Effects of putrescine and low temperature on the apoplastic antioxidant enzymes in the leaves of two wheat cultivars

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ABSTRACT

The effects of putrescine (a polyamine), low temperature and their combinations on the activities of apoplastic antioxidant enzymes were studied in the leaves of two wheat cultivars, winter (Dogu-88) and spring (Gerek-79). Fifteen-day-old wheat seedlings were treated with putrescine solutions (0.1, 1 and 10mM) prior to cold treatment (5/3°C). The activities of apoplastic catalase, peroxidase and superoxide dismutase were determined in the leaves both under normal and cold conditions at 1, 3 and 5 days. The results indicate that cold treatment significantly increased the activities of apoplastic catalase, peroxidase and superoxide dismutase in winter wheat while not generally affecting spring wheat. Under control conditions, the putrescine treatments were more effective in increasing the enzyme activities in winter wheat than in spring wheat. However, under cold conditions, the putrescine treatments surprisingly induced enzyme activities in spring wheat while generally reducing those in winter wheat leaves. The results show that putrescine may act as an agent inducing primary changes in the apoplastic antioxidant system of wheat leaves during reactive oxygen species-mediated damage caused by low temperature stress.

Keywords: oxidative damage; environmental stress; *Triticum aestivum*; cereals; plant stress metabolism

Low temperature stress is known to be one of several major factors limiting the growth and productivity of cereals. Significant changes occur in cellular membranes when plants are affected by cold stress. During response to cold stress, similarly to other stresses, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals are also produced within plant cells (Wise and Naylor 1987, Okuda et al. 1991). ROS are highly reactive and, in the absence of any protective mechanism, can be detrimental to the normal metabolism through oxidative damage to lipids, protein and other macromolecules (Rout and Shaw 2001). Antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), however, were shown to be the most important components in the scavenging system of the ROS (Kang et al. 2003, Li 2008). Plants have to maintain the activities of antioxidant enzymes in order to accommodate environmental stresses causing oxidative stress (Li 2008, Simova-Stoilova 2008). A correlation between the intracellular antioxidant capacity and cold tolerance was demonstrated in some plant species including wheat (Baek and Skinner 2003, Janda et al. 2003). In addition, the antioxidant enzymes in apoplastic spaces of plants under environmental stresses, such as cold, pathogen attack, ozone, and salt, were shown to play an important role in the regulation of stress response (Ranieri et al. 1996, Bolwell et al. 1999, Hernandez et al. 2001, Patykowski and Urbanek 2003, Atıcı and Nalbantoglu 2003, Tasgin et al. 2006).

Plant polyamines, on the other hand, can function as second messengers modulating various anatomical, biochemical and physiological processes in intracellular and extracellular areas of plants under environmental stresses (Kuznetsov et al. 2006, Alca'zar et al. 2006, Cavusoglu et al. 2008). Their metabolism, catabolism and related enzymes are also associated with the cell walls including apoplast, where lignification, suberization and wall stiffening occur (Bouchereau et al. 1999, Kuznetsov et al. 2006). The pro-oxidative role of polyamines is considered primarily in connection with the generation of H_2O_2 in plant apoplast,

Supported by the Research Fund of Atatürk University, Project No. BAP-2004/350.

which is required for the formation of suberin, lignin, and oxyproline proteins (Aronova et al. 2005). In addition, they play an important role in the antioxidative system and protect membranes from oxidative damage during response to environmental stresses (Kim et al. 2002, Verma and Mishr 2005, Kuznetsov et al. 2006, Shevyakova et al. 2006), but the mode of their involvement in these actions is not fully understood (Nayyar and Chander 2004, Aronova et al. 2005). Although putrescine (Put), an important polyamine, has been reported to affect the response of plants under cold stress, there are no studies, to our knowledge, elucidating the effects of exogenous polyamine treatments on the apoplastic antioxidant system in plants exposed to cold stress. Recently, Paramonova et al. (2003) showed that Put affected the apoplast ultrastructure and apoplastic POX activity in the leaf mesophyll of *Mesembryanthemum crystallinum* under salinity stress. We hypothesize that Put, an obligate precursor of other polyamines, may be involved in responses to cold stress of plants, by regulating the activities of the apoplastic antioxidant enzymes.

