

Exogenously applied polyamines ameliorate osmotic stress-induced damages and delay leaf rolling by improving the antioxidant system in maize genotypes

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Abstract: Leaf rolling (LR), particularly in crops, is a dehydration avoidance mechanism that results from water loss in plants under drought conditions. Two maize cultivars (*Zea mays* L.) differing in their tolerance to drought (cultivar Side was selected as stress-tolerant and cultivar Karaçay was selected as stress-sensitive) under PEG-induced drought stress were used in this study to understand the effects of polyamines (PAs) on LR response and the role of the antioxidant system in the delayed LR process. Aerial parts of the seedlings were excised prior to the experiment and then submerged in a Hoagland nutrient solution with or without 0.1 mM Putrescine and 0.1 mM Spermine for 17 h, after which they were submitted to osmotic stress treatments (5% PEG) for 24 h. LR was physically prevented in order to clarify the relationship between PAs and antioxidant systems in LR response. Plants were evaluated by measuring the accumulation of hydrogen peroxide (H₂O₂), lipid peroxidation, antioxidant enzyme activities, and nonenzymatic antioxidant levels. Exogenous applications of PAs prevented water loss and delayed LR in comparison with the control (seedlings treated only with PEG). PAs almost totally prevented increases in lipid peroxidation. Antioxidant enzyme activities in rolled leaves were induced in response to osmotic stress in both maize seedlings via PA application. Ascorbate, glutathione, and endogenous PA levels increased as a result of PA applications. H₂O₂ content was lower in PA-pretreated plants than in the control in both cultivars. The activity/effectiveness of antioxidant system components of the physical prevention of leaf rolling (PLR) group of plants were lower than those of the group of plants with rolled leaves, especially in sensitive Karaçay, whereas more induced H₂O₂ content and lipid peroxidation were determined in the PLR group. In addition, exogenous applications of PAs further increased the components of Side's antioxidant system in comparison with Karaçay. When considered together, the data suggest that exogenous PAs may have a H₂O₂-mediated regulatory role in the LR process through the induction of antioxidant machinery. A stimulated antioxidant system decreases oxidative stress damage from excess accumulation of H₂O₂, thus delaying LR. In summary, exogenous PAs could help plants invert the adverse effects of osmotic stress and might play a key role in providing tolerance in plants through modulating the antioxidant system and decreasing H₂O₂ levels and water loss in plants via LR, as an alternative drought-protection mechanism. In addition, maize cultivars with late LR by PA applications may be provided as an opportunity for improving yield potential in drought-prone areas.

Key words: Antioxidant systems, leaf rolling, osmotic stress, polyamines, *Zea mays*

1. Introduction

Plants under natural conditions frequently encounter various environmental stresses that have adverse effects on their growth and productivity (Gong et al., 2014). Leaf rolling (LR), particularly in crops, is a dehydration avoidance mechanism that results from water loss in plants under biotic and abiotic stress conditions. LR provides protection against damage resulting from excess radiation (Kadioğlu and Terzi, 2007). On the other hand, delayed LR may be connected to the ability of plants to maintain plant cell turgor by enhancing water uptake or minimizing water loss despite drought stress (Kadioğlu et al., 2012).

LR is an important and necessary mechanism, protecting photosynthetic machinery and reducing yield loss as a result of drought stress in comparison with prevention of leaf rolling (PLR) (Nar et al., 2009; Sağlam et al., 2014). The relationship between delayed LR and drought resistance was previously recorded in *Ctenanthe setosa* plants exposed to drought stress (Kadioğlu et al., 2011).

Similar to other environmental stresses in higher plants, osmotic stress caused by drought may disturb the redox state homeostasis and cause oxidative stress (Smirnoff, 1993). Reactive oxygen species (ROS) cause several alterations, especially in protein degradation, enzyme

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inactivation, and changes in the gene expression, and interfere with multiple important metabolism pathways (Mahajan and Tuteja, 2005). Plants develop functional antioxidant systems, including antioxidant enzymes and low-molecular-weight antioxidants, in order to protect themselves against the toxic effects of ROS. It has been put forth by many researchers that drought tolerance of plants is closely related to induced enzyme activities and the expression of genes involved in antioxidant systems (Sharma and Dubey, 2005; Xu et al., 2011; Liu et al., 2014) as well as high antioxidant capacities of the cells due to antioxidant metabolites.

Antioxidant machinery may not be adequate to minimize the adverse effects of oxidative damage under severe stress conditions. Thus, accumulation of osmotically active substances in plants is a crucial step in our understanding of how plants respond to various environmental stresses. Polyamines (PAs) are osmotically active substances known to be important for drought tolerance. PAs are a class of phytohormone-like aliphatic amine compounds; major types include triamine spermidine (Spd), tetramine spermine (Spm), and their precursor diamine, putrescine (Put). PAs are not only involved in the regulation of plant growth and physiological events (Kusano et al., 2007; 2008; Takahashi and Kakehi, 2010); they also play important roles in modulating plant defense response against multiple environmental stresses (Bouchereau et al., 1999). On the other hand, the effects of exogenous PAs on plant tolerance under osmotic stress have been studied in some plant species (Liu et al., 2004; Radhakrishnan and Lee, 2013). Wang et al. (2007) reported that exogenous PAs cause an increase in endogenous PA content, thus providing drought tolerance. Furthermore, it has been suggested that the stimulatory effect of exogenous PAs reduce free radicals or lipid peroxidation by acting as an antioxidant (Velikova et al., 2000).

Investigations concerning the effect of PAs on LR phenomena are rather rare. It was put forth in previous studies that PAs delayed LR in *C. setosa* during drought stress (Kadioğlu et al., 2002). It was also reported that exogenous PAs increase proline content as well as reduce sugar and soluble proteins during LR, which contributes to osmotic adjustments (Saruhan et al., 2006). Considering the important role of PAs in delaying LR, the questions regarding how PAs are able to control the delay in LR and the role that antioxidant systems play in delayed LR need to be clarified. In this study, we hypothesize that exogenous application of PAs can delay LR by activating the antioxidant system or regulating osmotic adjustment and can decrease ROS levels in plants under osmotic stress conditions. Therefore, we examined the efficacy of PAs on the antioxidant system as well as their relations with LR phenomena in the seedlings of two cultivars of maize

differing in their drought tolerance. In order to determine the relationship between PAs and LR, detached leaves of maize cultivars were exposed to a short period of osmotic stress. In addition, it has not yet been clarified how delayed LR affects the antioxidant system. Therefore, the effects of artificial/physical PLR on the antioxidant system under osmotic stress were also examined.

2. Materials and methods

2.1. Plant growth and stress applications

The seeds of maize (*Zea mays* L.) cultivars differing in tolerance to drought stress (tolerant Side and sensitive Karaçay) were obtained from the West Mediterranean Agricultural Research Institute, Antalya, Turkey. Seeds were sown in plastic pots containing a peat/sand mix (5:1). Seedlings were grown at a temperature of 23 ± 2 °C, relative humidity of 60 ± 5 %, and light intensity of approximately $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ in a climate chamber for 30 days. The pots were watered with distilled water every 2 days. The seedlings were excised from aerial parts and kept in distilled water in test tubes wrapped with aluminum foil for 1 h in order to ensure effective PA import for the seedlings, as well as to mitigate the effects of wound stress due to excision (Terzi et al., 2014, 2015). The detached seedlings were divided into three groups for each maize cultivar and were submerged in Hoagland nutrient solution (pH 6.0) with or without 0.1 mM Put and Spm for 17 h, after which they were submitted to osmotic stress treatments (5% polyethylene glycol (PEG)) for 24 h. In order to indicate a relation between LR and antioxidants, LR was artificially prevented in half of the leaves by clamping them with plastic wires at the beginning of the PEG-induced osmotic stress. Treatment groups were as follows: 5% 6000 PEG as a control, osmotic stress combined with a 0.1 mM Put application, and osmotic stress combined with a 0.1 mM Spm application. The fourth leaves of the plants were used for the following assay.

