DROUGHT STRESS

Alleviation of Water Deficit Stress Effects by Foliar Application of Ascorbic Acid on *Zea mays* L.

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Keywords
antioxidant enzymes; ascorbic acid; lipid peroxidation; oxidative stress; proline accumulation; *Zea mays* L.

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Abstract

The effects of water-deficit stress and foliar application of ascorbic acid were studied in leaves of *Zea mays* L. (single cross 704). The activity of antioxidant enzymes, superoxide dismutase, peroxidase, catalase and polyphenol oxidase was clearly increased by water-deficit stress. Foliar application of ascorbic acid reduced stress-induced and antioxidative enzymes activities. Proline and malondialdehyde levels were decreased in water-deficit stressed plants by ascorbic acid application. It seems that, ascorbic acid application helps the plants for better resistance under the stress by inactivation and scavenging of free radicals. Chlorophyll content was also decreased by water-deficit stress. The significant decrease of chlorophyll content was obtained in stressed plant than control. Ascorbate was oxidized to dehydroascorbate whereupon total ascorbate was decreased and dehydroascorbate was increased in leaves. The results of the present study indicated that ascorbic acid reduced the harmful effects of reactive oxygen species and improved plant resistance to water stress. In brief, ascorbic acid treatment reduced the damaging action of drought and decreased enzyme activity due to scavenging of reactive oxygen species; thereupon it may be effective for the improvement of stressed plants in arid and semi-arid regions.

Introduction

Water-deficit stress induces numerous biochemical and physiological responses in plants (Pattangual and Madore 1999). A common effect of water-deficit stress, similar to other environmental stresses, is oxidative damage to cells (Smirnoff 1998). Generation of reactive oxygen species (ROS) leads to lipid peroxidation (Chen et al. 2000); protein degradation (Jiang and Zhang 2001) and nucleic acid damage (Hagar et al. 1996). Under conditions of drought and other environmental stresses, ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals are generated (Zhu 2000). To prevent or mitigate oxidative damage from ROS, plant cells possess a non-enzymatic antioxidant system that includes ascorbate, gluthatione, tocopherols, carotenoids and flavonoids, and an enzymatic antioxidant system that includes superoxide dismutase (SOD, EC; 1.15.1.1), catalase (CAT, EC; 1.11.1.6), peroxidase (POX, EC; 1.11.1.7) and polyphenol oxidase (PPO, EC; 1.10.3.1) (Agarwal and Pandey 2004).

The ascorbic acid (AsA) is a small, water-soluble molecule, which acts as a primary substrate in the cyclic pathway for detoxification and neutralization of superoxide radicals and singlet oxygen (Noctor and Foyer 1998, Smirnoff 2005). Many oxidants, such as the hydroxyl radical, contain an unpaired electron and thus are highly reactive and damaging to plants at the molecular level. Ascorbate has a central role in photosynthesis, as its high concentration in chloroplasts implies. Ascorbate functions co-ordinately with glutathione and several enzymatic antioxidants to inactivate superoxide, which is produced by the Mehler reaction and photorespiration (Noctor and Foyer 1998). Ascorbate is also believed to detoxify singlet oxygen and hydroxyl radicals (Asada 1999, Chen and Gallie 2004). It has also been reported that exogenous

ascorbate can increase resistance to salt stress and reduce the level of oxidative stress (Shalata and Neumann 2001).

The primary scavenger is SOD, which converts $O_2^{\bullet}$, to H_2O_2, which is eliminated by ascorbate peroxidase in association with dehydroascorbate reductase and glutathione reductase (Asada 1994). Peroxidase plays an important role in eliminating H_2O_2, by utilizing ascorbate as its specific electron donor to reduce H_2O_2 to water, with the concomitant generation of monodehydroascorbate. H_2O_2 is also scavenged by CAT (Comba et al. 1998), which is a common enzyme in living organisms and catalyses the decomposition of hydrogen peroxide to water and oxygen. The polyphenol oxidase enzyme catalyses the *o*-hydroxylation of monophenols to *o*-diphenols. Changes in activities of various antioxidant enzymes under environmental stresses have been reported (Hernandez et al. 2000).

The objective of this work was to investigate the effects of exogenous application of AsA on some physiological response of maize plants to water-deficit stress. We investigate the protein and chlorophyll content, lipid peroxidation, proline accumulation, ascorbate and dehydroascorbate content, and activities of antioxidant enzymes in maize leaves under water deficit stress with and with out AsA application. We assumed that AsA could prevent cell damages by neutralize ROS.

