

Full Length Research Paper

Effects of exogenous ABA on antioxidant enzymes in detached citrus leaves treated by rapid freezing

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Accepted 8 July, 2011

Two-year-old seedlings of trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) and lemon (*Citrus limonia* Osbeck.) were sprayed with 0.1 mmol·L⁻¹ abscisic acid (ABA) and then their leaves were collected randomly to expose to freezing stress. Specifically, these detached leaves were treated at 0, -3, -6, -9 and -12°C for 1 h respectively. The concentration of malondialdehyde (MDA), percentage of electrolyte leakage (EL) and the activities of several antioxidant enzymes (superoxide dismutase, catalase and peroxidase) were determined spectrophotometrically. The results show that the percentage of electrolyte leakage in trifoliolate orange was lower than that in lemon, while the MDA concentration and the antioxidant activities of antioxidant enzymes in trifoliolate orange were higher than those in lemon. Pretreatment with the abscisic acid can significantly reduce the membrane damage caused by freezing stress. However, the lipid peroxidation damage caused by low temperature and the activities of antioxidant enzymes were irregularly influenced by ABA pretreatment. We were thus able to infer that exogenous ABA can enhance the cold tolerance of citrus by a process rather than by activating antioxidant enzymes.

Keywords: Antioxidant enzyme, short-term freezing stress, citrus, abscisic acid (ABA).

INTRODUCTION

Freezing is one of abiotic stresses that can cause irreversible damage to plant cells due to the mechanical forces generated by the formation of extracellular ice crystals, cellular dehydration and increased concentration of intracellular salts (Thakur et al., 2010). However, freezing tolerance of many plants can be improved by a process called cold acclimation. During cold acclimation process, ABA is thought to trigger large amounts of cellular responses in plants required by the development of freezing tolerance (Shilpi and Narendra, 2005). Also it has been reported that the application of exogenous ABA can improve the freezing tolerance in various plant species, including *Arabidopsis* (Mantyla et al., 1995),

patens (Minami et al., 2003; Nagao et al., 2005), chickpea (Nayyar et al., 2005) and wheat (Zabotin, 2009). These findings indicate that ABA may function in a common physiological process during the development of cold tolerance and freezing tolerance.

Oxidative stress may be a significant factor correlated with chilling-induced injuries (Fadzillah et al., 1996; O'Kane et al., 1996; Prasad et al., 1994; Srivalli et al., 2003). Redundant reactive oxygen species (ROS) will be generated in plant cells when plants cope with freezing exposure. To protect themselves from those toxic oxygen intermediates, some defense enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (PODs) in plants can be activated to eliminate the ROS. Treatment of *Populus cathayana* Rehd with ABA can increase the antioxidant activities of its antioxidant enzymes and the antioxidant levels (Yanwei et al., 2009).

Poncirus trifoliata (L.) Raf., a relatively interfertile citrus, is extremely cold tolerant and can survive freezes at -20°C if it is fully acclimated to cold (Yelenosky, 1985). It is widely used as the main rootstock of commercial citrus

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Abbreviations: ABA, Abscisic acid; POD, peroxidase; CAT, catalase; SOD, superoxide dismutase; EL, electrolyte leakage; MDA, malondialdehyde; ROS, reactive oxygen species.

production for its ability to enhance the grafted species' cold tolerance and resistance to some pathogens and insects. On the other hand, *Citrus limonia* Osbeck., one of the most cold-sensitive citrus species, can only be able to endure temperature slightly below freezing. Little has been known about the effects of short-term freezing stress on antioxidant enzymes of citrus pretreated with ABA. In this study, we investigate the changes of antioxidant enzymes' activities in ABA-treated leaves of *P. trifoliata* (L.) Raf. and *C. limonia* Osbeck with an aim to evaluate the role of ABA on freezing tolerance of citrus.

MATERIALS AND METHODS

Plant materials and exogenous ABA pretreating

Poncirus trifoliata (L.) Raf. (tolerant to freezing stress) and *C. limonia* Osbeck (susceptible to freezing stress) were transplanted to 20 cm diameter plastic pots containing a mixture of peat and perlite (3:1, v/v), growing for 40 days in a greenhouse at the temperature of 25 to 30°C under natural light condition with routine irrigation management, and biweekly fertilization with 0.3% solution of N-P-K fertilizer (15:15:15). Then the plants were sprayed twice a week with 0.1 mmol.L⁻¹ ABA solution containing 0.05% Tween-20. Meanwhile, another group of plants were sprayed with distilled water as duration. After three-week treatment, leaves were collected at random from each group, put into sealed plastic bags and exposed to freezing stress at 0, -3, -6, -9, -12°C for 1 h respectively.