The present study, therefore, was arranged to assess the role of exogenous Put treatments on the apoplastic antioxidant enzymes in the leaves of wheat cultivars with different cold tolerance, and to elucidate the mutual effects of Put and cold treatments on the apoplastic antioxidant system. Hence, CAT, POX and SOD activities were studied in the leaf apoplastic space of winter and spring wheat cultivars (Dogu-88 and Gerek-79, respectively) treated with different Put solutions prior to cold treatment.

MATERIAL AND METHODS

Winter (Dogu-88) and spring (Gerek-79) cultivars of wheat (*Triticum aestivum* L.) 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 wheat seeds were planted in sand in 25 cm pots, and were grown in a growth chamber under controlled environmental conditions during 15 days (22/18°C day/night temperature, 50% relative humidity and a photon flux density of 400 µmol/ms photosynthetic active radiation at 16-h photoperiod). The plants were watered routinely with Hoagland solution. Put solutions containing 0.01% Tween-20 (a surfactant) were freshly prepared at 0.1, 1 and 10mM concentrations (pH 6.5). These solutions were sprayed on the leaves of some 15-day-old plants while distilled water (pH 6.5) containing only Tween-20 on other plants (0.0mM Put). After the above applications were performed, some of the plants treated and non-treated by Put were transferred to cold conditions (5/3°C day/night temperature, 50% relative humidity and 16-h photoperiod) for another 5 days. Then, the plant leaves were harvested at 1, 3 and 5 days to determine the activities of apoplastic antioxidant enzymes after they were sprayed with Put solutions.

For extraction of apoplastic enzymes in leaves, harvested fresh leaves (7 g) were carefully cut into 2 cm lengths with a sharp bistoury, and rinsed in 6 changes of distilled water to remove cellular proteins from the cut ends. At the end of each rinsing, removing cellular proteins were calculated by measuring at a wavelength of A_{280}. The leaves were then vacuum-infiltrated for 15 min in 20mM ascorbic acid solution including 20mM $CaCl_2$. The leaves were blotted dry and placed vertically in a 20 ml syringe. 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 15 min (4°C). Proteins were precipitated from apoplastic extracts by adding 1.5 times the volume of ice-cold MeOH containing 1% HOAc and the samples were incubated overnight at –30°C. After centrifugation at 3500 g for 20 min (4°C), the protein pellets were washed with 100% ice-cold EtOH and 70% ice-cold EtOH (Tasgin et al. 2003, 2006). 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, Tatgýn et al. 2006).

In determination of enzyme activities, the dried apoplastic protein pellets obtained from the leaves (7 g) were dissolved in 1 ml, 0.2M phosphate buffer (pH 6.5). Then, the activities of CAT, POX and SOD in the apoplastic fractions were determined spectrophotometrically. CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50mM phosphate buffer (pH 7.5) containing 20mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 µmol H_2O_2/min (Upadhyaya et al. 1985). POX activity was measured by monitoring the increase in absorbance at 470 nm in 50mM phosphate buffer (pH 5.5) containing 1mM guaiacol and 0.5mM H_2O_2. One unit of POX activity was defined as the amount of enzyme that caused an increase in

absorbance of 0.01/min (Upadhyaya et al. 1985). SOD activity in apoplastic fractions was estimated by recording the decrease in optical density of nitro-blue tetrazolium (NBT) dye by the enzyme (Dhindsa et al. 1981). Three milliliters of the reaction mixture contained, 2μM riboflavine, 13mM methionine, 75μM NBT, 0.1mM EDTA, 50mM phosphate buffer (pH 7.8), 50mM sodium carbonate and 0.1 ml the apoplastic fraction. Reaction was started by adding 60 μl from 100μM riboflavin solution and placing the tubes under two 30W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. 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.