Materials and Methods

The experiment was conducted in a glasshouse at Faculty of Agriculture, Tarbiat Modares University (51°8′E and 35°43′N), Tehran, Iran during the spring and summer of 2007. The seeds of maize (*Zea mays* L. Single cross 704) were grown in plastic pots (22-l volume) that contained clay–loam soil (35 % Clay, 35 % Silt and 30 % Sand) in a glasshouse under conditions of 25/20 °C day/night temperature and photon flux density of 250 μmol m^{-2} s^{-1}. Five seeds were planted in each pot and after full germination; the number of plants was reduced to two seedlings per pot. The plants were irrigated at field capacity level.

Experimental design

The experimental design was a randomized complete block arrangement in $3 \times 2 \times 4$ factorial with three replications. There was a control group (S0) and two stress treatment groups (Sb, no irrigation during vegetative growth and Sa, no irrigation during flowering). Two time foliar applications of AsA (Vb, before flowering; and Va, after flowering) and four concentrations of AsA (0, 50, 100 and 150 ppm) were applied. In sum, there were 24 treatments in this experiment.

Water-deficit stress and ascorbic acid treatments

Water-deficit stress was created by stopping irrigation at the vegetative (V7; during the V7 growth stages the rapid growth phase and kernel row determination begins) and reproductive (R1; this stage begins when any silk is visible outside the husk) stages of growth. Plants were exposed to water-deficit stress for 8 days (at the end of stress, soil water potential was −1.3 MPa), which was followed by foliar application of AsA solution at end of water stress period. Foliar application was performed by backpack sprayer. The control plots were treated by distilled water. Time domain reflectometry was used to estimate soil moisture in all pots (TDR, FM-Trime-IMKO-Gmbh, Ettlingen, Germany). Tween 20 (1 drop per litre) was added to AsA solution to encourage adhesion to the leaves and the solution.

Sampling

One day after foliar application of AsA, leaves were sampled. After washing, leaves were frozen in liquid N_2 and stored at −80 °C until biochemical analysis.

Extract preparation

Frozen leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml ice-cold extraction buffer (25 mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 18 000 ***g*** for 30 min at 4 °C, and then the supernatant was passed through filter paper. The supernatant was used for the determination of enzyme activity and protein content as a crude extract. All procedures were carried out at 4 °C.

Determination of the activities of antioxidant enzymes in crude extracts

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 μl crude extract, 500 μl 10 mM H_2O_2 and 1400 μl 25 mM sodium phosphate buffer. The decrease in the absorbance recorded at 240 nm for 1 min by a spectrophotometer (Cintra GBC, Dandenong, Victoria, Australia). Catalase activity of the extract was expressed as ΔA mg^{-1} protein min^{-1}.

Superoxide dismutase activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 100 μl 1 μM riboflavin, 100 μl 12 mM L-methionine, 100 μl 0.1 mM EDTA (pH 7.8), 100 μl 50 mM Na_2CO_3 (pH 10.2), 100 μl 75 μM NBT in 2300 μl 25 mM sodium phosphate buffer (pH 6.8) and 200 μl crude enzyme extract, in a final volume of 3 ml. Glass test tubes that contained the reaction mixture were illuminated with a fluorescent lamp

(120 W), and identical tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme which caused 50 % inhibition of photochemical reduction of NBT. Superoxide dismutase activity of the extract was expressed as $\Delta A\ mg^{-1}$ protein min^{-1}.

Peroxidase and PPO activity were estimated by the method of Ghanati et al. (2002). Peroxidase enzyme activity was determined by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance at 470 nm was recorded in a spectrophotometer for 1 min. The reaction mixture contained 100 μl crude extract, 500 μl 5 mM H_2O_2, 500 μl 28 mM guaiacol and 1900 μl 60 mM potassium phosphate buffer (pH 6.1). POX activity of the extract was expressed as $\Delta A\ mg^{-1}$ protein min^{-1}.