MDA analysis

The concentration of the MDA, an ultimate product of lipid peroxidation, was measured according to the method adapted from Heath and Parker (1968) to show the degree of lipid peroxidation.

Determination of electrolyte leakage (EL)

Electrolyte leakage (EL) of the leaf samples during the ABA treatment was determined by the method of Minami et al. (2003).

Measurement of antioxidant enzymes

The activities of SOD, CAT and POD in the leaf samples were analyzed according to the methods of Madamanchi et al. (1994), Aebi (1984) and Ali et al. (2005) respectively.

Statistical analysis

The data shown are the means \pm S.D. of three separate experiments. Statistical significance was estimated using the Student's t-test and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Electrolyte leakage (EL)

Electrolyte leakage can reflect the degree of damage

resulted from stresses to plasmalemma. The percentages of electrolyte leakage in citrus leaves under the freezing stress are shown in Figure 1. As it presents, the percentages of electrolyte leakage occurred in trifoliolate orange (*P. trifoliata* (L.) Raf.) and lemon (*C. limonia* Osbeck.), both increased with the growing intensity of freezing stress. However, the latter increased greater than the former. In addition, electrolyte leakages of leaves pretreated with ABA were lower than that of the non-treated ones. Moreover, ABA displays its significant ($P < 0.05$) inhibition of the electrolyte leakage in lemon below -6°C while inhibits the electrolyte leakage in trifoliolate orange below -9°C.

Accumulation of MDA

MDA is a compound generated in lipid peroxidation caused by free radicals. As shown in Figure 2, the MDA content in trifoliolate orange and in lemon both increased with the increasing freezing intensity. Under the same condition, the MDA content in trifoliolate orange was relatively higher than that in lemon. In addition, exogenous ABA can aggravate trifoliolate orange's lipid peroxidation damage resulted from low temperature; while in lemon, exogenous ABA decreased its lipid peroxidation damage. However, there were no significant differences ($P > 0.05$) between the MDA content of the ABA-pretreated leaves and that of the non-treated ones.

Activities of antioxidant enzymes

Superoxide dismutase (SOD) activity

The SOD activities of the citrus leaves under different freezing stresses are listed in Figure 3. Figure 3 shows that SOD activities of trifoliolate orange leaves increased sharply with the gradually growing freezing intensity, while in the lemon leaves, the SOD activities were not affected significantly ($P > 0.05$) by the freezing stress. Moreover, the SOD activity in trifoliolate orange leaves was higher than that of lemon leaves, no matter whether pretreated with the ABA or not. Similar to the MDA content, there were no significant differences ($P > 0.05$) between the SOD activity of the ABA-pretreated leaves and the non-treated ones.

Catalase (CAT) activity

As shown in Figure 4, the CAT activities in leaves of trifoliolate orange and lemon were not significantly influenced by the freezing stress. Under the same condition, the CAT activity in trifoliolate orange was higher than that in lemon. It is interesting that the CAT activity in trifoliolate orange's ABA-treated leaves was significantly increased when they were frozen below -9°C.