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

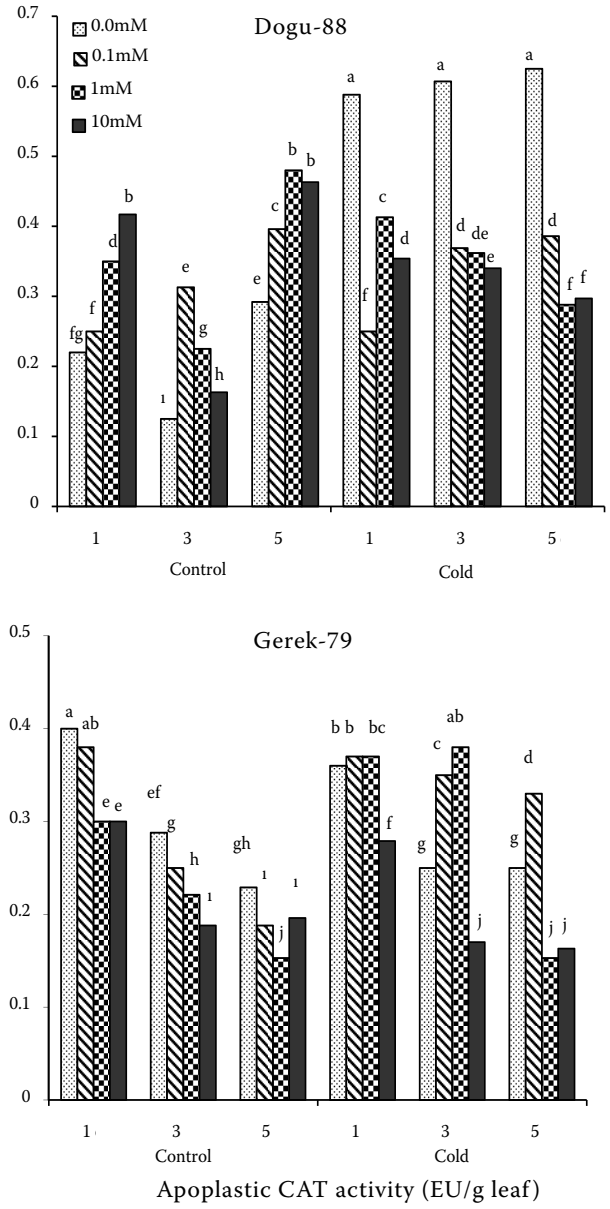

Figure 1. Effects of putrescine in different concentrations (0.1, 1 and 10mM) and cold (5/3°C) on apoplastic CAT activity. Means with different letters are significantly different at $P < 0.01$ based on Duncan's multiple range test

RESULTS AND DISCUSSION

We studied the separate and combined effects of Put (0.1, 1 and 10mM) and low temperature stress (5/3°C) on the activities of apoplastic CAT, POX and SOD in the leaves of winter wheat (Dogu-88) and spring wheat (Gerek-79). In addition, in order to determine the influence duration of the cold and the Put treatments, the activities of the antioxidant enzymes were followed at 1, 3 and 5 days after both treatments.

On all the days studied, the cold treatment (5/3°C) alone significantly ($P < 0.01$) increased apoplastic CAT activity in the winter wheat while it decreased slightly in the spring wheat leaves as compared with their respective controls (Figure 1). The results may demonstrate that the winter wheat rather than the spring wheat could regulate apoplastic CAT activity versus a sudden decrease in ambient temperature. This differs from the results of a recent study (Tasgin et al. 2006), which found that when cold was applied to winter wheat during an acclimation period in which temperature was gradually reduced from 20/18°C to 5/3°C, apoplastic CAT activity increased slightly at 10/5°C while decreasing at 5/3°C. The results suggest that gradual or sudden reductions in ambient temperature can produce important variations in antioxidative response to cold stress. However, although there are previous publications concerning the reduction of cellular CAT activity by cold treatment (Shim et al. 2003, Kuznetsov 2006), the results are inconclusive. Some researchers reported that cold does not inhibit CAT activity but may even stimulate activity (Prasad 1997, Shang et al. 2003). Baek and Skinner (2003) who studied the effects on different wheat lines proposed that CAT is more highly expressed in cold-tolerant wheat than cold-sensitive wheat during cold ac-