2.2. LR degree (%) and plant water status measurements

The degree of LR was measured according to Premachandra et al. (1993) as a percentage reduction in the width of the leaf's middle portion as a result of rolling. Water potential (Ψ_{leaf}) of individual leaves was measured using a thermocouple psychrometer at 27 ± 1 °C (PSYPRO; Wescor, Inc., Logan, UT, USA). Measurements of relative water content (RWC) in leaf tissue were determined according to Castillo (1996).

2.3. Estimation of lipid peroxidation

Malondialdehyde (MDA) content of leaf tissue was measured for lipid peroxidation according to the method of Heath and Packer (1968). A leaf sample of 0.5 g was homogenized in 10 mL of 0.1% (w/v) TCA, and 4 mL of 0.5% (w/v) TBA containing 20% (w/v) TCA was added to

1 mL of supernatant. The absorbance of the supernatant was measured at 532 and 600 nm. MDA concentration was determined by its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.4. Assay of hydrogen peroxide content

H_2O_2 level was determined according to method of Velikova et al. (2000). A tissue sample of 0.25 g was homogenized in 3 mL of 5% TCA with 0.1 g of activated charcoal at 0 °C. To a 0.5-mL aliquot of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.75 mL of 1 M KI were added. The absorbance was read at 390 nm and the H_2O_2 level was calculated as $\mu\text{mol g}^{-1} \text{ FW}$.

2.5. Measurements of antioxidant enzymes

A frozen leaf segment of 0.5 g was homogenized in 5 mL of extraction buffer (50 mM potassium phosphate buffer (pH 7.0) + 1 mM EDTA + 1% polyvinylpyrrolidone). For the APX assay, the extraction buffer was supplemented with 5 mM ascorbic acid. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the nitro blue tetrazolium photo reduction rate, following the method of Beauchamp and Fridovich (1971). Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (25 °C, $e = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) at 240 nm (Aebi, 1983). Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was measured by monitoring the increase in absorbance at 470 nm (25 °C, $e = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) due to guaiacol oxidation (Urbanek et al., 1991). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was estimated by monitoring ascorbate (ASC) oxidation at 290 nm (25 °C, $e = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) (Nakano and Asada, 1981). Glutathione reductase (GR, EC 1.6.4.2) activity was determined by measuring the decrease in absorbance of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (25 °C, $e = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), according to Foyer and Halliwell (1976). Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was determined by following the oxidation of NADH at 340 nm (25 °C, $e = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), according to Hossain et al. (1984). The assay of dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was carried out by measuring the increase in absorbance at 265 nm (25 °C, $e = 14 \text{ mM}^{-1} \text{ cm}^{-1}$) due to ascorbic acid formation (Hossain and Asada, 1984). All enzyme activities were given on a protein basis. Protein content was specified according to Bradford (1976).

2.6. Assay of nonenzymatic antioxidant contents

Glutathione (GSH) content was determined by measuring the mixture at 412 nm, following the spectrophotometric method of Griffith (1980), using an assay based on the enzymatic recycling of GSH. The ASC level of leaves was estimated as described by Liso et al. (1984).

2.7. PA analysis

A sample of lyophilized leaves of 20 mg was extracted in 5 cm³ 5% (w/v) perchloric acid for PA analysis. After

centrifugation, supernatants were collected, after which the pellet was washed several times with the same solution and incubated in 5% perchloric acid. Aliquots of the samples containing free plus conjugated and bound PAs were hydrolyzed in 6 M HCl at 110 °C for 18 h to convert the conjugated and bound forms of the PAs into the free form. 1,6-Hexanediamine (0.5 mM; 5 μL) was used as the internal standard, and after dansylation, PAs in the supernatant and hydrolysates were quantified using HPLC as described by Burtin et al. (1989).

2.8. Statistical analysis

The experiment had a completely randomized design. All analyses in this study were the means of three replicates. Statistical analysis was performed by one-way ANOVA using Duncan's multiple comparison test and a significance was determined at the 5% ($P \leq 0.05$) level.

3. Results

3.1. LR degree (%) and plant water status parameters

Low PA concentration (0.1 mM) was preferred for exogenous application of PAs because LR used as a visual symptom of plant water deficit was detected at PA concentrations higher than 0.1 mM (data not presented). In our study, exogenous applications of both Put and Spm delayed LR in both cultivars. On the other hand, exogenous Spd had no effect on LR. Therefore, only the effects of Put and Spm on LR phenomena were studied in this paper. It was found that the LR degree (%) of seedlings with exogenous PAs was less than that of the control under osmotic stress. Furthermore, there were also statistical differences in LR degree between both cultivars. For instance, LR degrees after 0.1 mM Put and Spm applications were 21% and 20% in Side, while it was 33% in the control. In Karaçay, LR degrees of the control, Put-applied, and Spm-applied plants were 43%, 32%, and 33%, respectively (Figure 1A).

We evaluated Ψ_{leaf} and RWC of both cultivars under osmotic stress after PA applications to understand how water status was affected. Exogenously applied PAs caused a decrease in the Ψ_{leaf} of stressed plants, but these decreases were much lower than those of control plants in rolled leaves of both cultivars. For instance, while Ψ_{leaf} of PEG-treated seedlings with LR was -1.57 MPa in Side, it was -1.31 and -1.29 MPa in Side seedlings pretreated with 0.1 mM Put and Spm, respectively. Similarly, while the RWCs of Put- and Spm-pretreated seedlings with LR were 69% and 70%, the value was 64% in the control. Ψ_{leaf} and RWC in Karaçay with LR were lower than those of Side. On the other hand, with respect to PLR, Ψ_{leaf} and RWC were more reduced in comparison with LR. However, compared with its control, there was an increase in water status after PA in seedlings with PLR, but this increase was not as great as in seedlings with LR (Figures 1B and 1C).

