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BRIEF COMMUNICATION

Effects of salicylic acid and salinity on apoplastic antioxidant enzymes in two wheat cultivars differing in salt tolerance

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Abstract

The effects of salicylic acid (SA) and salinity on the activity of apoplastic antioxidant enzymes were studied in the leaves of two wheat (*Triticum aestivam* L.) cultivars: salt-tolerant (Gerek-79) and salt-sensitive (Bezostaya). The leaves of 10-d-old seedlings grown at nutrient solution with 0 (control), 250 or 500 mM NaCl were sprayed with 0.01 or 0.1 mM SA. Then, the activities of catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) were determined in the fresh leaves obtained from 15-d-old seedlings. The NaCl applications increased CAT and SOD activities in both cultivars, compared to those of untreated control plants. In addition, the NaCl increased POX activity in the salt-tolerant while decreased in the salt-sensitive cultivar. In control plants of the both cultivars, 0.1 mM SA increased CAT activity, while 0.01 mM SA slightly decreased it. SA treatments also stimulated SOD and POX activity in the salt-tolerant cultivar but significantly decreased POX activity and had no effect on SOD activity in the salt-sensitive cultivar. Under salinity, the SA treatments significantly inhibited CAT activity, whereas increased POX activity. The increases in POX activity caused by SA were more pronounced in the salt-tolerant than in the salt-sensitive cultivar. SOD activity was increased by 0.01 mM SA in the salt-tolerant while increased by 0.1 mM SA treatment in the salt-sensitive cultivar.

Additional key words: catalase, NaCl stress, peroxidase, superoxide dismutase, *Triticum aestivum.*

Salt stress belongs to factors limiting the plant productivity. Similarly to other stresses, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals are also produced during salinity (Bartosz 1997, Meloni *et al.* 2003). ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Rout and Shaw 2001). Antioxidative enzymes are the most important components in the scavenging system of ROS. Superoxide dismutase (SOD) is a major scavenger of superoxide, and its enzymatic action results in the formation of H_2O_2 which is then scavenged by catalase (CAT) and a variety of peroxidases (POX) (Noctor and Foyer 1998). A correlation between the intracellular antioxidant capacity and NaCl tolerance has been demonstrated in some plant species including wheat (Dionisio-Sese and Tobita 1998, Sairam and Srivastava 2002, Agarwal and Pandey 2004, Mandhania *et al.* 2006).

The effects of environmental stresses on the antioxidant system in the apoplastic space have been studied by some researchers, and it has been suggested that this compartment is important in plant response to abiotic stresses (Luwe 1996, Vanacker *et al.* 1998, Atici and Nalbantoglu 2003). In plant cells subjected to stresses, initial events occur mostly in apoplastic space (Vanacker *et al.* 1998, Atici and Nalbantoglu 2003, Taşkın *et al.* 2003, 2006). On the other hand, some researches have showed that adverse environmental factors are capable of inducing the synthesis of ROS in apoplastic space of plants (Luwe 1996, Ranieri *et al.* 1996, Vanacker *et al.* 1998, Hernandez *et al.* 2001, Taşgın *et al.* 2006). Hernandez *et al.* (2001) showed different sensitivity of leaf apoplastic antioxidant enzymes to NaCl in two pea cultivars.

Salicylic acid (SA) is a natural and hormone-like signal molecule for the activation of plant defenses, and

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Abbreviations: CAT - catalase; POX - peroxidase; SOD - superoxide dismutase; SA - salicylic acid.
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regulates a large variety of physiological processes in plants (Klessig and Malamy 1994, Wang and Li 2006). In addition, some studies have reported a major role of SA in modulating the plant response to most abiotic stresses including salt stress (Minibayeva *et al.* 2003, Sakhabutdinova *et al.* 2004, Taşgın *et al.* 2003, 2006). Exogenous SA can regulate the activities of intracellular antioxidant enzymes such as SOD, POX and increase plant tolerance to environmental stresses (Senaratna *et al.* 2000, Sakhabutdinova *et al.* 2004). In addition, SA treatment can regulate the activities of apoplastic antioxidant enzymes in plant leaves exposed to cold stress and it plays an important role in the induction of stress tolerance by affecting apoplastic proteins (Taşkın *et al.* 2003, 2006). However, no data have been recorded about the role played by SA on apoplastic antioxidant capacity in plants grown under salinity.

