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Time-dependent effect of salicylic acid on alleviating cold damage in two barley cultivars differing in cold tolerance

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Abstract: The time-dependent effect of salicylic acid (SA) on alleviating cold damage in the Tokak (tolerant) and Akhisar (sensitive) varieties of barley (*Hordeum vulgare*) was investigated. Seedlings subjected to 0.0, 0.1, and 1 mM SA for 7 days were transferred to cold (7/5 °C) on days 7, 14, 21, and 28 for 3 days. It was found that 0.1 mM SA had a greater alleviating effect, decreasing the freezing injury in both varieties for at least 10 days after the application. Moreover, 0.1 mM SA had a greater effect on alleviation in Tokak by increasing the ice nucleation activity of apoplastic proteins extracted from the leaves. SA was effective for at least 24 days in decreasing the effect of freezing temperature by regulating apoplastic proteins in the leaves after the application. Furthermore, 0.1 and 1 mM SA had an alleviating effect by decreasing the lipid peroxidation level in both varieties for at least 24 days after the application. It can be seen that SA treatment can play a positive role in alleviating cold damage in barley. Therefore, it is suggested that results from SA treatments may contribute to research related to diminishing cold damage in agricultural applications.

Key words: Apoplastic protein, cold, freezing injury, ice nucleation activity, lipid peroxidation, salicylic acid

1. Introduction

The stresses imposed by temperature have important implications for agriculture. Plants differ in their resistance to chilling and freezing temperatures (Levitt, 1980). Low temperature is one of the most important stress factors limiting the growth and productivity of cereals (Janda et al., 2003). Freezing is lethal to most cellular organisms. Dehydration of the intracellular environment and physical damage by ice crystals are major causes of freezing injury and death (Levitt, 1980). Cold tolerance is due to the capacity to avoid intracellular ice formation, to withstand extracellular ice formation, and to decrease peroxidation of unsaturated fatty acids in phospholipids (Tasgin et al., 2003; Xu et al., 2006). Freezing-tolerant plants exhibit injury only at temperatures lower than the temperature at which extracellular ice formation begins (Antikainen, 1996). Plants produce several compounds to protect cells from fatal intracellular and intercellular ice formation. Several different types of overwintering plants accumulate sugars, amino acids, and antifreeze compounds, including antifreeze proteins in the apoplastic (extracellular) region (Atici & Nalbantoglu, 1999, 2003; Yu et al., 2001; Tasgin et al., 2003, 2006; Griffith & Yaish, 2004).

Searches for signal molecules show that stress tolerance is an important step in bettering our understanding of how plants acclimate to an adverse environment. Some studies indicate that salicylic acid (SA) is synthesised endogenously and is a hormone-like signal molecule for the activation of plant defences (Hayat & Ahmad, 2007). The role of SA in plant growth and development, flowering, ion uptake, stomatal regulation, and photosynthesis has been investigated (Pancheva et al., 1996; Popova et al., 1997; Uzunova & Popova, 2000). Several studies also supported a major role for SA in modulating the plant's response to several abiotic and biotic stresses, such as ultraviolet light, drought, salt, temperature, heavy metals, and plant pathogenesis (Gaffney et al., 1993; Janda et al., 1999; Senaratna et al., 2000; Ding et al., 2002; Kang et al., 2003; Tasgin et al., 2003, 2006; Ananieva et al., 2004; Guo et al., 2007, 2009; Mutlu et al., 2009; Kadioglu et al., 2011; Saruhan et al., 2012; Mutlu & Atici, 2012). Recent studies have reported the effects of SA on cold damage.

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These studies demonstrated that SA treatment increased the chilling tolerance of maize (Janda et al., 1999; Horvath et al., 2002), tomato (Ding et al., 2002), banana (Kang et al., 2003), winter wheat (Tasgin et al., 2003, 2006), red globe grape (Li et al., 2005), mustard (Setia et al., 2006), cucumber (Xia et al., 2007), radish (Biao, 2006), grass (Wang et al., 2009), and eggplant (Chen et al., 2011). However, the molecular events involved in SA signalling are not yet fully understood. Therefore, it is important to study how SA enhances the cold tolerance of plants.