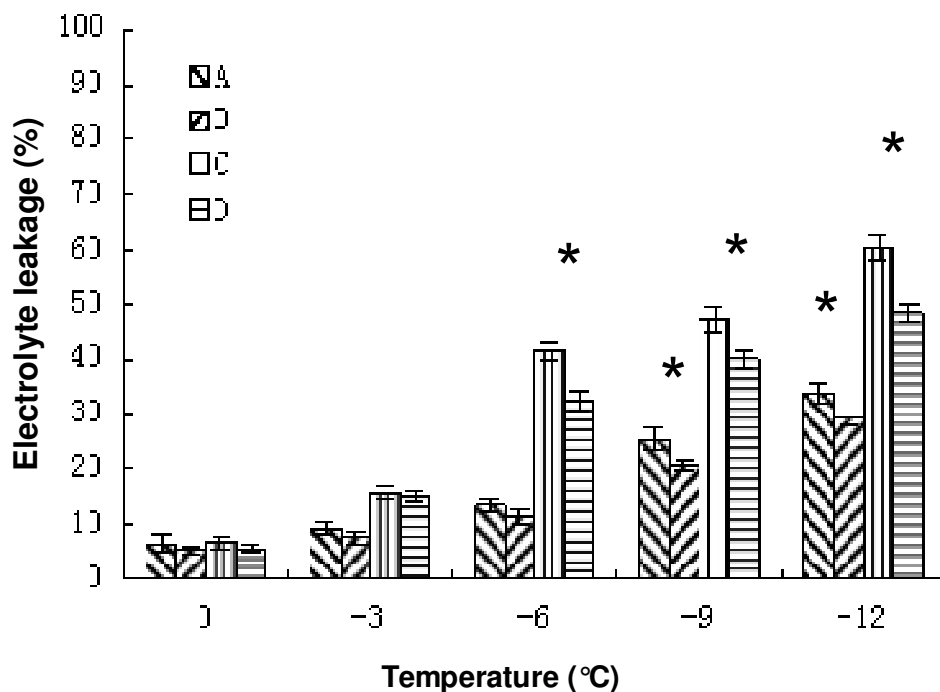


Figure 1. Electrolyte leakage of *Poncirus trifoliata* (L.) Raf. (A and B) and *Citrus limonia* Osbeck (C and D) frozen at 0, -3, -6, -9, -12°C for 1 h respectively. *Poncirus trifoliata* (L.) Raf. and *Citrus limonia* Osbeck. were pretreated with 0.1 mmol.L⁻¹ ABA (B and D) and water (CK) (A and C). Data is shown as means ± S.E. of three replications. Asterisks indicate values that differ significantly from the control at P<0.05 (Student's *t*-test).

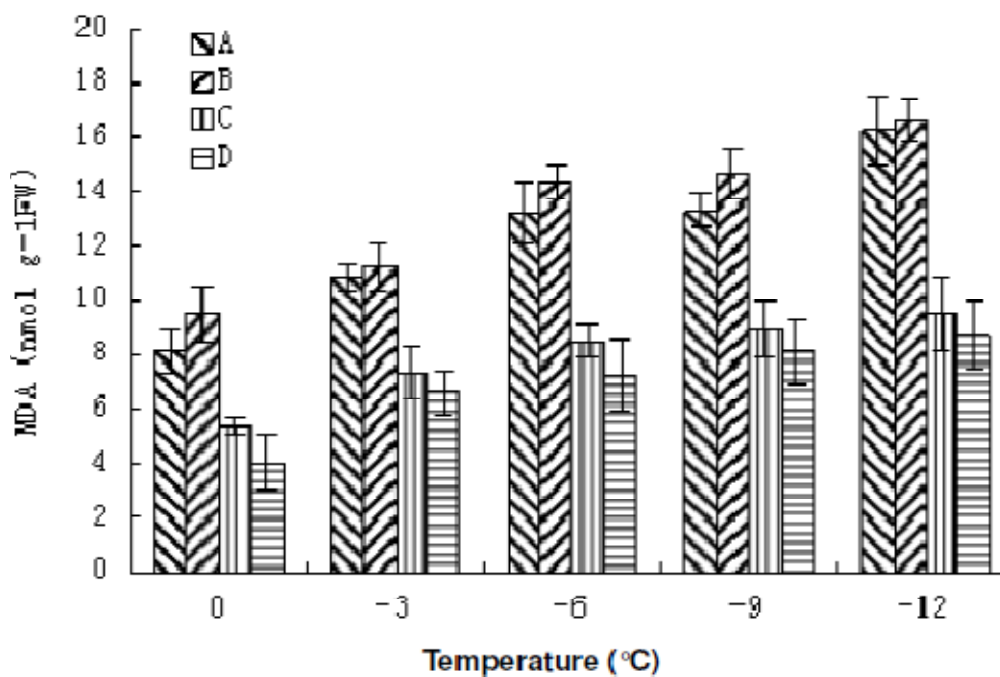


Figure 2. Malondialdehyde (MDA) concentration of *Poncirus trifoliata* (L.) Raf. (A and B) and *Citrus limonia* Osbeck (C and D) frozen at 0, -3, -6, -9, -12°C for 1 h respectively. *Poncirus trifoliata* (L.) Raf. and *Citrus limonia* Osbeck. were pretreated with 0.1 mmol.L⁻¹ ABA (B and D) and water (CK) (A and C). Data is shown as means ± S.E. of three replications.