The objective of this work was to study the effects of SA and NaCl treatment on the apoplastic antioxidant enzymes in wheat leaves and to elucidate the roles of both SA and the apoplastic antioxidant enzymes in plants under saline conditions.

Triticum aestivum L. salt-sensitive cv. Bezostaya and salt-tolerant cv. Gerek-79 were used. Before sowing, the plant seeds were surface-sterilized for 10 min with 10:1 water/bleach (commercial NaOCl) solution and then washed five times with distilled water. The plants were grown hydroponically in a growth chamber under controlled environmental conditions for 15 d (day/night temperature of 22/20 °C, relative humidity of 75 %, and a 16-h photoperiod with photosynthetically active radiation at photon flux density of 400 μmol m^{-2} s^{-1}). In the standard nutrient solution (Hoagland) NaCl was added to 10-d-old seedlings to adjust to 250 and 500 mM concentration. SA solutions (0.01 and 0.1 mM, pH 6.5) were sprayed once on the leaves. Distilled water was used to spray control plants. After 5 d the plant leaves were harvested to determine the apoplastic antioxidant enzymes.

Apoplastic proteins were extracted as described in Hon *et al.* (1994). Harvested fresh leaves (7 g) were carefully cut with a sharp bistoury into 2 cm lengths, and rinsed 6 times in distilled water to remove cellular proteins from the cut ends. At the end of each rinsing, the removing cellular proteins were calculated by measuring absorbance by spectrophotometer (*Shimadzu UV-1700*, Japan) at a wavelength of 280 nm (A_{280}). The leaves were then vacuum-infiltrated for 15 min in 20 mM ascorbic acid and 20 mM $CaCl_2$ solution. The leaves were blotted dry and placed vertically in a 20-cm^3 syringe. The syringes were placed in centrifuge tubes. The apoplastic extract was collected from the bottom of the tubes after the centrifugation at 1 500 *g* for 20 min (4 °C). Proteins were precipitated from apoplastic extracts by adding 1.5 times the volume of ice-cold MeOH containing 1 % acetic acid (HOAc) and incubated the samples overnight at -28 °C. After centrifugation at 3 500 *g* for 20 min (4 °C), the protein pellets were washed with 100 % ice-cold EtOH and 70 % ice-cold EtOH. Contamination of apoplastic extract by cytoplasm constituents, as monitored by the activity of glucose-6-phosphate dehydrogenase was always less than 1% in relation to the catabolic fraction (Patykowski and Urbanek 2003, Taşkın *et al.* 2006).

The dried apoplastic protein pellets obtained from the leaves (7 g) were dissolved in 1 cm^3, 0.2 M phosphate buffer (pH 6.5). The CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 μmol H_2O_2 per min (Upadhyaya *et al.* 1985). The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM H_2O_2. One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per min (Upadhyaya *et al.* 1985). The SOD activity in apoplastic fractions was estimated by recording the decrease in absorbance of nitro-blue tetrazolium dye by the enzyme (Dhindsa *et al.* 1981). The reaction mixture contained 2 μM riboflavine, 13 mM methionine, 75 μM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 cm^3 the apoplastic fraction. Reaction was started by adding 0.06 cm^3 100 μM riboflavin solution and placing the tubes under two 30 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Reaction was stopped by switching off the light. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme (Sairam and Srivastava 2002).

All experiments were performed 6 times and the average of the values was used. Data were analyzed by analysis of variance, and means were compared by Duncan's multiple range test.