Polyphenol oxidase enzyme activity was determined by the oxidation of catechol in the presence of H_2O_2. The increase in absorbance at 410 nm was recorded in a spectrophotometer for 1 min. The reaction mixture contained 100 μl crude enzyme, 500 μl 5 mM H_2O_2, 500 μl 0.02 M catechol and 1900 μl 100 mM sodium phosphate buffer (pH 6.5). The activity of PPO enzymes of the extract was expressed as $\Delta A\ mg^{-1}$ protein min^{-1}. The activity of each enzyme was expressed on protein basis. Protein concentration of the crude extract was measured by the method of Bradford (1976).

Determination of protein content in crude extracts

The protein content of the crude extract was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976). One millilitre of Bradford solution was added to 100 μl crude extract and absorbance recorded at 595 nm for estimate of total protein content. The protein concentration was calculated from a BSA standard curve.

Determination of malondialdehyde in crude extract

The level of membrane damage was determined by measuring the amount of malondialdehyde which is the end product of lipid peroxidation according to De Vos et al. (1991) method. In brief, samples were homogenized in 10 % trichloroacetic acid (w/v) and aliquots of the filtrates were heated (100 °C for 30 min) in 0.25 % thiobarbituric acid. The amount of MDA in the samples was determined from the absorbance at 532 nm, followed by correction for non-specific absorbance at 600 nm in a spectrophotometer. Concentration of MDA determined by extinction coefficient MDA (ε = 155 $\mu M\ cm^{-1}$).

Determination of proline content in crude extract

Proline content of leaves was determined according to a modification of the method of Bates et al. (1973). Samples of leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3 % w/v), and then centrifuged at 18 000 ***g*** for 15 min. Two millilitres of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 h at 100 °C and then allowed to cool to room temperature. Four millilitres of toluene was then added to the tubes and then mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min, to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve, and was expressed as mmol g^{-1} fresh weight.

Determination of chlorophyll content in leaf extract

Chlorophyll was extracted in 80 % acetone from the leaf samples according to the method of Arnon (1949). Extracts were filtrated and content of total chlorophyll was determined by spectrophotometry at 645 and 663 nm, respectively. The content of chlorophyll was expressed as mg g^{-1} fresh weight according to Equation 1 (Arnon 1949).

$$\text{Total chlorophyll} = [20.2(D645) + 8.02(D663)] \times \frac{V}{1000W} \quad (1)$$

Determination of ascorbate and dehydroascorbate content in leaves

Leaves oxidized ascorbate (dehydroascorbate) content was determined according to a modification of the method of Law et al. (1983). Frozen leaves (0.2 g) were homogenized in 3 ml 5 % (w/v) sulphosalicylic acid. After centrifugation, the supernatant was adjusted to pH 5.5–6.5 by adding 800 μl 150 mM phosphate buffer (pH 6.4) and 33 μl 5 M NaOH. Aliquots of samples were then sequentially mixed with 100 μl water, 200 μl 10 % (w/v) trichloroacetic acid, 200 μl 44 % (v/v) H_3PO_4, 200 μl 4 % (w/v) bipyridyl (dissolved in 70 % ethanol) and 100 μl 3 % (w/v) $FeCl_3$. After 60 min incubation at 30 °C, the absorbance of the solution was measured at 525 nm in a spectrophotometer. For measuring the total ascorbate content, the oxidized fraction was reduced by adding 50 μl 10 mM dithiothreitol (DTT). The solution was incubated for 15 min at room temperature, and the surplus DTT was subsequently inactivated by adding 50 μl 0.5 % (w/v) *N*-ethylmaleimide. The concentration of total ascorbate and oxidized ascorbate was determined by using a calibration curve of their respective standards.

Statistical analysis of the data

All data were analysed from analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute 2002). The assumptions of variance analysis were tested by insuring that the residuals were random, homogenous, with a normal distribution about a mean of zero. Duncan's multiple range tests was used to measure statistical differences between treatment methods and controls.