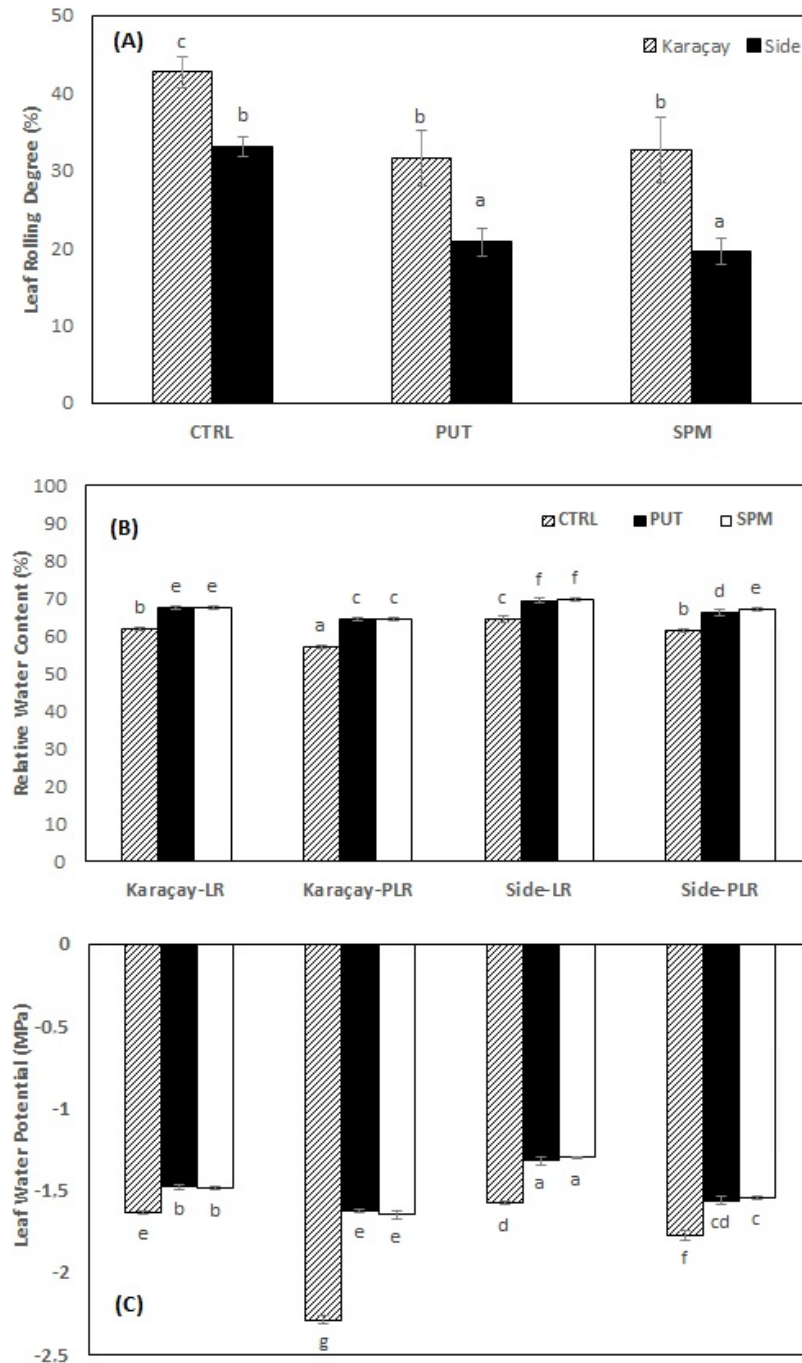


Figure 1. Effect of exogenous PAs on leaf rolling degree (%) (A), relative water content (RWC) (B), and leaf water potential (Ψ_{leaf}) (C) in rolled leaves and artificially prevented leaves by clamping of detached maize seedlings under osmotic stress. The seedlings were submitted to three treatments: osmotic stress treatment with 5% polyethylene glycol 6000 (PEG) (CTRL, control); pretreated with 0.1 mM Put and osmotically stressed (PUT); and pretreated with 0.1 mM Spm and osmotically stressed (SPM). LR – Leaf rolling, PLR – prevention of leaf rolling. Vertical bars indicate standard deviation. Different letters represent significant differences at $P \leq 0.05$.

3.2. Estimation of lipid peroxidation and hydrogen peroxide content

The lipid peroxidation levels of both cultivars, estimated in terms of MDA content, are shown in Figure 2. MDA content

diminished in the rolled leaves of both cultivars under osmotic stress after Put and Spm applications compared to their controls. PLR induced more lipid peroxidation than LR. There was a decrease in MDA content in seedlings

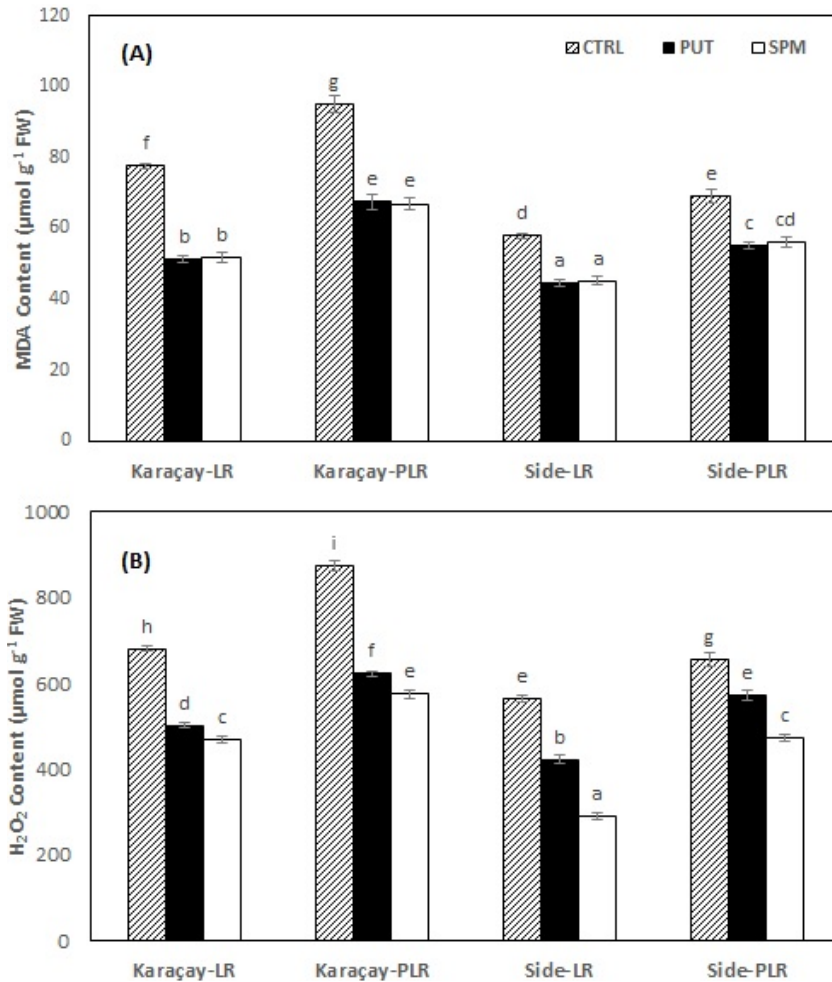


Figure 2. Effects of exogenous PAs on lipid peroxidation (A) and H_2O_2 (B) content in rolled leaves and artificially prevented leaves by clamping of detached maize seedlings under osmotic stress. The seedlings were submitted to three treatments: osmotic stress treatment with 5% polyethylene glycol 6000 (PEG) (CTRL, control); pretreated with 0.1 mM Put and osmotically stressed (PUT); and pretreated with 0.1 mM Spm and osmotically stressed (SPM). LR – Leaf rolling, PLR – prevention of leaf rolling. Vertical bars indicate standard deviation. Different letters represent significant differences at $P \leq 0.05$.

with PLR after PA application compared with the control, but this decrease was not as great as that of seedlings with LR (Figure 2A).

PA applications, particularly Spm, prevented H_2O_2 production in rolled leaves of both cultivars compared to their controls. In other words, exogenously applied PAs caused a significant decline in endogenous H_2O_2 content in seedlings with LR under stress conditions. In addition, PLR increased H_2O_2 content more than LR did. There was also a decrease in H_2O_2 content after PA application in seedlings with PLR compared with the control, which was not as great as that of seedlings with LR (Figure 2B).