In both the salt-sensitive and the salt-tolerant wheat cultivars grown at the control conditions, apoplastic CAT activity was increased by 0.1 mM SA while slightly reduced ($P > 0.01$) by 0.01 mM SA (Fig. 1*A*). The result implies that SA can cause as depended on concentration both the increase and the decrease of the CAT activity in wheat leaves grown in control conditions. In our previous study 0.01 mM treatment of SA decreased apoplastic CAT activity in winter wheat but 0.1 mM SA was not used in that study (Taşgın *et al.* 2006). Although there are some publications describing the decrease of total CAT activity by exogenous SA treatment (Shim *et al.* 2003, Shi *et al.* 2006), some other studies also showed that SA treatment did not inhibit CAT activity (Tenhaken and Rubel 1997, Ding *et al.* 2007), and also stimulated it (He *et al.* 2005, Agarwal *et al.* 2005). On the other hand, the relatively low concentrations (0.01 - 0.05 mM) of SA can act as a moderate stress, having an effect on the oxidative

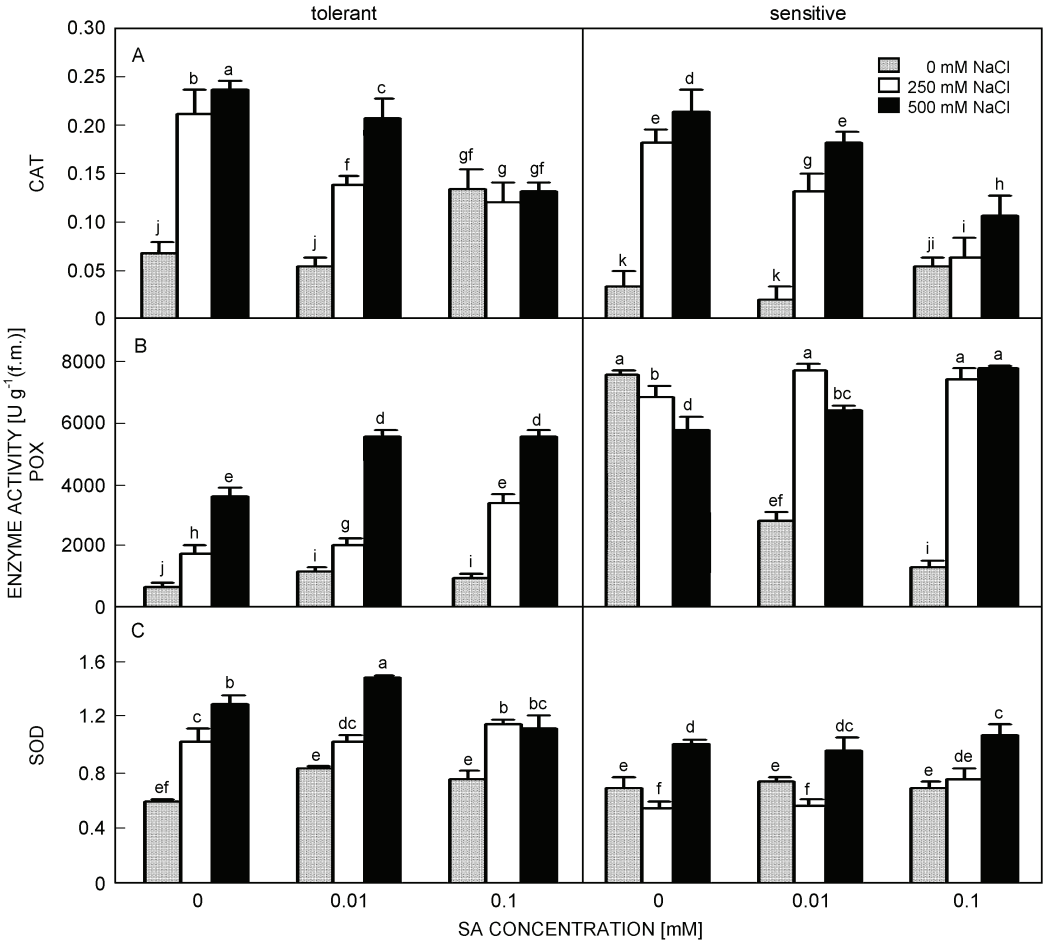

Fig. 1. Effects of salicylic acid (SA) and NaCl on apoplastic CAT, POX and SOD activities in the leaves of two wheat (*Triticum aestivam* L.) cultivars: salt-tolerant (Gerek-79) and salt-sensitive (Bezostaya). *Vertical bars* represent the standard error of the mean of six replicate. Means with different letters are significantly different at $P < 0.01$ based on Duncan's multiple range test.