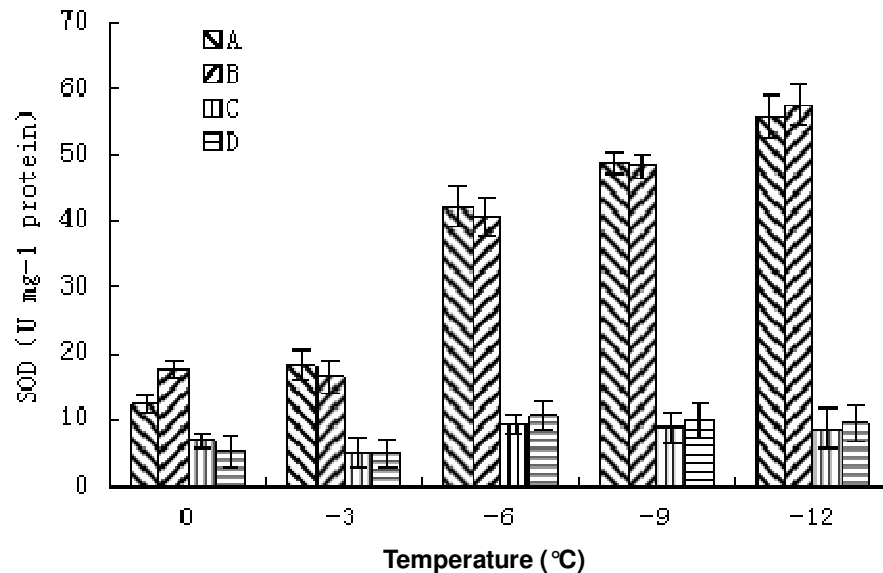


Figure 3. Superoxide dismutase (SOD) activity of *Poncirus trifoliata* (L.) Raf. (A and B) and *Citrus limonia* Osbeck. (C and D) frozen at 0, -3, -6, -9, -12°C for 1 h respectively. *Poncirus trifoliata* (L.) Raf. and *Citrus limonia* Osbeck. were pretreated with 0.1 mmol.L⁻¹ ABA (B and D) and water (CK) (A and C). Data is shown as means ± S.E. of three replications.

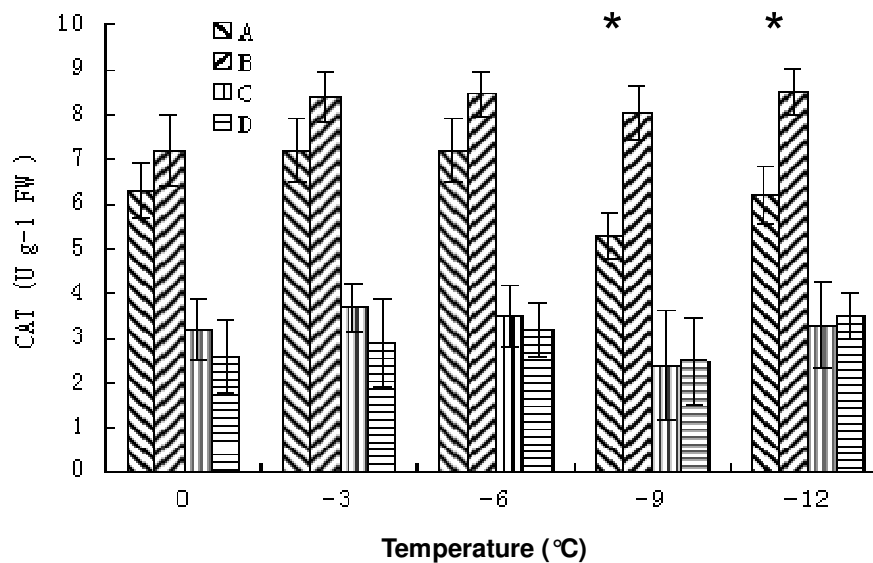


Figure 4. Catalase (CAT) activity of *Poncirus trifoliata* (L.) Raf. (A and B) and *Citrus limonia* Osbeck. (C and D) frozen at 0, -3, -6, -9, -12°C for 1 h respectively. *Poncirus trifoliata* (L.) Raf. and *Citrus limonia* Osbeck. were pretreated with 0.1 mmol.L⁻¹ ABA (B and D) and water (CK) (A and C). Data is shown as means ± S.E. of three replications. Asterisks indicate values that differ significantly from the control at P<0.05 (Student's t-test).