Results and Discussion

The results demonstrated that CAT activity increased in response to water-deficit stress particularly after flowering. Catalase activity was higher in control plants than in those exposed to different AsA concentration (Fig. 1). The lowest levels of CAT activity were in the S0 group treated with 100-ppm AsA at vegetative phase and in Sb group treated with 150-ppm AsA. Foliar application of AsA at vegetative phase significantly decreased CAT activity in stressed plants, except of 50-ppm that did not show any significant different compared to control treatment. In addition, exogenous AsA application at reproductive phase decreased CAT activity in Sb group compared to control. Similar results observed for Sa group under AsA application at vegetative phase (Vb). Superoxide dismutase activity was increased in the Sa group without AsA application when compared to S0 group (Fig. 2). In non-stressed plants, the application of 150-ppm AsA at vegetative and reproductive phases resulted in lower SOD activity than in the control (none-stressed and without foliar application). Superoxide dismutase activity in the leaves of water-deficit stressed plants at vegetative phase treated with 100-and 150 ppm ascorbate was lower than that of controls (without foliar application). Applying of 150-ppm AsA at reproductive stage of water stressed plants, decreased the activity of SOD to the level of control plants. In Sb group, PPO activity was decreased by application of 150-ppm AsA solution compared to control without foliar application. In Sb group usage of 50, 100 and 150-ppm AsA decreased PPO activity in plants compared to the control (Fig. 3). In Sa group foliar application at reproductive phase decreased PPO activity. The greatest peroxidase activity was found in the stressed-Sa group that was not treated with AsA. When 150-ppm AsA applied at reproductive phase, POX activity significantly decreased (Fig. 4). The lowest levels of POX activity were found in the unstressed plants.

It has been demonstrated that water-deficit stress induces oxidative stress in plant tissues. Exposes chloroplasts to excessive excitation energy, may increase generation of ROS and induce oxidative stress (Gossett et al. 1994b). Similar results have been obtained when sorghum plants are subjected to water-deficit stress (Zhang and

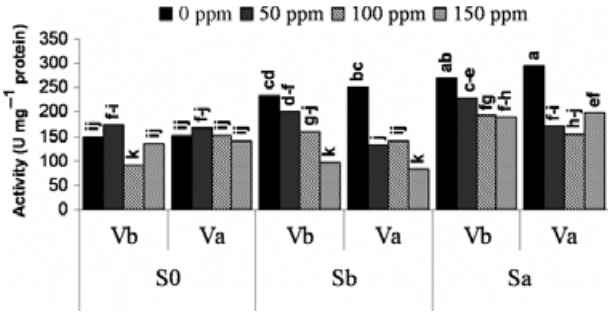

Fig. 1 Changes in catalase activity due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

Kirkham 1996). To overcome the effects of oxidative stress, plants make use of a complex antioxidant system. Relatively higher activities of ROS scavenger enzymes have been reported in many stressed plants, which suggest that the antioxidant system plays an important role in plants against environmental stresses (Henrique et al. 2005).

Superoxide dismutase may function as a ROS scavenger, by converting $O_2^{\bullet-}$ to H_2O_2 (Alscher et al. 2002). Hydrogen peroxide is converted to oxygen and water by CAT and POX, which use ascorbate as a hydrogen donor (Hegedus et al. 2001). Recent studies have demonstrated that over-expression of mitochondrial SOD in transgenic *Arabidopsis thaliana* (Wang et al. 2004) and chloroplastic Cu/Zn SOD in transgenic *Nicotiana tabacum* (Badawi et al. 2004) can lead to enhanced stress tolerance. Similar results have been found in *Triticum aestivum* (Sairam and Srivastava 2001) and *Lycopersicon* spp. (Mittova et al.

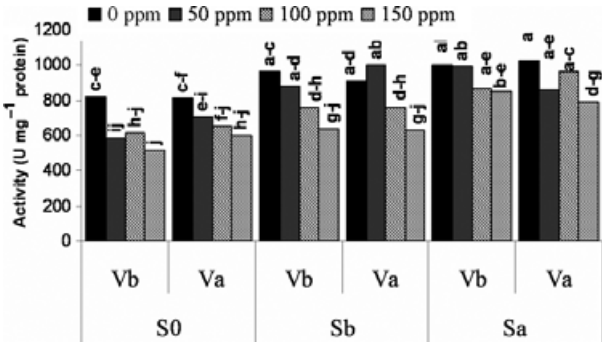

Fig. 2 Changes in superoxide dismutase activity due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

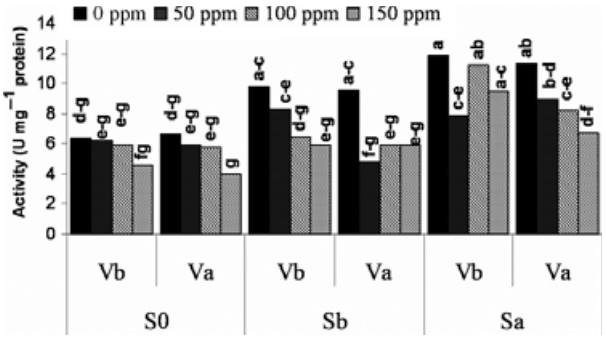

Fig. 3 Changes in polyphenol oxidase activity due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