status of plant similar to that of stress-acclimating processes (Horváth *et al.* 2007b) and SA can also initiate as a signal molecule several protective resistance mechanisms against pathogens, chilling and heat stress (Lamb 1994, Janda *et al.* 1999, Horváth *et al.* 2007a,b). SA application itself may also cause oxidative stress to plants, partially through the accumulation of H_2O_2 (Kawano and Muto 2000, Minibayeva and Gordon 2003, Horváth *et al.* 2007b) and excessive H_2O_2 in plant tissues can cause unrecoverable membrane damage (Rao *et al.* 1997). We conclude that the induced increase of the apoplastic CAT under high SA concentrations can be considered as an important mechanism in the apoplastic defense strategy. The CAT activity was also increased by the NaCl treatments (250 and 500 mM) in the both cultivars (Fig. 1*A*). This increase became more expressive with the increase of the NaCl concentrations. The stimulation of total CAT activity by salt stress has been observed in many plant species (Ghorbanli *et al.* 2004, Mandhania *et al.* 2006). It can be concluded that the increase of the apoplastic CAT activity can be to decrease the level of excessive H_2O_2 produced in the apoplast during the salt stress, because CAT activity seems to be crucial for the removal of H_2O_2 during salt stress in plants. In addition, exogenous SA treatment decreases apoplastic CAT activity induced by NaCl in the wheat plants. Similarly, SA treatment decreased apoplastic CAT activity in winter wheat under cold stress (Taşgın *et al.* 2006). These results also demonstrate that SA can play a regulating role in plant apoplast during the oxidative burst caused by salt stress.

The applications of both SA and NaCl significantly stimulated apoplastic POX activity in the tolerant cultivar (Gerek-79) while SA decreased it in the control of salt-sensitive cultivar (Bezostaya), compared to respective controls (Fig. 1*B*). Some studies showed that both salt stress and exogenous SA resulted in higher activity of cellular POX in plant cells (Agarwal and Pandey 2004, Sakhabutdinova *et al.* 2004, Mandhania *et al.* 2006). It was also determined that SA treatment increased apoplastic POX activity in wheat root cells (Minibayeva *et al.* 2003) and the winter wheat leaves (Taşgın *et al.* 2006) grown under control conditions. Also, salinity caused an increase of apoplastic POX activity in resistant cultivar of bell pepper while a decrease in sensitive cultivar (Turhan *et al.* 2006).

The SA treatments stimulated apoplastic SOD activity more in the leaves of the salt-tolerant cultivar than in the salt-sensitive cultivar (Fig. 1*C*). Although there are no reports elucidating the effect of exogenous SA treatment on apoplastic SOD, it has been shown that the activity of intracellular SOD was increased by SA treatment (Sakhabutdinova *et al.* 2004, Agarwal *et al.* 2005). The increase in apoplastic SOD activity of the salt-tolerant cultivar in response to SA can be related to induction of antioxidant responses that protect the plant from damage. Our data on the SOD activity support the above reasoning (Fig. 1*C*). In our study, NaCl (250 and 500 mM) also increased the SOD activity in the salt-tolerant cultivar whereas only 500 mM salt in the salt-sensitive one, compared to respective controls (Fig. 1*C*). This observation is consistent with that of Turhan *et al.* (2006) who showed that salinity caused increase in apoplastic SOD activity in resistant cultivar of pepper while decrease in sensitive cultivar. Under salinity, low SA (0.01 mM) treatment generally increased the SOD activity of the salt-tolerant cultivar while high concentration (0.1 mM) could increase it in the salt-sensitive cultivar (Fig. 1*C*).