3.3. Antioxidant enzymes

PEG-induced osmotic stress affected all antioxidant enzyme activities in both maize cultivars. There was no difference in SOD activity between the groups of PA

applications and their controls in both cultivars. Moreover, SOD activity in both maize cultivars of seedlings with PLR was lower than in those with LR (Figure 3A).

Under osmotic stress, PA applications significantly enhanced CAT activity in rolled leaves of both cultivars in comparison to their controls. In the Spm-treated plants, CAT activity was higher than in Put-treated plants. After exogenous applications of Put and Spm, CAT activities increased by 57% and 94% in Side seedlings under osmotic stress, respectively. In Karaçay, the activities in Put- and Spm-treated plants increased by 16% and 52%, respectively. CAT activities in both maize cultivars were reduced in seedlings with PLR in comparison with LR. However, there was an increase in CAT activity after PA application in seedlings with PLR in comparison with the control, but this increase was not as great as that in plants with LR (Figure 3B).

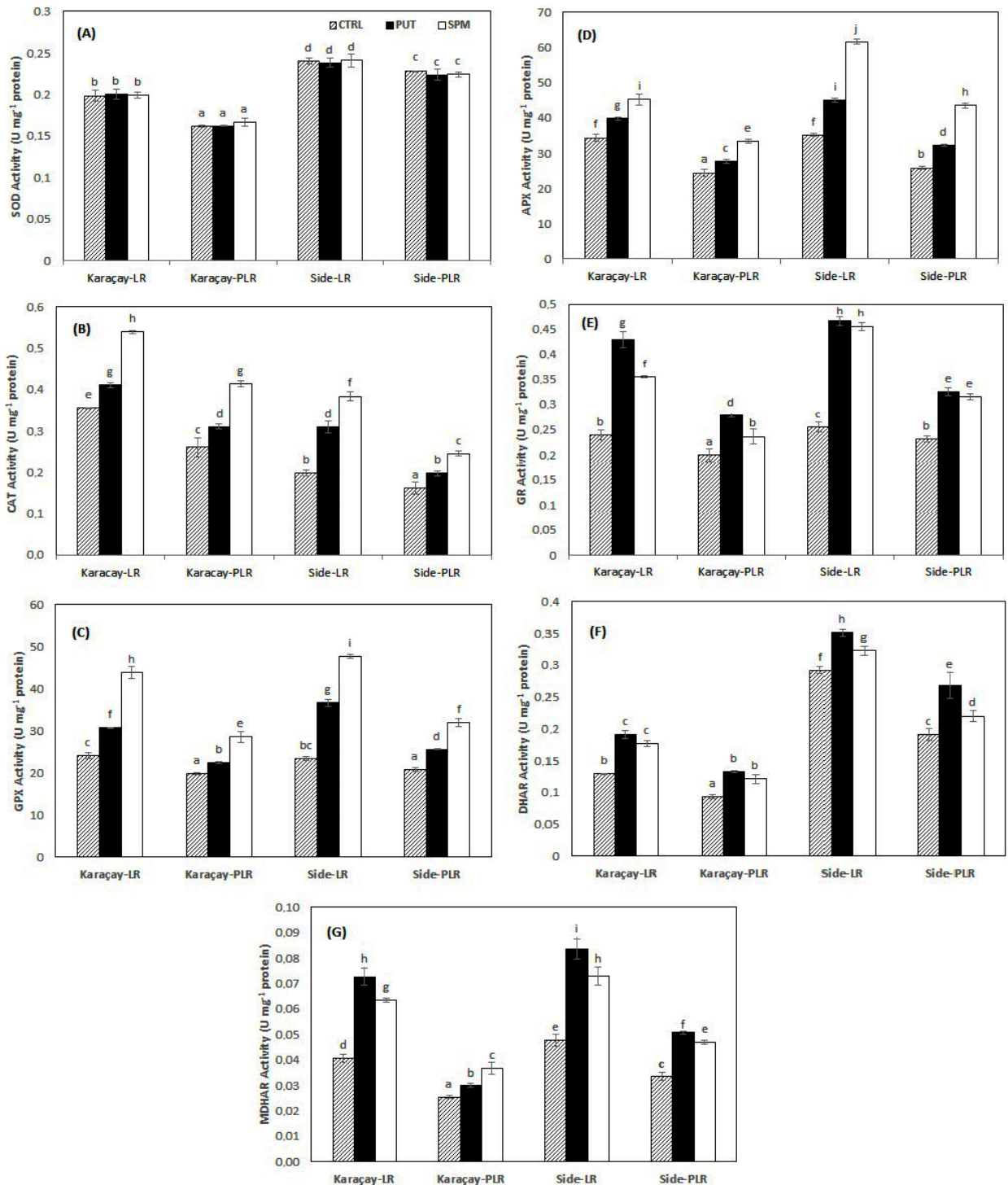


Figure 3. Effects of exogenous PAs on SOD (A), CAT (B), GPX (C), APX (D), GR (E), DHAR (F), and MDHAR (G) enzyme activities in rolled leaves and artificially prevented leaves by clamping of detached maize seedlings under osmotic stress. The seedlings were submitted to three treatments: osmotic stress treatment with 5% polyethylene glycol 6000 (PEG) (CTRL, control); pretreated with 0.1 mM Put and osmotically stressed (PUT); and pretreated with 0.1 mM Spm and osmotically stressed (SPM). LR – Leaf rolling, PLR – prevention of leaf rolling. Vertical bars indicate standard deviation. Different letters represent significant differences at $P \leq 0.05$.

GPX activity was induced by PA applications in seedlings with LR under osmotic stress. These increases were respectively 57% and 103% in Side and 27% and 82% in Karaçay after exogenous applications of both Put and Spm in comparison with their controls. GPX activity was of the greatest significance in the rolled leaves of Spm-applied plants. PA applications resulted in a decrease in GPX activity in plants with PLR in comparison with LR. Following Put and Spm applications, GPX activities in Side were 25.6 and 31.8 U/mg protein in seedlings with PLR, while these values were 36.6 and 47.6 U/mg protein in seedlings with LR, respectively (Figure 3C).

The combination of PA applications and osmotic stress resulted in an increase of APX in the rolled leaves of both cultivars. Generally, Side displayed greater APX activity than Karaçay for all the treatments. After Put application, APX activity increased by 28% in Side and 16% in Karaçay. After application with Spm, APX activity increased by 75% in Side and 32% in Karaçay. Compared to LR, APX activity was reduced in both cultivars with PLR. After Put and Spm applications, APX activity in Side plants with PLR was 32.1 and 43.5 U/mg protein, while it was 45.1 and 61.6 U/mg protein, respectively, in plants with LR (Figure 3D).

When compared with their corresponding controls, PA applications significantly increased GR, DHAR, and

MDHAR activities in rolled leaves of both cultivars under osmotic stress. All three enzyme activities in Put-treated plants were greater in comparison with those of the Spm-applied plants. GR activity in the Spm-treated drought-tolerant cultivar was increased by 84% but the increase in the Put-treated cultivar under stress conditions was 88%. MDHAR and DHAR enzyme activities underwent similar GR enzyme activity changes. In addition, GR, MDHAR, and DHAR activities in both maize cultivars were reduced by PLR in comparison with LR. There were also increases in all three enzyme activities after PA applications in plants with PLR in comparison with their controls, but these increases were not as drastic as those in plants with LR (Figures 3E–3G).