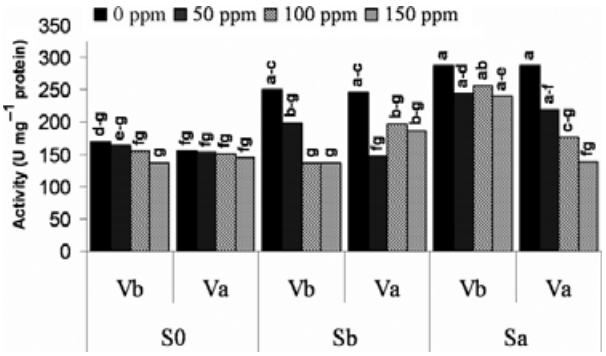

Fig. 4 Changes in peroxidase activity due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

2002). Even though high-SOD activity protects plants against superoxide radicals, it cannot be considered solely responsible for membrane protection against peroxidation. However, when the plants were subjected to water-deficit stress (Sa and Sb group), the increase in CAT activity was higher than that in control plants (S0 group). Our results showed that leaf CAT activity increased with water-deficit stress, and that this increase was more conspicuous after flowering. In addition, activities of CAT, POX and PPO enzymes were also affected by water-deficit stress. The same results have been found for other plant species (Mittova et al. 2002). Increase of antioxidant enzymes activity due to salt stress has been reported (Dolatabadian et al. 2008).

AsA is a key component of the ascorbate–glutathione cycle (Noctor and Foyer 1998) and plays a protective role against ROS (Noctor and Foyer 1998). Ascorbate acts as an antioxidant by being available for energetically favourable oxidation. Many oxidants, such as the hydroxyl radical, contain an unpaired electron and thus are highly reactive and damaging to plants at the molecular level. This is due to their interaction with nucleic acids, proteins and lipids. Although SOD is not the only H_2O_2-producing enzyme in plant tissues, the balance between the activity of this enzyme and that of other the H_2O_2-scavenging enzymes in cells is considered crucial for determining the levels of $O_2^{\bullet}$ and H_2O_2 in cells (Badawi et al. 2004).

We observed that leaf activity of CAT, SOD, PPO and POX increased with water-deficit stress in all treatment groups (Sa and Sb). However, when AsA was applied to plants, the activity of enzymes was decreased. This view is further supported by the arguments that major detoxification of ROS produced during photosynthesis is mediated by CAT, POX and SOD and by reductive processes involving the major redox buffers of plant cells such as ascorbate and glutathione (Foyer and Noctor 2003). Furthermore, such a mechanism may be important in certain abiotic stresses which decrease stomatal conductance such as drought, salinity, high temperature, etc. (Foyer and Noctor 2003). However, the highest enzyme activity was seen in plants after flowering. Antioxidant enzymes play an important role in providing a plant defence mechanism against water-deficit-stress-induced oxidative damage. Therefore, induction of this antioxidative defence mechanism may be assumed to reflect the plant response required to overcome oxidative injury induced by environmental stress. These findings about the antioxidant enzymes activity suggest that an accumulation of ROS might have occurred in response to water stress that was reduced by enhanced antioxidant enzymes activity and endogenous AsA level due to the application of AsA.

The protein content of water deficit stressed plants that were not treated with AsA was low, particularly at after flowering stress (Fig. 5). The application of AsA (100 and 150 ppm) increased the protein content in the leaves of stressed. The reduction of protein content in the leaves of the stressed plants was probably due to injurious effects of ROS (Noctor and Foyer 1998) because the exogenous AsA may prevent the protein degradation.