In conclusion, apoplastic CAT and SOD activities were increased by the NaCl applications in both cultivars, whereas POX activity increased only in the salt-tolerant. The apoplast-associated POX enzyme increased in response to the salt stress according to salt sensitivity of cultivars. SA at different concentration had different effects on the CAT activity. However, both the SA treatments stimulated SOD and POX activity especially in the salt-tolerant cultivar. Under salinity, the SA treatments inhibited CAT activity, whereas increased POX and SOD activities. These increases were generally more pronounced in the salt-tolerant than in the salt-sensitive cultivar. The results suggest that apoplastic CAT, POX and SOD enzymes play an important role in the $O_2^{\bullet-}$ and H_2O_2 scavenging in apoplastic spaces of wheat leaves, and these enzymes are also related, at least in part, to salt-induced oxidative stress tolerance of wheat.

References

Agarwal, S., Pandey, V.: Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. - Biol. Plant. **48**: 555-560, 2004.

Agarwal, S., Sairam, R.K., Srivastava, G.C., Meena, R.C.: Changes in antioxidant enzymes activity and oxidative stress by abscisic acid and salicylic acid in wheat genotypes. - Biol. Plant. **49**: 541-550, 2005.

Atici, Ö., Nalbantoglu, B.: Antifreeze proteins in higher plants. - Phytochemistry **64**: 1187-1196, 2003.

Bartosz, G.: Oxidative stress in plants. - Acta Physiol. Plant. **19**: 47-64, 1997.

Dhindsa, R.A., Plumb-Dhindsa, P., Thorpe, T.A.: Leaf senescence correlated with increased permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. - J. exp. Bot. **126**: 93-101, 1981.

Ding, Z.S., Tian, S.P., Zheng, X.L., Zhou, Z.W., Xu, Y.: Responses of reactive oxygen metabolism and quality in mango fruit to exogenous oxalic acid or salicylic acid under chilling temperature stress. - Physiol. Plant. **130**: 112-121, 2007.

Ghorbanli, M., Ebrahimzadeh, H., Sharifi, M.: Effects of NaCl and mycorrhizal fungi on antioxidative enzymes in soybean. - Biol. Plant. **48**: 575-581, 2004.

He, Y., Liu, Y., Cao, W., Huai, M., Xu, B., Huang, B.: Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in Kentucky bluegrass. - Crop Sci. **45**: 988-995, 2005.

Hernandez, J.A., Ferrer, M.A., Jimenez, A., Barcelo, A.R., Sevilla, F.: Antioxidant systems and $O_2^{\bullet-}/H_2O_2$ production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. - Plant Physiol. **127**: 817-831, 2001.

Hon, W.C., Griffith, M., Chong, P., Yang, D.C.S.: Extraction and isolation of antifreeze proteins from winter rye (*Secale cereale* L.) leaves. - Plant Physiol. **104**: 971-980, 1994.

Horwáth, E., Pál, M., Szalai, G., Páldi, E., Janda, T.: Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought stress on wheat plants. - Biol. Plant. **51**: 480-487, 2007a.

Horwáth, E., Szalai, G., Janda, T.: Induction of abiotic stress tolerance by salicylic acid. - J. Plant Growth Regul. **26**: 290-300, 2007b.

Janda, T., Szalai, G., Tari, I., Páldi, E.: Hydroponic treatment with salicylic acid decreases the effect of chilling injury in maize (*Zea mays* L.) plants. - Planta **208**: 175-180, 1999.

Kang, G., Wang, C., Sun, G., Wang, Z.: Salicylic acid changes activities of H_2O_2-metabolizing enzymes and increases the chilling tolerance of banana seedlings. - Environ. exp. Bot. **50**: 9-15, 2003.

Kawano, T., Muto, S.: Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. - J. exp. Bot. **51**: 685-693, 2000.

Klessig, D.F., Malamy, J.: The salicylic acid signal in plants. - Plant mol. Biol. **26**: 1439-1458, 1994.

Lamb, C.J.: Plant disease resistance genes in signal perception and transduction. - Cell **76**: 419-422, 1994.

Luwe, M.: Antioxidants in the apoplast and symplast of beech (*Fagus sylvatica* L.) leaves: seasonal variations and responses to changing ozone concentration in air. - Plant Cell Environ. **19**: 321-328, 1996.

Mandhania, S., Madan, S., Sawhney, V.: Antioxidant defense mechanism under salt stress in wheat seedlings. - Biol. Plant. **50**: 227-231, 2006.