In the present study, the effect of exogenous SA on alleviating cold damage in 2 barley cultivars differing in cold tolerance was investigated by determining the freezing injury, ice nucleation activity of apoplastic proteins, and lipid peroxidation levels. After SA application, the effect time of SA was also determined on these parameters.

2. Materials and methods

2.1. Plant material

Barley seeds (*Hordeum vulgare* L.), Tokak (tolerant) and Akhisar (sensitive), were planted in sand in 15-cm pots. They were maintained in a 20/18 °C (day/night) growth chamber with a 12-h day length for 7 days to initiate germination. After 7 days, SA solution (0.1 and 1 mM) was sprayed onto the leaves of certain plants. All the plants (both with and without SA treatment) were transferred to cold (7/5 °C) on days 7, 14, 21, and 28 for 3 days. The leaves were harvested to determine freezing injury (0.1 g), ice nucleation activity (7 g), and lipid peroxidation (0.5 g) on days 10, 17, 24, and 31.

2.2. Determination of freezing injury

Freezing injuries in the leaves were determined by using a freezing bath and an electrical conductivity bridge (Atici & Nalbantoglu, 1999). Fresh leaves were cut into 2-cm lengths and rinsed in 6 changes of water to remove cellular proteins from the cut ends. At the end of each rinsing, the removed cellular proteins were measured at a wavelength of A_{280}. Next, the leaves (0.1 g) were placed in each of the 15 tubes and the tubes were positioned in a freezing bath (low temperature circulator LTD 6 G, Grant Instruments, Cambridge, UK). After equilibration at –1 °C for 30 min, the temperature was lowered stepwise by 1 °C intervals from –1 to –15 °C. The tubes were allowed to equilibrate at each temperature for 15 min. The tubes were then removed from the freezing bath one by one at each temperature and 4 mL of HPLC-grade water was added to each tube containing the frozen leaves. These tubes were stored at 4 °C for 24 h. The conductivity (ion leakage value) of the solution in each tube was measured at room temperature with an electrical conductivity bridge. The obtained ion leakage value (µS/cm) was used to express freezing injury.

2.3. Determination of ice nucleation activity

Apoplastic proteins obtained from barley leaves were used to determine ice nucleation activity (Atici & Nalbantoglu, 1999). Fresh leaves (7 g) were cut into 2-cm lengths, and rinsed in 6 changes of water to remove cellular proteins from the cut ends. At the end of each rinse, the removed cellular proteins were calculated by measuring at a wavelength of A_{280}. The leaves were then vacuum-infiltrated for 30 min in 20 mM ascorbic acid + 20 mM $CaCl_2$ solution. The leaves were blotted dry and placed vertically in 20-mL syringes. The syringes were placed in centrifuge tubes. The apoplastic extract was collected from the bottom of the tubes after the leaves were centrifuged at 1500 × *g* for 20 min. Proteins were precipitated from the apoplastic extracts by adding 1.5 times the volume of ice-cold MeOH containing 1% HOAc and incubating the samples overnight at –20 °C. After centrifugation for 20 min at 3500 × *g*, the protein pellets were washed with 100% ice-cold EtOH and 70% ice-cold EtOH. The dried protein pellets in the Eppendorf tubes were dissolved in 1 mL of HPLC-grade water and the tubes were then positioned in the freezing bath. After equilibration at –1 °C for 30 min, the temperature was lowered stepwise by 0.5 °C intervals. The tubes were allowed to equilibrate at each temperature for 10 min. The tubes were then removed from the freezing bath after the apoplastic protein solution in each tube had been frozen. The freezing temperature was used as a threshold for ice nucleation activity.

2.4. Determination of lipid peroxidation

The level of lipid peroxidation was measured as described by Heath and Packer (1968) with slight modifications (Ananieva et al., 2002). In 3 mL of 0.1% TCA was homogenised 0.5 g of leaf material, followed by centrifugation at 15,000 × *g* for 30 min at 4 °C. To 0.5 mL aliquot of the supernatant was added 1 mL of reagent (0.5% thiobarbituric acid (TBA) in 20% TCA, w/v). For a negative control, 0.5 mL of 0.1% TCA and 1 mL of reagent were added. The test-tubes were heated at 95 °C for 30 min and then quickly cooled in an ice bath. After cooling and centrifugation to give a clear supernatant, the absorbance of the supernatant at 532 nm was read and the value for the nonspecific absorption at 600 nm was subtracted. The level of malondialdehyde (MDA) was estimated by using the mmol/L extinction coefficient of 155 mmol/(L cm) (Mutlu et al., 2011).