The MDA content was higher in water-deficit stressed plants than in non-stressed plants (Fig. 6). Application of AsA reduced MDA levels in Sb and Sa groups. We found that water-deficit stress-induced progressive accumulation of MDA in the leaves. Malondialdehyde is a decomposition product of the polyunsaturated fatty acids of biological membranes (Gossett et al. 1994a). Cell membrane stability has been widely used to differentiate stress-tolerant and -susceptible cultivars of some crops (Blum and Ebercon 1981). However, lipid peroxidation is not

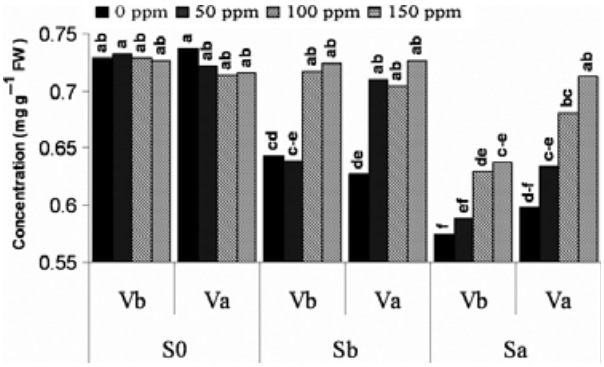

Fig. 5 Changes in protein content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

the only cause of oxidative stress damage, because ROS may also damage macromolecules, such as DNA and proteins (Pastori and Foyer 2002). Increase of MDA content has been reported by Sairam and Saxena (2000) in wheat plants under water-stress condition.

The total chlorophyll content was increased when stressed plants in the vegetative phase were treated with AsA after flowering. The total chlorophyll content was lower in stressed plants than in the non-stressed plants (Fig. 7). In plants, symptoms of oxidative stress include loss of chlorophyll and protein and a decline in membrane permeability, all of which lead to progressive reduction in photosynthetic capacity. Water-deficit stress leads to an increase in ROS in chloroplasts and destruction of chlorophyll molecules. Singlet oxygen molecules and $O_2^{\bullet-}$ radicals predominantly attack double-bond-containing compounds, thus damaging the chloroplast membrane system and photosynthetic reaction centres (Zhang et al. 2003). AsA is a detoxifier and neutralizer of superoxide radicals, it can enhance leaf chlorophyll content.

The proline content in the Sa treatment group that was not treated with AsA was high (Fig. 8). In S0 plants, application of AsA did not have any effects on the proline content in their leaves. At vegetative phase applying of 100 and 150-ppm AsA on stressed plants decreased proline accumulation compared to control treatment (without AsA application). In addition, foliar application of 100 and 150-ppm AsA at before and after flowering on Sa group plants decreased proline accumulation. It is well established that the concentration of proline, which makes up to 80 % of the total amino acid pool, increases up to 100 times the normal level in many plants when they are stressed. The accumulation and protective effect of proline has been observed in many higher plants. The function of proline in stressed plants is often explained by its property as an osmolyte (Saradhi et al. 1995). Other possible positive roles of proline under stress have been proposed and include the stabilization of proteins (Anjum et al. 2000), the scavenging of hydroxyl radicals (Smirnoff and Cumbes 1989) and the regulation of cytosolic pH (Venekamp 1989). In the current study, we found that water-deficit stress increased the leaf proline content, which might have contributed to osmotic adjustment and allowed the plant to maintain turgor pressure and adapt to limited water availability. Application of AsA scavenged ROS and prevented biosynthesis of extra proline.

Ascorbate assay showed that maximum ascorbate content was related to S0 treatments with application of 100 and 150-ppm AsA in both of growth stages of plants

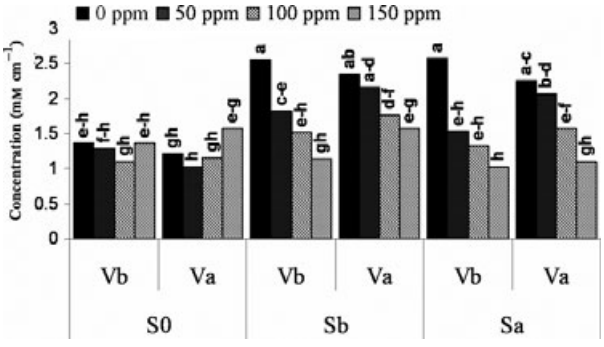

Fig. 6 Changes in malondialdehyde content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

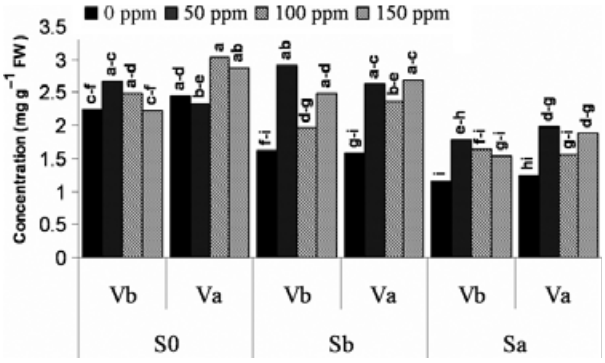

Fig. 7 Changes in total chlorophyll content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