3.4. Antioxidant substances

The effects of osmotic stress and PA applications on the nonenzymatic antioxidants of the two maize cultivars are shown in Figure 4. Antioxidant substances were synthesized more in PA-treated rolled leaves than in seedlings with PLR. In plants with LR, Put and Spm applications enhanced the ASC content compared to their controls. On the other hand, ASC content was reduced by PLR in comparison with LR. There was an increase in ASC content after PA application in plants with PLR in

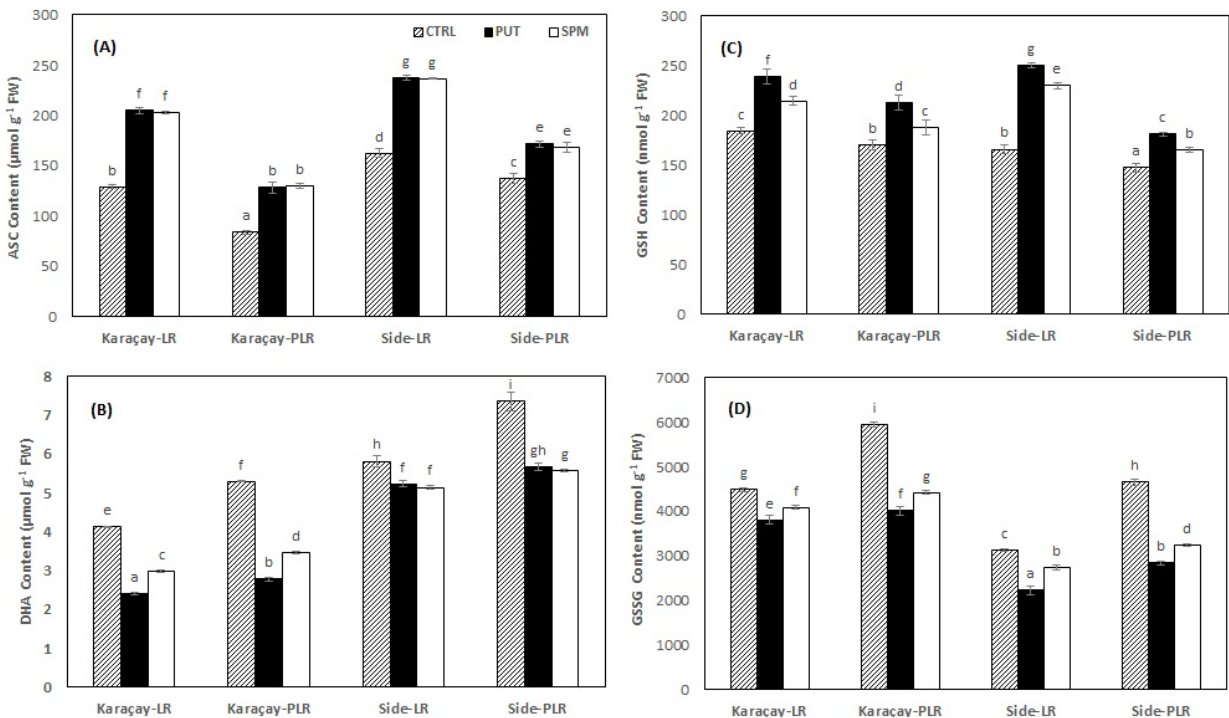


Figure 4. Effects of exogenous PAs on the changes in ASC (A), DHA (B), GSH (C), and GSSG (D) contents in rolled leaves and artificially prevented leaves by clamping of detached maize seedlings under osmotic stress. The seedlings were submitted to three treatments: osmotic stress treatment with 5% polyethylene glycol 6000 (PEG) (CTRL, control); pretreated with 0.1 mM Put and osmotically stressed (PUT); and pretreated with 0.1 mM Spm and osmotically stressed (SPM). LR – Leaf rolling, PLR – prevention of leaf rolling. Vertical bars indicate standard deviation. Different letters represent significant differences at $P \leq 0.05$.

comparison with their controls, but this increase was not as significant as that of plants with LR (Figure 4A).

There was an opposite trend for DHA content. PA application significantly decreased DHA content in rolled leaves of maize seedlings in comparison with their controls. In addition, more DHA content was induced in plants with PLR than with LR. There was also a decrease in DHA after PA application in seedlings with PLR in comparison with their controls, but this decrease was not as large as that of seedlings with LR (Figure 4B).

Compared to their controls, PA application significantly increased GSH content in rolled leaves in both cultivars under osmotic stress conditions. GSH contents after Put and Spm applications increased by 51% and 39%, respectively, in Side and by 30% and 16%, respectively, in Karaçay. On the other hand, GSH content in plants with PLR was lower than that in plants with LR for both cultivars. Compared with controls, there was an increase in GSH following PA application in seedlings with PLR, but this increase was not as significant as that of plants with LR. The increases in GSH were 23% and 12% in Side following Put and Spm applications, respectively, in plants with PLR compared with the control. There was also a similar trend in GSH content in Karaçay (Figure 4C).

There was an opposite trend for GSSG content. PA applications caused a decrease in GSSG content in plants with LR. After Put and Spm applications, GSSG decreased by 28% and 12% in Side plants with rolled leaves and by 15% and 9% in Karaçay plants with rolled leaves, respectively. However, GSSG content was reduced by PLR in comparison with LR. There was also a decrease in GSSG after PA application in plants with PLR in comparison with their controls, but this decrease was not as great as that in plants with LR (Figure 4D).

3.5. Endogenous PA content

Endogenous PA content (free Put and Spm) in both cultivars was enhanced significantly after pretreatment with Put and Spm under osmotic stress (Figures 5A and 5B). The increase in endogenous PA content in drought-tolerant Side was greater than in drought-sensitive Karaçay. After Put application, endogenous Put and Spm contents of Side increased by 101% and 206% in comparison with its control under osmotic stress. Put content of Karaçay increased by 60% in comparison with its control, but Spm content did not change. There was also a similar trend in endogenous PA content following Spm application in Side. Put content of Karaçay following Spm application decreased by 49%, while Spm content increased by 115% in comparison with its control under osmotic stress (Figures 5A and 5B).