climation. The increase of apoplastic CAT activity by the cold treatment in winter wheat may be a result of its greater tolerance of cold conditions, suggesting that winter wheat can scavenge better the excessive H_2O_2 produced in apoplast during the low temperature than spring wheat.

In the present study, under the control treatment the Put treatments (0.1, 1 and 10mM) also significantly ($P < 0.01$) increased apoplastic CAT activity at 1, 3 and 5 days in the winter wheat while being reduced in the spring wheat leaves, compared to respective controls (Figure 1). The results indicate that Put applied under control conditions had an effect on CAT activity similar to cold treatment. It has been similarly reported that treatment with polyamines significantly promoted the activity

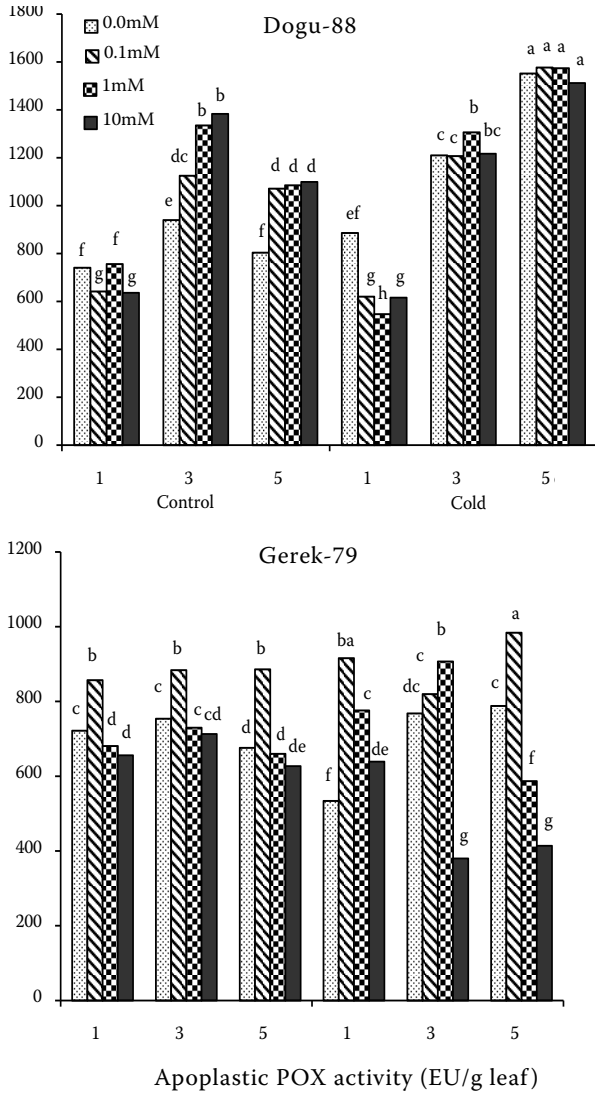

Figure 2. Effects of putrescine in different concentrations (0.1, 1 and 10mM) and cold (5/3°C) on apoplastic POX activity. Means with different letters are significantly different at $P < 0.01$ based on Duncan's multiple range test