2.5. Statistical analysis

All experiments were performed 3 times and the average of the values was used. The data were analysed by analysis of variance, and means were compared using Duncan's multiple range test.

3. Results and discussion

In the present study, for the first time, the time-dependent effect of SA on alleviating cold damage in 2 barley varieties (tolerant and sensitive) was investigated by determining freezing injury, ice nucleation activity, and lipid peroxidation levels. To achieve this aim, barley seedlings with SA treatments (0.0, 0.1, and 1 mM) were transferred to a cold facility (7/5 °C) and the values obtained from 0.1 and 1 mM SA treatments were compared to values from the 0.0 mM SA treatment (cold control).

Freezing injury values in the leaves are presented in Figure 1. For simple quantitative comparisons, the highest freezing injury values obtained from the groups (days 10, 17, and 24) of plants without SA treatment (cold control) were identified as 100% freezing injury. The average values of % freezing injuries in plants without SA treatment were calculated using values between a temperature giving at least 50% injury and –15 °C (Table). The average values of % freezing injuries in plants with SA treatment were calculated by using values between a temperature giving at

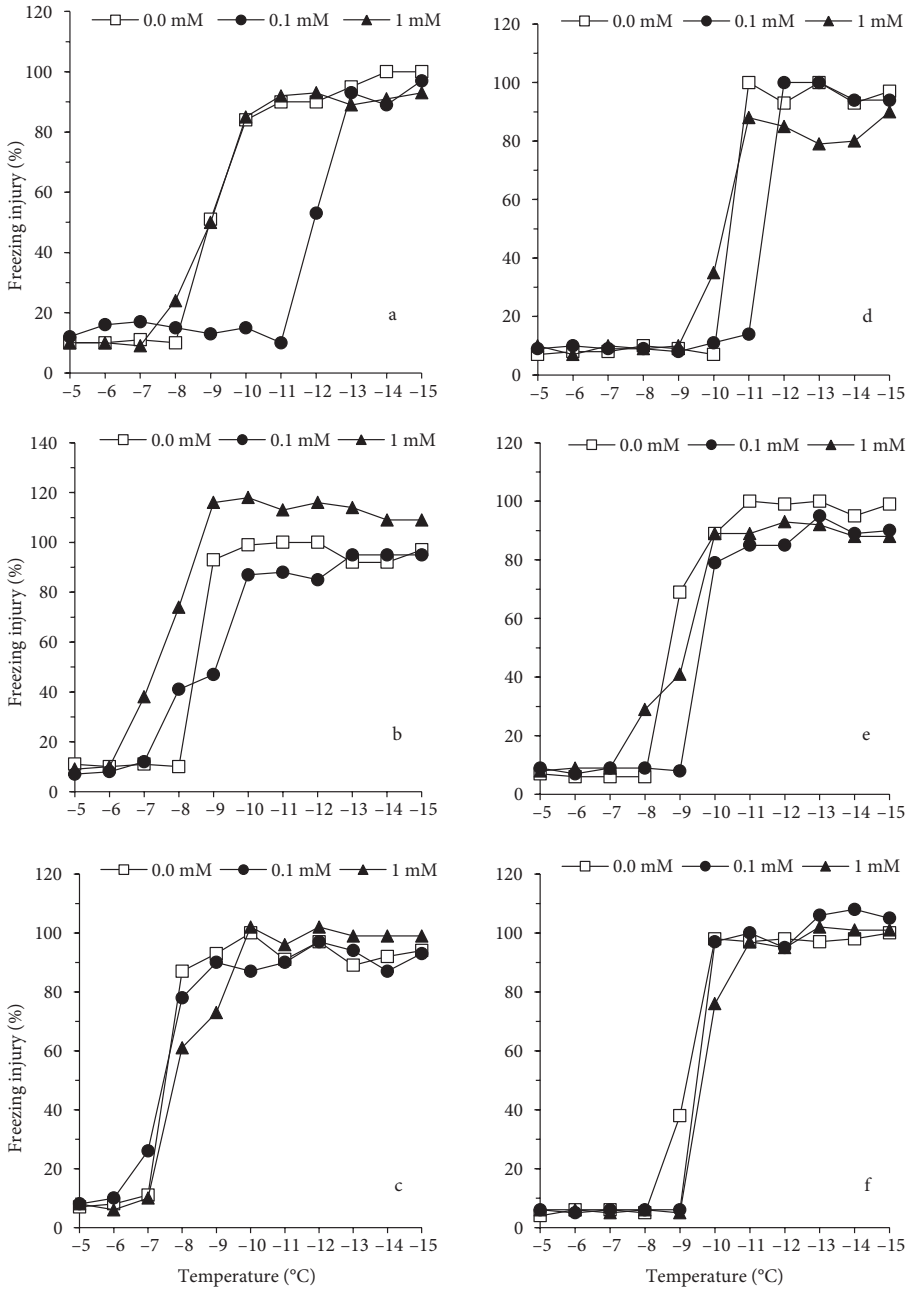

Figure 1. Freezing injury % values in barley leaves with SA treatment transferred to cold conditions (a: 10-day, b: 17-day, c: 24-day for Tokak and d: 10-day, e: 17-day, f: 24-day for Akhisar). Each value in the graph shows the average of 3 experiments.

Table. Average values of % freezing injuries at barley leaves with SA treatment transferred to cold conditions. Values in a column followed by the same letter are not significantly different at $P < 0.05$ level as determined by Duncan's multiple range test.