4. Discussion

PAs play vital roles in the defense reactions of plants to several types of environmental stress. However, the effect

of PAs on the antioxidant system and their relations with LR phenomena have not been fully elucidated. This research suggests that exogenous applications of PAs may help minimize the adverse effects of osmotic stress in maize seedlings. In the current study, application of PAs delayed LR in both cultivars. Moreover, there were also differences between the two maize cultivars in terms of LR degree (Figure 1A). The degree of LR was less in the tolerant cultivar, Side, than in the sensitive cultivar, Karaçay. In addition, LR degree of PA-applied seedlings was less than that of their control groups (PEG-induced osmotic stress only). RWC and Ψ_{leaf} , which are the most important indicators of plant water status, were enhanced in seedlings of both cultivars with rolled leaves compared to plants with PLR, but they were greater in Side plants with rolled leaves than in Karaçay. These RWC and Ψ_{leaf} results are in accordance with other studies showing that tolerant varieties are better at maintaining RWC and Ψ_{leaf} under drought stress (Fernandez and Castrillo, 1999; Guoth et al., 2009). Furthermore, in comparison with their controls, PA-applied seedlings also exhibited higher RWC and Ψ_{leaf} during LR, which indicated that applications of PAs enhanced the osmotic stress tolerance of the maize cultivars, especially in Side. Similarly, Farooq et al. (2009) put forth that exogenous applications of PAs increase Ψ_{leaf} and RWC of rice plant leaves under drought stress. It is thought that PAs have the ability to improve plant water status. Therefore, such an ameliorative effect on RWC and Ψ_{leaf} may have been due to the role of PAs in osmotic adjustment under stressful environmental conditions, as reported earlier in different studies (Farooq et al., 2009, 2010). Thus, PAs may contribute to the osmotic stress acclimation of maize cultivars. It was reported that plants with LR have a greater resistance to drought and high temperatures, and that they have higher water-use efficiency (Kadioğlu et al., 2012). Since the decrease in Ψ_{leaf} occurred due to PLR, it could be concluded that LR might be a water-saving regulatory mechanism under osmotic stress. Moreover, exogenous applications of PAs recovered leaf water status. This could be carried out by osmotic adjustment (PA accumulation) induced via PA applications.

Numerous studies have demonstrated that the application of PAs prevents lipid peroxidation under various environmental stresses (Arasimowicz-Jelonek et al., 2009; Farooq et al., 2009; Yiu et al., 2009). In this study, we found that exogenous PAs could effectively suppress increases in MDA caused by osmotic stress in both cultivars. PLR induced more lipid peroxidation than LR. In comparison with Karaçay, Side seedlings with rolled leaves that were exposed to exogenous applications of Put or Spm successfully resisted osmotic stress, proof of the reduced MDA content. Lower MDA levels observed in

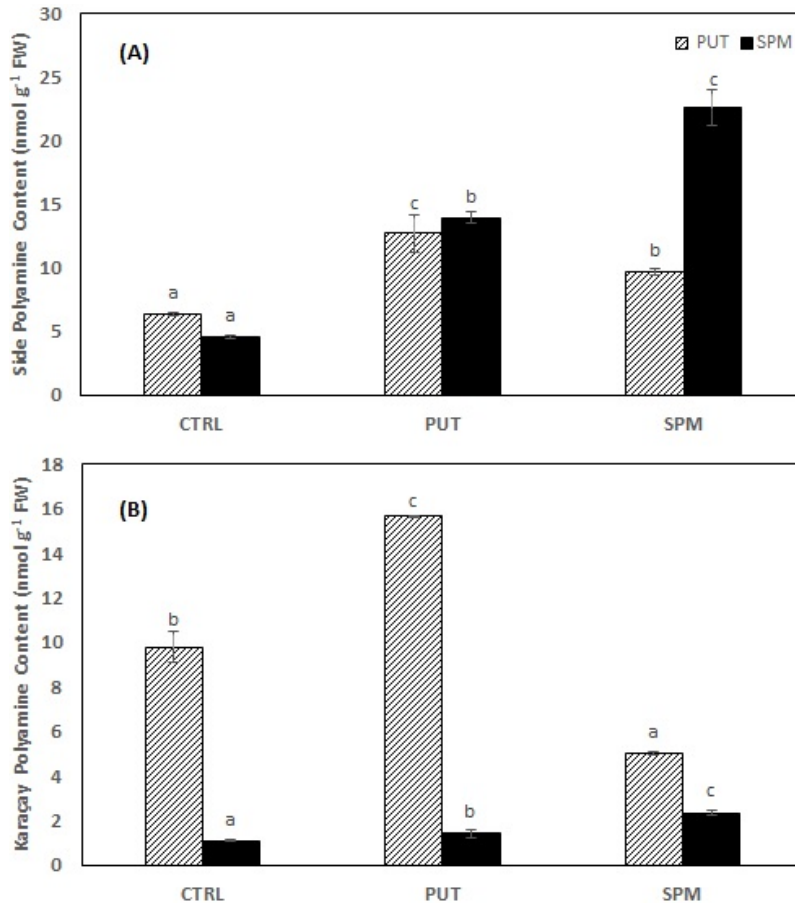


Figure 5. Effects of exogenous PAs on endogenous polyamines contents (A, B) in rolled leaves of detached maize seedlings under osmotic stress. The seedlings were submitted to three treatments: osmotic stress treatment with 5% polyethylene glycol 6000 (PEG) (CTRL, control); pretreated with 0.1 mM Put and osmotically stressed (PUT); and pretreated with 0.1 mM Spm and osmotically stressed (SPM). Vertical bars indicate standard deviation. Different letters represent significant differences at $P \leq 0.05$. Each of the polyamines was evaluated among themselves.

PA-pretreated rolled Side leaves suggests better protection from oxidative damage. The better protection in Side seems to result from a more efficient antioxidative system, while the significant increase in MDA content in leaves of Karaçay appears to arise from lower antioxidant enzyme activity, which will be discussed later.

Plants produce excessive ROS when exposed to multiple environmental stresses. The enhanced production of ROS causes oxidative damage through membrane destruction, protein damage, and enzyme inhibition, leading to cell death (Smirnov, 1993). However, PAs can protect biological membranes and critical macromolecules from oxidative damage because they have the potential to act as ROS scavengers (Besford et al., 1993) and thus can maintain biological membranes' stability under stress conditions (Roberts et al., 1986). In the present study, exogenous applications of PAs significantly decreased H_2O_2 content in seedlings of both cultivars with rolled

leaves under osmotic stress. A strong negative relationship between some antioxidant enzymes (especially CAT, GPX, and APX) and H_2O_2 proves that PA applications enhance antioxidant enzyme activities, which results in H_2O_2 scavenging. Pretreatment with PAs, especially Spm, remarkably prevented H_2O_2 accumulation in Side plants with rolled leaves, where it alleviated osmotic stress injury. These results support the idea that PAs also act as free radical scavengers. PA applications may mitigate the effects of osmotic stress, as suggested by the substantially lowered H_2O_2 and MDA contents. Our findings are similar to those of Farooq et al. (2009), who suggested that PAs specifically enhance certain enzyme activities, resulting in the elimination of H_2O_2 and improved membrane structures under drought stress in rice seedlings. Compared with LR, increases in H_2O_2 and MDA content of plants with PLR demonstrated that LR might protect the plant from photodamage by reducing leaf exposure to light. Moreover,

it would be appropriate to draw attention to H_2O_2 and LR degree. Endogenous concentrations of H_2O_2 decreased in parallel with the decrease in LR just after PA applications. On the other hand, the degree of LR and endogenous H_2O_2 increased under osmotic stress in our study. In some previous studies, H_2O_2 -mediated delay in LR as a result of leaf hydration maintenance and stimulation of antioxidant systems was observed in *C. setosa* (Sağlam et al., 2014) and maize (Terzi et al., 2014) under osmotic stress. Similarly, exogenous applications of PAs might regulate endogenous H_2O_2 content by the antioxidant system. Thus, the decrease/relief of stress resulted in LR delay. However, it is necessary to carry out further work to clarify that phenomenon.