Meloni, D.A., Oliva, M.A., Martinez, C.A., Cambraia, J.: Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. - Environ. exp. Bot. **49**: 69-76, 2003.

Minibayeva, F.V., Gordon, L.K.: Superoxide production and the activity of extracellular peroxidase in plant tissues under stress conditions. - Russ. J. Plant Physiol. **50**: 411-416, 2003.

Minibayeva, F., Mika, A., Luthje, S.: Salicylic acid changes the properties of extracellular peroxidase activity secreted from wounded wheat (*Triticum aestivum* L.) roots. - Protoplasma **221**: 67-72, 2003.

Noctor, G., Foyer, C.H.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol.

Plant mol. Biol. **49**: 249-279, 1998.

Patykowski, J., Urbanek, H.: Activity of enzymes related to H_2O_2 generation and metabolism in leaf apoplastic fraction of tomato leaves infected with *Botrytis cinerea*. - J. Phytopathol. **151**: 153-161, 2003.

Ranieri, A., Durso, G., Nali, C., Lorenzini, G., Soldatini, G.F.: Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. - Physiol. Plant. **97**: 381-387, 1996.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C.B.: Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes: salicylic acid-mediated oxidative damage requires H_2O_2. - Plant Physiol. **115**: 137-149, 1997.

Rout, N.P., Shaw, B.P.: Salt tolerance in aquatic macrophytes: possible involvement of the antioxidative enzymes. - Plant Sci. **160**: 415- 423, 2001.

Sairam, R.K., Srivastava, G.C.: Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. - Plant Sci. **162**: 897-904, 2002.

Sakhabutdinova, A.R., Fatkhutdinova, D.R., Shakirova, F.M.: Effect of salicylic acid on the activity of antioxidant enzymes in wheat under conditions of salination. - Appl. Biochem. Microbiol. **40**: 501-505, 2004.

Senaratna, T., Touchell, D., Bunn, E., Dixon, K.: Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. - Plant Growth Regul. **30**: 157-161, 2000.

Shi, O., Bao, Z., Zhu, Z., Ying, O., Qian O.: Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence, and antioxidant enzyme activity in seedlings of *Cucumis sativa* L. - Plant Growth Regul. **48**: 127-135, 2006.

Shim, I.S., Momose, Y., Yamamoto, A., Kim, D.W., Usui, K.: Inhibition of catalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. - Plant Growth Regul. **39**: 285-292, 2003.

Taşgın, E., Atici, Ö., Nalbantoglu, B., Popova, L.P.: Effects of salicylic acid and cold treatments on protein levels and on the activities of antioxidant enzymes in the apoplast of winter wheat leaves. - Phytochemistry **67**: 710-715, 2006.

Taşgın, E., Atici, Ö., Nalbantoglu, B.: Effects of salicylic acid and cold on freezing tolerance in winter wheat leaves. -Plant Growth Regul. **41**: 231-236, 2003.

Tenhaken, R., Rubel, C.: Salicylic acid is needed in hypersensitive cell death in soybean but does not act as a catalase inhibitor. - Plant Physiol. **115**: 291-298, 1997.

Turhan, E., Karni, L., Aktas, H., Deventurero, G., Chang, D.C., Bar-Tal, A., Aloni, B.: Apoplastic anti-oxidants in pepper (*Capsicum annuum* L.) fruit and their relationship to blossom-end rot. - J. hort. Sci. Biotech. **81**: 661-667, 2006.

Upadhyaya, A., Sankhla, D., Davis, N., Sankhla, N., Smith, B.N.: Effect of paclobutrazol on the activities of some enzymes of activated oxygen metabolism and lipid peroxidation in senescing soybean leaves. - J. Plant Physiol. **121**: 453-461, 1985.

Vanacker, H., Carver, T.L.W., Foyer, C.H.: Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. - Plant Physiol. **117**: 1103-1114, 1998.

Wang, L.J., Li, S.H.: Salicylic acid-induced heat or cold tolerance in relation to Ca^{2+} homeostasis and antioxidant systems in young grape plants. - Plant Sci. **170**: 685-694, 2006.