	SA (mM)	Tokak	Akhisar
10-day	0.0	87 bc	97 ab
	0.1	53 d	80 dc
	1.0	85 c	84 c
17-day	0.0	96 b	93 b
	0.1	85 c	76 d
	1.0	114 a	83 dc
24-day	0.0	93 bc	98 ab
	0.1	90 bc	102 a
	1.0	91 bc	95 ab

least 50% injury without SA treatment and –15 °C (Table). The average value(s) in Tokak were decreased by 39% on day 10 and 12% on day 17 by 0.1 mM SA and were increased by 19% on day 17 by 1 mM SA (Table and Figure 1). The average values in Akhisar were decreased by 18% at both days 10 and 17 by 0.1 mM SA, and 14% and 11% on days 10 and 17 by 1 mM SA, respectively (Table; Figure 1). Neither of the SA treatments changed their average values on day 24 (Table; Figure 1) or day 31 (unpublished data) in the varieties. The results are consistent with previous studies in which SA treatment increased resistance to chilling or cold injury in maize (Janda et al., 1999), tomato (Ding et al., 2002), banana (Kang et al., 2003), winter wheat (Tasgin et al., 2003), red globe grape (Li et al., 2005), mustard (Setia et al., 2006), cucumber (Xia et al., 2007), radish (Biao, 2006), and grass (Wang et al., 2009). It can be seen from the results that 1 mM SA treatment increased freezing injury on day 17 in the variety with cold tolerance (Tokak), while it decreased on days 10 and 17 in the sensitive variety (Akhisar). Therefore, it is concluded that 1 mM SA presents different effects in the 2 barley varieties. However, 0.1 mM SA treatment has a greater effect on alleviating cold damage by decreasing the freezing injury in both barley varieties. Our study also shows that the effect of SA on freezing injury can last for at least for 10 days after the application.