of intracellular CAT (Shunzhi et al. 2003, Li et al. 2004). The induced increase of the apoplastic CAT by Put can be regarded as an important mechanism in the apoplastic defence strategy. On the other hand, under cold conditions, all the Put treatments significantly ($P < 0.01$) decreased CAT activity in the winter cultivar on all the days studied, compared to respective controls. In the spring cultivar, however, the 0.1 and 1mM concentrations of Put increased the CAT activity while 10mM Put generally decreased activity (Figure 1). This different results for winter wheat may be a result of polyamine toxicity in the case of high polyamine concentration, because winter wheat under cold conditions would probably exhibit the combined effects of both high biosynthesis of polyamines and the exogenous Put treatment, which is manifested in their peroxidative effect as a result of intensive oxidative polyamine degradation in the apoplast (Kuznetsov et al. 2006). In addition, the decrease of CAT activity by Put + cold treatment in the winter cultivar may also be a result of the different cold response among cultivars different in cold tolerance. We conclude that in regulation of apoplastic CAT activity against cold stress the Put treatments were more effective for winter wheat than for spring wheat.

The cold treatment (5/3°C) alone increased significantly ($P < 0.01$) the apoplastic POX activity in the winter wheat leaves while not significantly affecting ($P > 0.01$) activity in the spring wheat (Figure 2). This supports the findings of Tasgin et al. (2006) who suggested that apoplastic POX activity in winter wheat was increased by cold acclimation. The Put treatments alone applied to winter wheat leaves under control conditions significantly ($P < 0.01$) increased POX activity on days 3 and 5 but not day 1 (Figure 2). In spring wheat under the same conditions, POX activity only increased on the days studied by the 0.1mM Put concentration, but decreased ($P > 0.01$) in response to the other two concentrations. Some findings in literature also point out that Put activated POX activity bound to the cell wall in plants under control conditions (Paramonova et al. 2003, Kuznetsov et al. 2006). Under the cold treatment, the Put treatments significantly ($P < 0.01$) reduced POX activity on day 1, but did not significantly ($P > 0.01$) affect activity on the other days (3 and 5) in winter wheat. In the spring cultivar, however, the low concentrations (0.1 and 1mM) of Put were shown to increase POX activity rather than the high concentration (10mM) (Figure 2). The result implies that Put can play a significant role

in response to cold by increasing apoplastic POX activity, especially in a cold-sensitive cultivar. It is suggested that POX activity was not increased in the winter wheat because it could probably achieve a sufficient increase of POX activity during cold acclimation (Figure 2).

In the present study, the cold treatment alone increased apoplastic SOD activity in winter wheat leaves on all the days studied but did not significantly ($P > 0.01$) affect activity in spring wheat leaves, compared to respective controls (Figure 3). Winter wheat probably scavenges more effectively the superoxide radical (O_2^-) causing oxidative damage in apoplast during cold stress. The increase of cellular SOD activity has been shown to be related to improved cold tolerance (Westhuizen 1998). Salt stress causing oxidative stress also elevated the level of apoplastic SOD in resistant cultivar of pepper rather than its sensitive cultivar (Turhan et al. 2006). Under control conditions, all the Put treatments alone increased significantly ($P < 0.01$) SOD activity in winter wheat leaves (Figure 3). However, in spring wheat under the same conditions, it increased SOD activity on days 1 and 3 while being decreased on day 5. It was observed that Put treatments generally elevated apoplastic SOD activity in each of the wheat cultivars. Under the cold treatment, all the Put treatments increased SOD activity on day 1, but caused an unexpected decrease on the other days (3 and 5) in winter wheat. In the cold-sensitive cultivar, however, the Put treatments caused important increases in SOD activity during cold period (Figure 3). In addition, the increased SOD activity was more conspicuous in the spring cultivar, suggesting that Put may promote O_2^- scavenging ability of the spring cultivar during cold stress. Put can play a significant role in response to cold by increasing apoplastic SOD activity.