Plants have very well-organized detoxification systems that scavenge toxic ROS to restrict oxidative damage under environmental stress conditions (Talaat, 2014). Antioxidant enzymes, as a part of the detoxification system, are major components in plant defense mechanisms (Lai et al., 2007). In this study, it is clear that PAs induce all antioxidant enzyme activities in rolled leaves of both cultivars. Compared to Karaçay, we also found that antioxidant enzyme activities of Side were further enhanced by PAs. Under osmotic stress, these increases in antioxidant enzyme activities of Side showed that Side has a more effective induced H_2O_2 -scavenging capacity. On the other hand, Karaçay's sensitivity to drought may be associated with its lower antioxidant enzyme activity, resulting in higher H_2O_2 accumulation. The higher stress tolerance of Side may be related to an increased level of antioxidant enzyme activities. The variability in the antioxidation pathway responses between these two maize cultivars also may be useful in understanding the underlying mechanism of stress tolerance. It has also been determined in this study that antioxidant enzyme activities in both maize cultivars were reduced by PLR in comparison with LR. This indicates that PAs stimulate antioxidant enzyme activity more in rolled leaves, thus protecting the seedlings from oxidative damage.

ASC-GSH pathway enzymes such as APX, GR, DHAR, and MDHAR are important in the control of ROS. PA applications with osmotically stressed plants (especially rolled leaves of Side plants) enhanced the activities of MDHAR, DHAR, and GR. These antioxidant enzyme activities in plants usually depend on antioxidant substances, such as ASC, GSH, tocopherol, and polyphenols (Ksouri et al., 2007); ASC and GSH especially play a major role in the signal transduction pathway in higher plants under stress conditions (Foyer and Noctor, 2011). Increased MDHAR and DHAR activities as a result of exogenous PA application suggest that there may be the generation of reduced ASC, which maintains higher APX activity under osmotic stress because of the fact that APX requires ASC as a substrate. Further, these increases in

enzyme activities indicate that the efficient maintenance of the ASC and GSH pool is required for running the ASC-GSH pathway for effective detoxification (Talaat, 2014). Compared to LR, decreases in antioxidant enzyme activities of seedlings with PLR indicate that LR may have an important role in reducing the adverse effects of oxidative stress.

Certain compounds, such as ASC and GSH, may be induced by PAs in plants (Ksouri et al., 2007; Shu et al., 2013; Li et al., 2014). The data indicate that Put and Spm applications elevated ASC content in rolled leaves under osmotic stress, especially in Side. It is clear that high ASC accumulation plays a crucial role in maintaining the antioxidant activity that protects plants from free radical injury (Zhou et al., 2009). Increasing reduced ASC levels plays a crucial role in the protection of APX activity. Similar to the change in ASC levels, the GSH levels in PA-applied plants were greater than that of their controls. On the other hand, there was an opposite trend for GSSG levels. The data may be related to changes in GR and DHAR activities. Exogenous PAs enhanced GR activity, resulting in high reduced GSH content and low oxidized GSH content in both cultivars as compared to control. In addition, PAs may increase osmotic stress tolerance of maize cultivars by promoting the ASC-GSH cycle. As compared to seedlings with LR, changes in ASC and GSH contents of seedlings with PLR indicate that LR may have an important role in reducing oxidative stress-induced effects and maintaining the ASC-GSH redox state of plants under abiotic and biotic stress conditions.

In this study, endogenous PAs level were induced by PA applications. During exposure to osmotic stress, Side exhibited high levels of free Put and Spm after exogenous application of PAs, whereas there was only a slight increase of PAs in Karaçay, suggesting that a high level of endogenous PAs is a major factor responsible for tolerance in maize. Indeed, stress-tolerant plants generally have a greater capacity to synthesize PAs in response to stress than sensitive plants, resulting in a two- to threefold rise in endogenous PA levels compared to unstressed plants (Kasukabe et al., 2004). Similar to our present results, Nayyar et al. (2005) reported that, following exogenous PA application, levels of endogenous PAs increased in soybean and chickpea seedlings. Our results suggest that PAs may have a key role in delaying LR and providing tolerance in plants exposed to osmotic stress. It is possible that endogenous PAs may play a crucial role in enhancing antioxidant capacity. In fact, Wi et al. (2006) reported that an increase in PA levels of transgenic tobacco induced a significant level of gene expression of antioxidant enzymes and thus PAs may play a major role in abiotic stress tolerance in plants.

In conclusion, exogenous PAs could diminish the injurious effects of osmotic stress and might have a role in drought tolerance in plants by modulating the antioxidant

system and decreasing the H_2O_2 level and water loss to some extent. In other words, PAs may have a H_2O_2 -mediated regulatory role in LR through the induction of the antioxidant defense system. The induced components of the antioxidant system reduce oxidative stress damage resulting from excess accumulation of H_2O_2 and then delay LR. Moreover, increases in endogenous PA content as well as the antioxidant system as a result of PA applications indicate that PAs may have a key role in delaying LR.

Furthermore, changes in all parameters in rolled and mechanically opened leaves suggested that LR is an advantageous mechanism protecting photosynthesis and reducing yield loss under drought stress by maintaining leaf hydration and inducing antioxidant machinery via exogenous PAs. As a result of the findings of the present study, we suggest the use of drought-tolerant maize cultivars with delayed LR response onset, which further increases antioxidant activity under drought conditions.

References

- Aebi H (1983). Catalase. In: Bergmeyer H, editor. *Methods of Enzymatic Analysis*. Weinheim, Germany: Weinheim-Verlag Chemie, pp. 273-286.
- Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Kubis J (2009). Interaction between polyamine and nitric oxide signaling in adaptive responses to drought in cucumber. *J Plant Growth Regul* 28: 177-186.
- Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44: 276-287.
- Besford RT, Richardson CM, Tiburcio AF (1993). Effect of polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically stressed oat leaves. *Planta* 189: 201-206.
- Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999). Polyamines and environmental challenges: recent development. *Plant Sci* 140: 103-125.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
- Burtin D, Martin-Tanguy J, Paynot M, Rossin N (1989). Effects of the suicide inhibitors of arginine and ornithine decarboxylase activities on organogenesis, growth, free polyamines and hydroxycinnamoyl putrescine levels in leaf explants of *Nicotiana xanthi* N.C. cultivated in vitro in a medium producing callus formation. *Plant Physiol* 89: 104-110.
- Castillo FJ (1996). Antioxidative protection in the inducible CAM plant *Sedum album* L. following the imposition of severe water stress and recovery. *Oecologia* 107: 469-477.
- Farooq M, Wahid A, Lee D (2009). Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. *Acta Physiol Plant* 31: 937-945.
- Farooq M, Wahid A, Lee DJ, Cheema SA, Aziz T (2010). Comparative time course action of the foliar applied glycinebetaine, salicylic acid, nitrous oxide, brassinosteroids and spermine in improving drought resistance of rice. *J Agro Crop Sci Z Acker Pflanzenbau* 196: 336-345.
- Fernandez D, Castrillo M (1999). Maize leaf rolling initiation. *Photosynthetica* 37: 493-497.
- Foyer CH, Halliwell B (1976). Presence of glutathione and glutathione reductase in chloroplast: a proposed role in ascorbic acid metabolism. *Planta* 133: 21-25.
- Foyer CH, Noctor G (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol* 155: 2-18.
- Gong B, Li X, Bloszies S, Wen D, Sun S, Wei M, Li Y, Yang F, Shi Q, Wang X (2014). Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*. *Free Radical Biol Med* 71: 36-48.
- Griffith OW (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106: 207-221.
- Guoth A, Tari I, Galle A, Csizsar J, Pecsvaradi A, Csuz L, Erdei L (2009). Comparison of the drought stress responses of tolerant and sensitive wheat cultivars during grain filling: changes in flag leaf photosynthetic activity, ABA levels and grain yield. *J Plant Growth Regul* 28: 167-176.
- Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125: 189-198.
- Hossain MA, Asada K (1984). Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. *Plant Cell Physiol* 25: 85-92.
- Hossain MA, Nakano Y, Asada K (1984). Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol* 25: 385-395.
- Kadioğlu A, Saruhan N, Sağlam A, Terzi R, Acet T (2011). Exogenous salicylic acid alleviates effects of long term drought stress and delays leaf rolling by inducing antioxidant system. *Plant Growth Regul* 64: 27-37.
- Kadioğlu A, Terzi R (2007). A dehydration avoidance mechanism: leaf rolling. *Bot Rev* 73: 290-302.
- Kadioğlu A, Terzi R, Saruhan N, Sağlam A (2012). Current advances in the investigation of leaf rolling caused by biotic and abiotic stress factors. *Plant Sci* 182: 42-48.
- Kadioğlu A, Turgut R, Palavan Ünsal N, Saruhan N (2002). Effect of polyamines on leaf rolling during drought stress in *Ctenanthe setosa* (Rosc.) Eichler. *Israel J Plant Sci* 50: 19-23.

- Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S (2004). Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol* 45: 712-722.
- Ksouri R, Megdiche W, Debez A, Falleh H, Grignon C, Abdelly C (2007). Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritima*. *Plant Physiol Biochem* 45: 244-249.
- Kusano T, Berberich T, Tateda C, Takahashi Y (2008). Polyamines: essential factors for growth and survival. *Planta* 228: 367-381.
- Kusano T, Yamaguchi K, Berberich T, Takahashi Y (2007). The polyamine spermine rescues *Arabidopsis* from salinity and drought stresses. *Plant Signal Behav* 2: 251-252.
- Lai QX, Bao ZY, Zhu ZJ, Qian QQ, Mao BZ (2007). Effects of osmotic stress on antioxidant enzymes activities in leaf discs of P_{SAG12}⁻ IPT modified gerbera. *J Zhejiang Uni-Sci B* 8: 458-464.
- Li X, Gong B, Xu K (2014). Interaction of nitric oxide and polyamines involves antioxidants and physiological strategies against chilling-induced oxidative damage in *Zingiber officinale* Roscoe. *Sci Hortic Amsterdam* 170: 237-248.
- Liso R, Calabrese G, Bitonti MB, Arrigoni O (1984). Relationship between ascorbic acid and cell division. *Exp Cell Res* 150: 314-320.
- Liu HP, Dong BH, Zhang YY, Liu ZP, Liu YL (2004). Relationship between osmotic stress and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings. *Plant Sci* 166: 1261-1267.
- Liu J, Li J, Su X, Xia Z (2014). Grafting improves drought tolerance by regulating antioxidant enzyme activities and stress-responsive gene expression in tobacco. *Environ Exp Bot* 107: 173-179.
- Mahajan S, Tuteja N (2005). Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys* 444: 139-158.
- Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22: 867-880.
- Nayyar H, Kaur S, Singh S, Kumar S, Singh KJ, Dhir KK (2005). Involvement of polyamine in the contrasting sensitivity of chickpea (*Cicer arietinum* L.) and soybean (*Glycine max* (L.) Merrill.) to water deficit stress. *Bot Bull Acad Sin* 46: 333-338.
- Premachandra GS, Saneoka H, Fujita K, Ogata S (1993). Water stress and potassium fertilization in field grown maize (*Zea mays* L.): effects of leaf water relations and leaf rolling. *J Agron Crop Sci* 170: 195-201.
- Radhakrishnan R, Lee IJ (2013). Ameliorative effects of spermine against osmotic stress through antioxidants and abscisic acid changes in soybean pods and seeds. *Acta Physiol Plant* 35: 263-269.
- Roberts DR, Dumdroff EB, Thompson JE (1986). Exogenous polyamines alter membrane fluidity in bean leaves: a basis for potential misinterpretation of their true physiological role. *Planta* 167: 395-401.
- Sağlam A, Kadioğlu A, Demiralay M, Terzi R (2014). Leaf rolling reduces photosynthetic loss in maize under severe drought. *Acta Bot Croat* 73: 315-332.
- Saruhan N, Turgut Terzi R, Kadioğlu A (2006). The effects of exogenous polyamines on some biochemical changes during drought stress in *Ctenanthe setosa* (Rosc.) Eichler. *Acta Biol Hung* 57: 221-229.
- Sharma P, Dubey RS (2005). Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. *J Plant Physiol* 162: 854-864.
- Shu S, Yuan LY, Guo SR, Sun J, Yuan YH (2013). Effects of exogenous spermine on chlorophyll fluorescence, antioxidant system and ultrastructure of chloroplasts in *Cucumis sativus* L. under salt stress. *Plant Physiol Biochem* 63: 209-216.
- Smirnoff N (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol* 125: 27-58.
- Takahashi T, Kakehi JI (2010). Polyamines: ubiquitous polycations with unique roles in growth and stress responses. *Ann Bot* 105: 1-6.
- Talaat NB (2014). Effective microorganisms enhance the scavenging capacity of the ascorbate-glutathione cycle in common bean (*Phaseolus vulgaris* L.) plants grown in salty soils. *Plant Physiol Biochem* 80: 136-143.
- Terzi R, Kadioğlu R, Kalaycıoğlu E, Sağlam A (2014). Hydrogen peroxide pretreatment induces osmotic stress tolerance by influencing osmolyte and abscisic acid levels in maize leaves. *J Plant Interact* 9: 559-565.
- Terzi R, Kalaycıoğlu E, Demiralay M, Sağlam A, Kadioğlu A (2015). Exogenous ascorbic acid mitigates accumulation of abscisic acid, proline and polyamine under osmotic stress in maize leaves. *Acta Physiol Plant* 37: 43.
- Urbanek H, Kuzniak-Gebarowska E, Herka H (1991). Elicitation of defense responses in bean leaves by *Botrytis cinerea* polygalacturonase. *Acta Physiol Plant* 13: 43-50.
- Velikova V, Yordanov I, Edreva A (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants, protective role of exogenous polyamines. *Plant Sci* 151: 59-66.
- Wang X, Shi G, Xu Q, Hu J (2007). Exogenous polyamines enhance copper tolerance of *Nymphoides peltatum*. *J Plant Physiol* 164: 1062-1070.
- Wi SJ, Kim WT, Park KY (2006). Overexpression of carnation S-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep* 25: 1111-1121.
- Xu L, Han L, Huang B (2011). Antioxidant enzyme activities and gene expression patterns in leaves of Kentucky bluegrass in response to drought and post-drought recovery. *J American Soc Hort Sci* 136: 247-255.
- Yiu JC, Juang LD, Fang DYT, Liu CW, Wu SJ (2009). Exogenous putrescine reduces flooding-induced oxidative damage by increasing the antioxidant properties of Welsh onion. *Sci Hort Amsterdam* 120: 306-314.
- Zhou SZ, Guo K, Elbaz AA, Yang ZM (2009). Salicylic acid alleviates mercury toxicity by preventing oxidative stress in roots of *Medicago sativa*. *Environ Exp Bot* 65: 27-34.