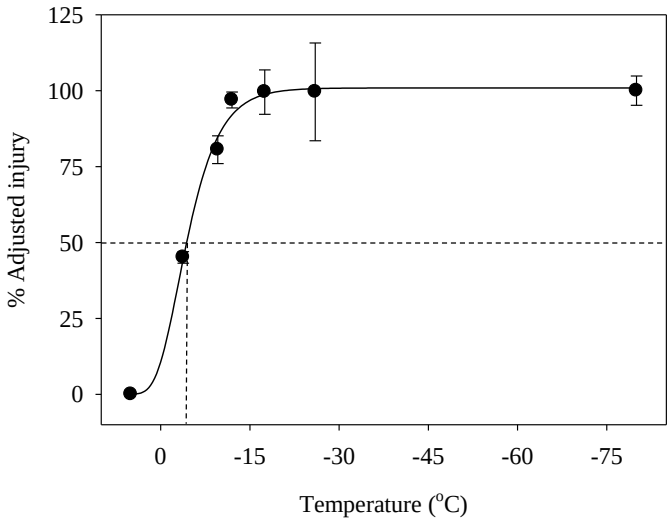

Fig. 16. Estimation of LT_{50} using triphenyl tetrazolium chloride analysis in wood tissues of 5-year-old branches of 'Janghowonhwangdo' peach trees using the Gompertz function fitted to means of the percent adjusted injury data per treatment temperature during cold acclimation. Data are means ± SE (n = 4).

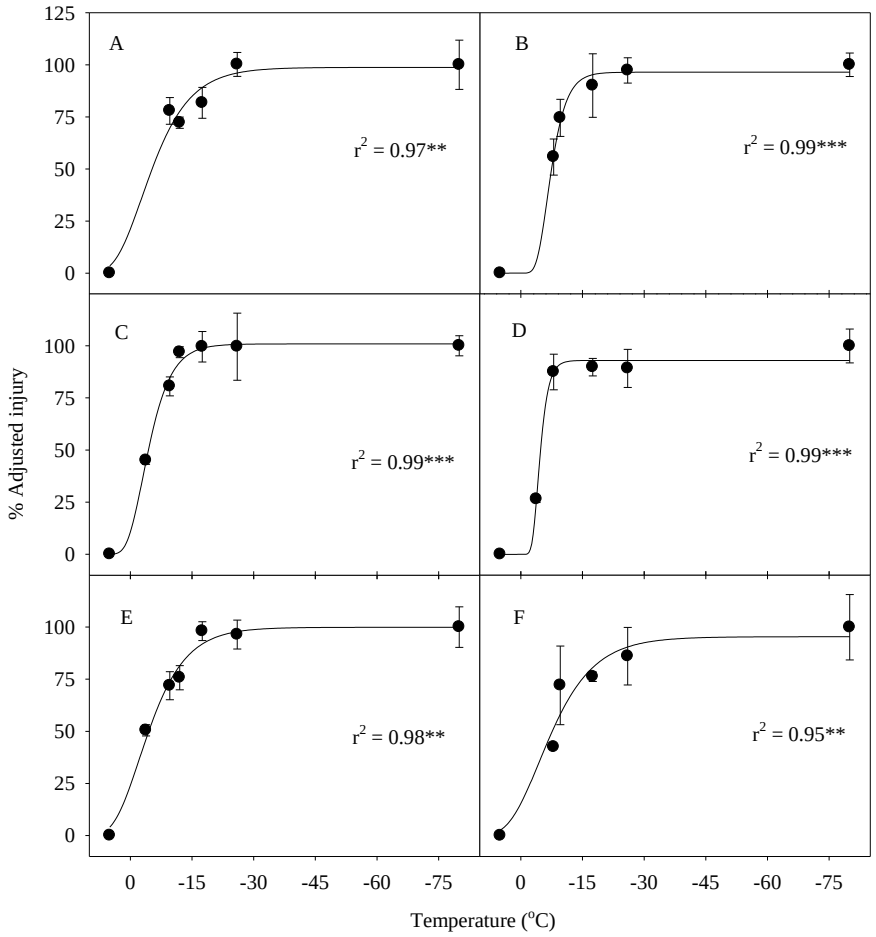

Fig. 17. Estimation of LT_{50} using triphenyl tetrazolium chloride analysis in shoots, and bark and wood tissues of 5-year-old branches on 'Janghowonhwangdo' and 'Hikawa Hakuho' peach trees using the Gompertz function fitted to means of the percent adjusted injury data per treatment temperature. A, B, shoots; C, D, bark tissues; E, F, wood tissues. Data are means ± SE (n = 4).