Peroxidases (PODs) activity

As shown in Figure 5, there was no significant effect put

by the ABA treatment on the PODs activities both in the leaves of trifoliolate orange and lemon under freezing stress for 1 h. The highest PODs activities in these two

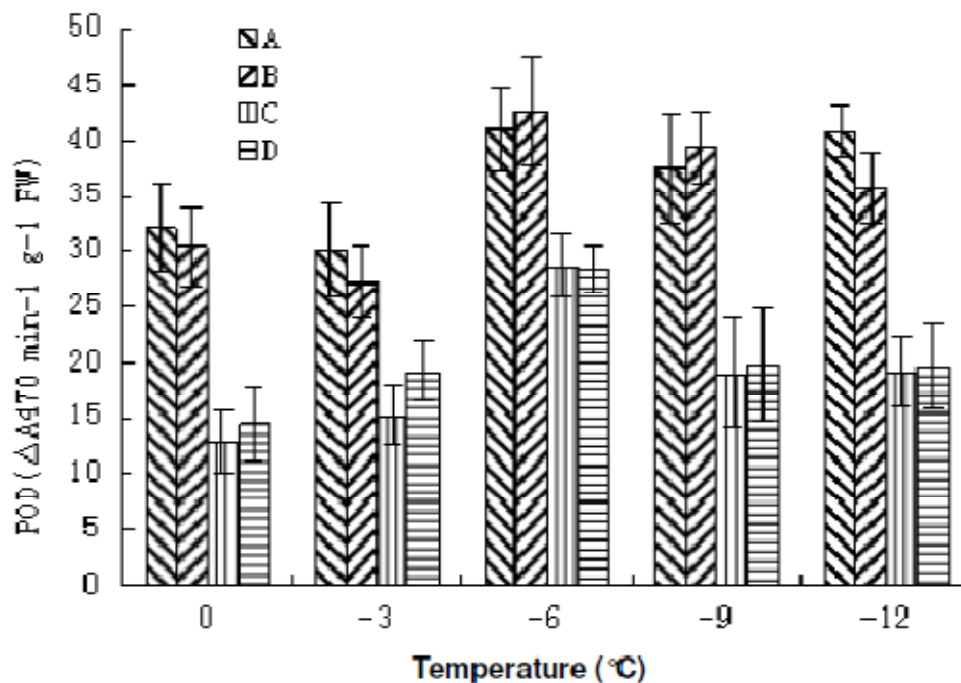


Figure 5. Peroxidases (PODs) activity of *Poncirus trifoliata* (L.) Raf. (A and B) and *Citrus limonia* Osbeck. (C and D) frozen at 0, -3, -6, -9, -12°C for 1 h respectively. *Poncirus trifoliata* (L.) Raf. and *Citrus limonia* Osbeck. were pretreated with 0.1 mmol·L⁻¹ ABA (B and D) and water (CK) (A and C). Data is shown as means ± S.E. of three replications.

species were both found in one hour at -6°C. Under the same condition, the PODs activity of the trifoliolate orange leaves was higher than that in the lemon. However, the activities of PODs in trifoliolate orange and lemon did not experienced distinctive changes by exogenous ABA pretreatment.

DISCUSSION

Abscisic acid (ABA), as a common phytohormone, plays an essential role in protecting plants from a variety of environmental stresses, such as drought, salt, and cold. ABA has been implicated in perception and transduction of cold stimulus (Plieth et al., 1999; Mishra et al., 2006). Several cold-regulated genes are known to be expressed in response to ABA (Kurkela and Borg-Franck, 1990). An exogenous application of ABA has been found to substitute for cold acclimation (Monroy and Dhindsa, 1995; Xing and Rajashekar, 2001; Nayyar and Kaushal, 2002). In our experiments, exogenous application of ABA can effectively reduce the electrolyte leakage in frozen leaves of citrus, no matter how high it is in *P. trifoliata* (L.) Raf. (tolerant to freezing stress) or in *C. limonia* Osbeck (susceptible to freezing stress). In addition, the application of exogenous ABA has also been reported to significantly increase the activities of SOD, CAT, POD and so on (Jiang et al., 2001). In our study, we observed

that 0.1 mmol·L⁻¹ ABA solution can dramatically increase the activities of CAT in *P. trifoliata* (L.) Raf., but did not regularly strengthen the activities of SOD and POD. However, the activities of SOD, CAT, and PODs in *C. limonia* Osbeck. were irregularly influenced by ABA pretreatment. Based on those findings, it suggested that effects of exogenous ABA on frozen tolerance may not be associated with antioxidant enzymes in citrus.