The ice nucleation activity values of apoplastic proteins extracted from the leaves are presented in Figure 2. An increase in the ice nucleation activity value expresses a decrease in the freezing temperature. The value(s) in Tokak increased by 1.7, 0.8, and 1.4 °C with 0.1 mM SA treatment on days 10, 24, and 31, respectively, and increased by 0.9 °C with 1 mM SA treatment on day 31 (Figure 2). The value(s) in Akhisar increased by 2 °C with 1 mM SA treatment on day 10 and decreased by 1 °C with 0.1 mM SA treatment on day 17 and 1.5 °C with 0.1 and 1 mM SA treatments on day 31 (Figure 2). It can be seen from the results that 0.1 mM SA treatment increased ice nucleation activity on days 10, 24, and 31 in the variety with cold tolerance (Tokak), while it decreased on days 17 and 31 in the sensitive variety (Akhisar). It is concluded that 0.1 mM SA shows different effects in the 2 barley varieties and has a greater effect on alleviating cold damage by increasing the ice nucleation activity in Tokak. This result is consistent with our previous study in which SA treatment caused a significant increase in the ice nucleation activity in winter wheat leaves under cold conditions (Tasgin et al., 2003). The present study also shows that SA can be effective for at least 24 days at a decreasing freezing temperature by regulating apoplastic proteins in the leaves after the application. It was reported that apoplastic proteins including antioxidant enzymes and antifreeze proteins were highly correlated with frost tolerance in winter oat, rye, and wheat (Livingston & Henson, 1998; Yu et al., 2001; Tasgin et al., 2006). Therefore, SA can increase freezing tolerance in barley leaves by affecting apoplastic antifreeze proteins.

Lipid peroxidation values in the leaves are presented in Figure 3. Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Xu et al., 2006; Mutlu et al., 2011; Baloğlu et al., 2012; Sekmen et al., 2012). The values in Tokak decreased by 21%, 15%, and 10% with 0.1 mM SA treatment and 18%, 16%, and 13% with 1 mM SA treatment on days 10, 24, and 31, respectively (Figure 3). The values in Akhisar decreased by 11%, 22%, 13%, and 10% with 0.1 mM SA treatment on all days and 13%, 12%, and 20% with 1 mM SA treatment on days 10, 17, and 24, respectively (Figure 3). It can be seen that 0.1 and 1 mM SA treatments can have an effect on alleviating cold damage by decreasing the lipid peroxidation level in both varieties. The results are consistent with previous studies in which SA pre-treatment decreased the MDA content in red globe grape (Li et al., 2005), *Gerbera jamesonii* Adlam (Lan et al., 2005), radish (Biao, 2006), cucumber (Xia et al., 2007), maize (Ping & Rui, 2007), orange (Huang et al., 2008), and grass (Wang et al., 2009) under chilling or cold stress. Our study also shows that the effect of SA on the lipid peroxidation level can be for at least 24 days (with the exception of 1 mM SA for 31 days in Akhisar) after the application. Some recent studies indicated that low storage temperature and exogenous SA can reduce lipid peroxidation by regulating the antioxidant system during storage of navel oranges (Huang et al., 2008), watermelon (Yang et al., 2008), and bamboo (Luo et al., 2011). Therefore, our results show that SA treatment may provide a useful means of maintaining