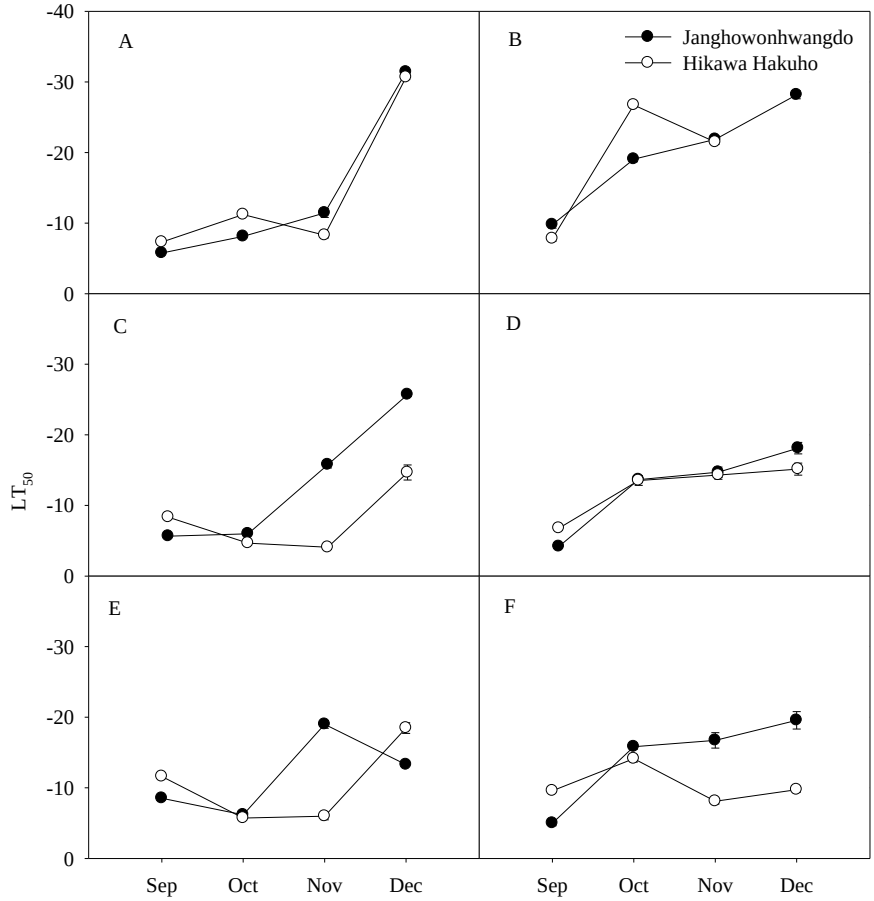

Fig. 18. Changes of cold hardiness in shoots, and bark and wood tissues of 5-year-old branches of ‘Janghowonhwangdo’ and ‘Hikawa Hakuho’ peach trees in different tissues from September to December, 2014, according to electrolyte leakage (A, C, E) and triphenyl tetrazolium chloride (B, D, F) analyses. A, B, shoots; C, D, bark tissues; E, F, wood tissues. Data are means ± SE (n = 4).

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ABSTRACT IN KOREAN

본 연구는 복숭아나무 ‘장호원황도’와 ‘일천백봉’ 두 품종의 신초와 5년생 가지를 대상으로 조직별 원줄기의 내한성을 측정할 수 있는 적절한 방법을 찾고자 저온 순화 동안 육안 판별법, 열분석법, 전해질 누출법, TTC 분석법을 수행하였다. 육안판별법은 간단히 조직 갈변 현상은 관찰하였으나, 온도에 따른 피해 양상이 매번 달라 수치화 하기 어려웠으며, 열분석은 세포 밖에서 결빙을 나타내는 고온 열방출점은 −5에서 −10℃ 범위 내에서 일관성 있게 발생하였다. 하지만 세포 내 공간의 결빙을 나타내는 저온 열방출점은 확인하기 어렵고 재현성이 낮아 복숭아나무의 조직별 원줄기 내한성을 측정하기에 적합하지 않았다. 반면에 전해질 누출법과 TTC 분석법은 분석 결과를 바탕으로 50% 피해가 발생하는 온도인 LT_{50} 값으로 내한성을 정량화 할 수 있어 복숭아나무 내한성 측정에 적합한 것으로 판단되었다. 하지만 전해질 누출법의 경우, 온도 처리 시작 전에 수피와 목질부를 분리해야 하고, 작은 조각으로 잘라야 하는 점에서 실험상 오차 요인을 내포하고 있었다. 이러한 결과를 통해 복숭아나무의 조직별 원줄기의 내한성을 측정하는 데에 있어서 전해질 누출법과 TTC 분석법이 적합한 것으로 판단되었다.