Under stress conditions, oxidative damage will occur and trigger overproduction of ROS (Okuda et al., 1991; Agarwal et al., 2005). To protect themselves from the toxic oxygen intermediates, plants are able to change the activity level of enzymes, such as SOD, CAT, and PODs to eliminate the ROS. Some genotypes' have stronger tolerance to environmental stresses and are associated with higher activities of antioxidant enzymes. According to some studies, the capacity of antioxidant enzyme is a significant indicator in the responsive mechanism for plants to overcome low temperature stress (Bowler et al., 1992; Fadzillah et al., 1996; O'Kane et al., 1996; Miyake and Yakota, 2000; Keles and O'ncel, 2002; Guo et al., 2006; Yang et al., 2008; Liu et al., 2009;). In this study, higher activities of antioxidant enzyme, including SOD, CAT and POD, were observed in *P. trifoliata* (L.) Raf. than those in *C. limonia* Osbeck.

Lipid peroxidation induced by ROS is considered as an important mechanism of membrane deterioration. MDA is produced by the peroxidation of membrane lipids. Many

studies have shown that accumulation of MDA is negatively correlated with the increased activities of antioxidant enzyme. However, in our study, compared with *C. limonia* Osbeck., higher concentration of MDA was observed in *P. trifoliata* (L.) Raf. The ROS concentration of plants exposed at low temperatures depends on its balance between producing and eliminating. On one hand, ROS, acting as intermediate signaling molecules, regulates the expression of genes associated with antioxidant defense mechanisms (Vranova et al., 2002; Neill et al., 2002). There are reports suggesting that hormones are located at the down stream of the ROS. Meanwhile, ROS acts as the second messenger in many hormone-signaling pathways (Chen et al., 1993; Orozco-Cardenas et al. 2001). Under low-temperature stress conditions, ROS were generated and antioxidant enzymes were activated. On the other hand, ROS are dangerous cytotoxic molecules which can cause damage to lipids.

Membranes play a key role in enduring chilling injury in plant cell (Thomashow, 1998). Electrolyte leakage is widely used as an indicator for membrane damages inducing by various stresses (Odlum and Blake, 1996). MDA has been used extensively as an indicator for free radical production and membrane injury under various abiotic stress conditions (Alexieva et al., 2001). Many investigations have demonstrated that the increasing rate of MDA content was positively correlated with the variation of electrolyte leakage (Sun et al., 2006). But in our study, when the data of *P. trifoliata* (L.) Raf. and *C. limonia* Osbeck. were analyzed separately, we found that the increased electrolyte leakage is positively correlated with the accumulation of MDA, However, compared with *C. limonia* Osbeck., higher concentration of the MDA and the lower electrolyte leakage were observed in *P. trifoliata* (L.) Raf. There was coherent relationship between the MDA content and the electrolyte leakage. It was believed that chilling injury would lead to lipid degradation. But the most essential and common mechanism is intercellular ice formation. The real freeze-induced harm is the ice formation rather than low temperatures (Shilpi and Narendra, 2005). Since *P. trifoliata* (L.) Raf. is one of the most anticold in all citrus, *C. limonia* Osbeck. is one of the most cold-sensitive species, we conclude that the degree of electrolyte leakage is a more reliable index of freezing-injured than the concentration of MDA test when gauging the injury degree of plant cells under freezing stress.

ACKNOWLEDGEMENTS

This research was supported by project of Natural Science Foundation of Hunan Province, China (No. 08JJ3066) and Scientific and Technological Project of Hunan Province, China (No. 2009NK3096). We thank Hua Xiang for his technical assistance and Qian Li, Xu Wu, Wei-qi Kuang and Peng Deng for their help in the

laboratory works.

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