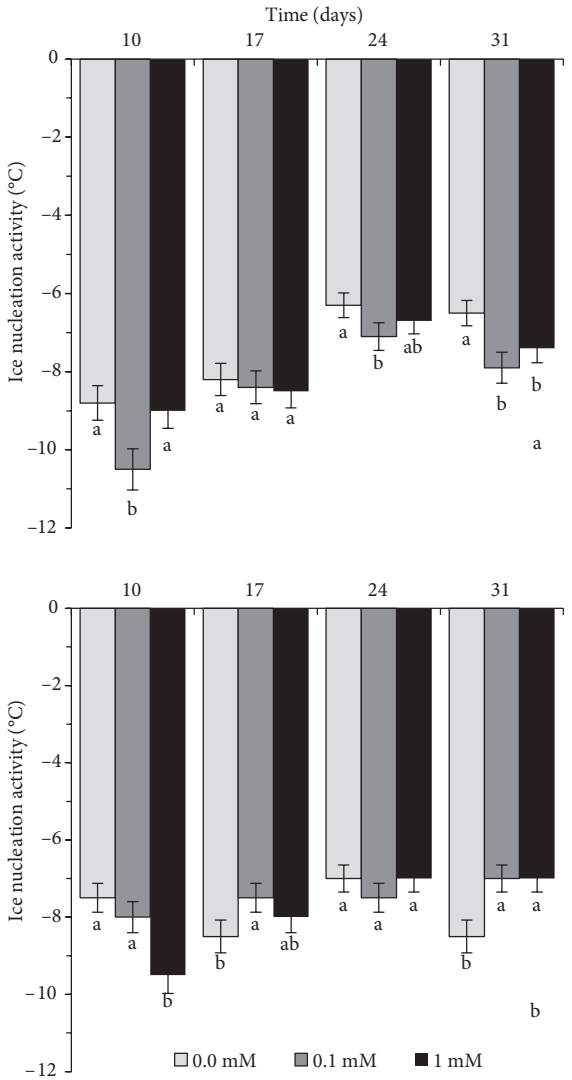

Figure 2. Ice nucleation activities of apoplastic proteins extracted from barley leaves with SA treatment transferred to cold conditions. (a: Tokak and b: Akhisar). Values in a group followed by the same letter are not significantly different at $P < 0.05$ level as determined by Duncan's multiple range test. Bars are the mean ± SE.

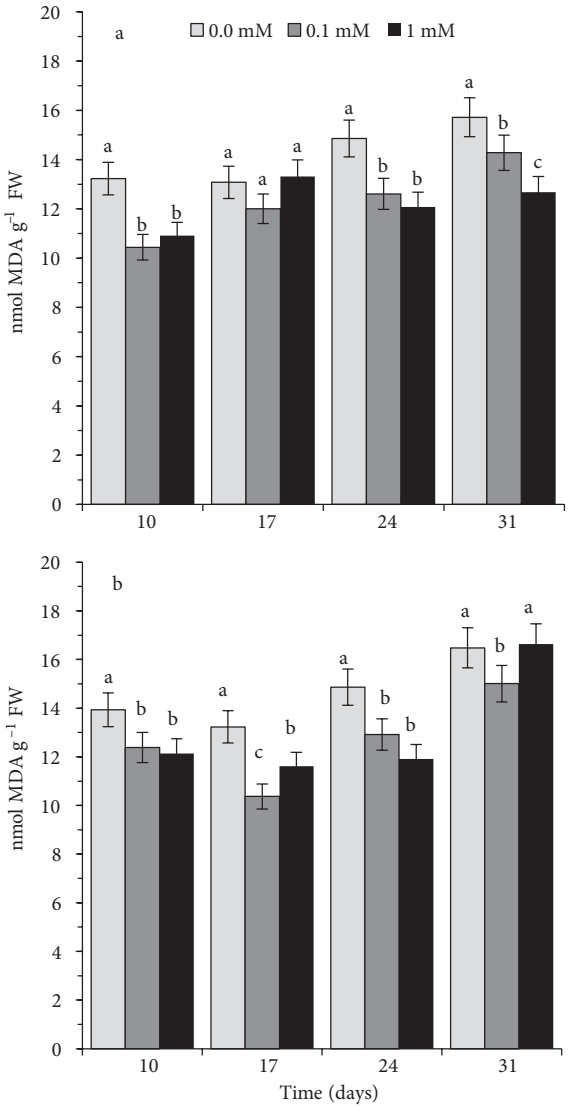

Figure 3. Lipid peroxidation levels in barley leaves with SA treatment transferred to cold conditions. (a: Tokak and b: Akhisar). Values in a group followed by the same letter are not significantly different at $P < 0.05$ level as determined by Duncan's multiple range test. Bars are the mean ± SE.

beneficial antioxidant activity before a plant is exposed to cold conditions.

4. Conclusion

The present study shows that SA treatment may play a positive role in alleviating cold damage by decreasing freezing injury, increasing ice nucleation activity, and decreasing lipid peroxidation in 2 barley cultivars differing in cold tolerance. It was also found that the tolerant variety is more protected by 0.1 mM SA treatment for at least 24 days. Our results can contribute to research related to diminishing cold damage in agriculture applications. In addition, it is concluded that endogenous SA produced in barley may be involved in alleviating cold damage. However, the effect of cold stress on the endogenous SA content and the role of endogenous SA during the development of cold tolerance are still unknown and require further research.

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