In conclusion, the cold treatment (5/3°C) increased the activities of apoplastic CAT, POX and SOD in winter wheat while not generally affecting spring wheat. Under control conditions, the Put treatments (0.1, 1 and 10mM) were more effective in increasing the activities of these enzymes in winter wheat than in spring wheat. However, under cold conditions, Put unexpectedly induced the enzyme activities in spring wheat while generally decreasing them in winter wheat leaves. This result may indicate that Put may take place of cold acclimation in winter wheat under control conditions; or that the antioxidative enzyme activities in winter wheat under the cold treatment decreased by the exogenous Put treatments can arise from a high amount of Put accumulated over much in winter wheat. It has been reported that in chilling-tolerant cultivars of rice and wheat, the level of Put significantly increased after exposure to chilling. In chilling-sensitive cultivars, however, the level of Put either increased slightly or decreased significantly after exposure to chilling (Bouchereau et al. 1999). This also shows that a high concentration of Put supplied both endogenously and exogenously in cold condition can cause a toxic effect to the apoplastic antioxidative system. However, since spring wheat cannot supply a high endogenous Put concentration, exogenous Put treatment could better regulate its apoplastic antioxidative response

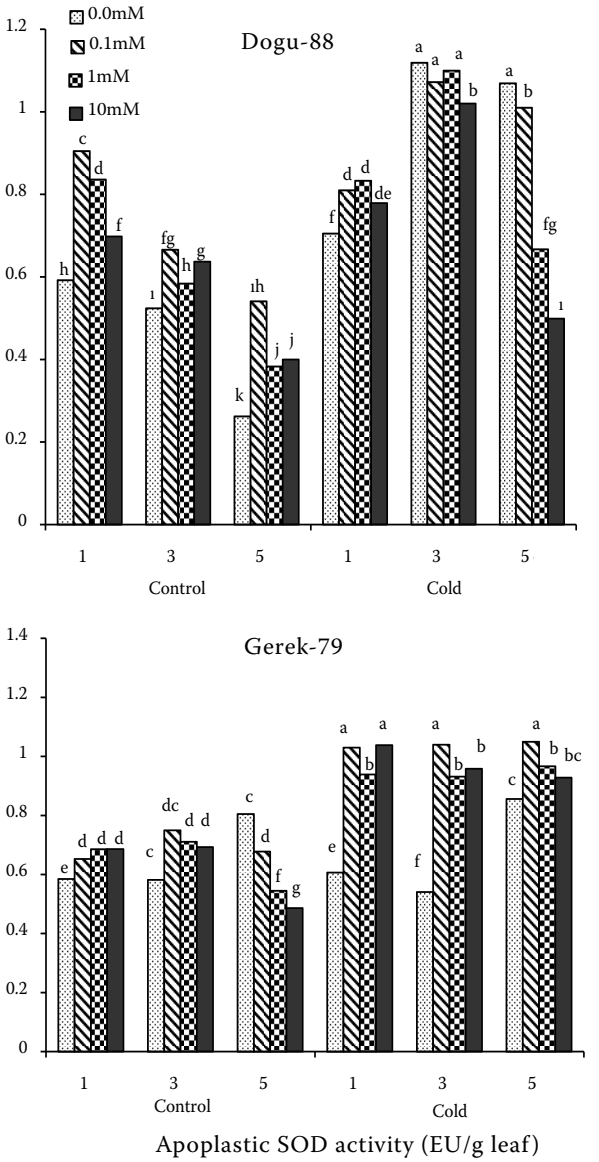

Figure 3. Effects of putrescine in different concentrations (0.1, 1 and 10mM) and cold (5/3°C) on apoplastic SOD activity. Means with different letters are significantly different at $P < 0.01$ based on Duncan's multiple range test

to cold stress. Exogenous Put treatment, hence, can be involved in antioxidative-mediated reactions in the apoplast, resulting in overcoming of oxidative stress during cold treatment. Based on these findings, we suggest that there may be different cultivar-specific mechanisms of regulation of apoplastic antioxidant enzymes by Put.

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Received on December 29, 2009

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