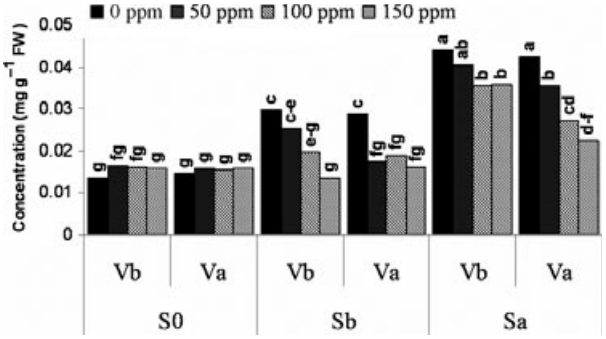

Fig. 8 Changes in proline content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

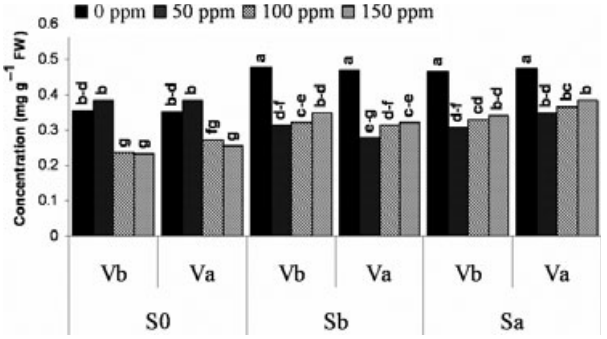

Fig. 10 Changes total dehydroascorbate content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

(Fig. 9). The ascorbate content in these plants which were not treated with AsA had the lowest amount of ascorbate in the leaves compared to all other plants. In general, foliar application of AsA increased the ascorbate content in the leaves of all plants whether they were treated in the vegetative or reproductive stages. Although the dehydroascorbate content was raised in the water-deficit stressed plants that were not treated with AsA, water-deficit stressed plants that had been treated with AsA had lower levels of dehydroascorbate in their leaves (Fig. 10). The lowest levels of dehydroascorbate were found in the unstressed plants that were treated with 100- and 150-ppm AsA. Water-deficit stressed plants that were treated with AsA at both of phases had less dehydroascorbate levels in their leaves than the plants that were not treated with AsA.

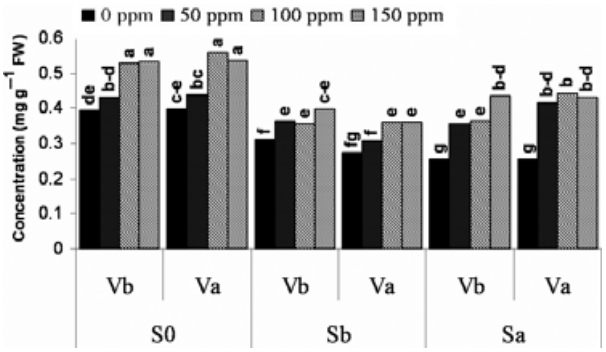

Fig. 9 Changes in ascorbate content due to water-deficit stress and foliar application of ascorbic acid. S0, no water-deficit stress; Sb, water-deficit stress during vegetative growth; Sa, water-deficit stress during flowering; Vb, ascorbic acid application before flowering; Va, ascorbic acid after flowering. All the values followed by the same letter are not statistically different at the $P < 0.05$ probability level.

In this work, the ascorbate pool decreased because of water-deficit stress, whereas, dehydroascorbate increased, which suggests that the ascorbate pool can be reduced by oxidative stress when the capacity of the regenerative system is exceeded. ROS oxidize ascorbate to monodehydroascorbate firstly and then to dehydroascorbate (Noctor and Foyer 1998).

Our results showed that AsA was involved in the physiological response to water-deficit stress, by changes in activity of antioxidant enzymes, such as CAT, SOD, PPO and POX. We conclude that AsA directly decreases the effects of water stress, prevents increased antioxidant enzyme activity during stress, and increases drought tolerance.

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