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Biochemical changes of common bean (*Phaseolus vulgaris* L.) to pretreatment with salicylic acid (SA) under water stress conditions

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Abstract

Salicylic acid (SA) is one of the important signal molecules which modulating plant responses to environmental stresses including drought. An experiment was therefore, conducted to evaluation the effect of exogenous SA on the lipid peroxidation, antioxidant enzymes activities and proline content of common bean under water stress conditions during 2011 in Iran. Results showed that drought increased membrane lipid peroxidation via increase of malondialdehyde (MDA) content as well as some antioxidant enzymes activities such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and proline level. Nonetheless, seeds soaking in SA (especially 0.5 mM) alleviated drought injuries by way of decrease of lipid peroxidation through reduce of MDA content and further increase in antioxidant enzymes activities especially SOD and proline level. Results signify that exogenous SA could help reduce the adverse effects of drought stress and might have a key role in common bean tolerance to drought by decreasing oxidative damage via further activities of antioxidant enzymes and more proline accumulation.

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Introduction

Common bean (*Phaseolus vulga*ris L.) is the most important food legume; however, drought stress results in significant seed yield reductions in 60% of global bean production areas (White *et al.,* 1994). A common effect of drought stress is oxidative damage. Exposure to drought results in a loss of balance between the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot-}$) and singlet oxygen ($^1O_2$) and their scavenging (Smirnoff, 1998). If not effectively and rapidly removed from plants excessive levels of ROS can damage a wide range of cellular macromolecules such as lipids, proteins and DNA and ultimately cause cell death. To protect the subcellular components from ROS accumulation, plants respond with an induction of enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and gluthatione reductase (GR), as well as antioxidant metabolites such as glutathione and ascorbate (Asada, 1999). Lipid peroxidation which leads to impairment of membrane functions is the system most easily ascribed to oxidative damage and also most frequently measured (Sairam *et al.,* 2002). One of the potentially important mechanisms of drought tolerance is osmotic adjustment, which can be achieved from the accumulation of compatible solutes. Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. Generally, they protect plants from stress through different courses, including contribution to cellular osmotic adjustment, detoxification of ROS, protection of membrane integrity and stabilization of enzymes/proteins (Yancey *et al.,* 1982; Bohnert and Jensen, 1996). Furthermore, because some of these solutes also protect cellular components from dehydration injury, they are commonly referred to as osmoprotectants. These solutes include proline, sucrose, polyols, trehalose, glycinebetaine, alaninebetaine, prolinebetaine, hydroxyprolinebetaine and pipecolatebetaine (Rhodes and Hanson, 1993).

Under severe stress conditions the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative damage. Therefore, synthesis of signal molecules in plants is an important step in understanding plant responses to environmental stresses. Salicylic acid (SA) acts as an endogenous signal molecule responsible for inducing abiotic stresses tolerance in plants. SA has been shown to regulate a large variety of physiological processes in plants (Saruhan *et al.,* 2011). Recent studies have demonstrated the major role of SA in modulating plant responses to abiotic stresses such as drought, salt, chilling, heat, ultraviolet, heavy metals (Senaratna *et al.,* 2000; Singh and Usha, 2003; Yusuf *et al.,* 2008; Yalpani *et al.,* 1994; Krantev *et al.,* 2008) and disease resistance (Raskin, 1992). Hayat *et al.,* (2008) studied the growth of water stressed tomato plants in response to exogenously applied salicylic acid. The results of their experiments revealed a significant decline in photosynthetic parameters, membrane stability index, leaf water potential, activities of the enzymes nitrate reductase and carbonic anhydrase, chlorophyll and relative water contents with a concomitant increase in proline content and the activities of antioxidant enzymes (CAT, POX and SOD). However, the treatment of these stressed plants with lower concentrations of salicylic acid significantly enhanced the aforesaid parameters thereby improved tolerance of the plants to drought stress.

Various chemicals such as osmoprotectants, growth regulators and stress signaling molecules are being successfully used to induce the tolerance against several biotic and abiotic stresses (Farooq *et al.,* 2010). It is hypothesized that exogenous SA application can diminish the harmful effects of drought stress and oxidative injury with the increase activities of antioxidant enzymes and proline accumulation. The present study therefore was carried out to investigate

the impact of different doses of exogenous SA to induce drought tolerance in common bean.

Materials and methods

This study was done in agricultural research farm of the Islamic Azad University, Shahre-Rey Branch (Longitude, latitude and altitude are 51° 28´ E, 35° 35´ N, and 1000 m, respectively) in south of Tehran, Iran during 2011. This region is located in an arid climate where the summer is hot and dry and the winter is cool and dry. The mean annual rainfall and temperature are 201.7 mm and 20.4° C. Soil texture of research farm was sandy clay loam with pH of 7.8, nitrogen 0.091%, phosphorus 9.1 ppm, potassium 350 ppm and EC of 2.8. The experiment was laid out in a split plot on the basis of randomized complete block design with four replications. The main plots included two irrigation regimes (I0: irrigation after 50 mm evaporation from class A pan and I1: irrigation after 100 mm evaporation from class A pan, as control and water stress conditions, respectively) and the sub plots included five concentrations of salicylic acid (0, 0.25, 0.5, 0.75 and 1 mM). Common bean seeds (cv. Derakhshan) were soaked for 6 h in salicylic acid solutions. Seeds before treatment were sterilized with sodium hypochlorite solution (1%) for 5 min and then washed thoroughly with distilled water. Each sub plot had four planting rows with length of 5 m. Distances on and between rows were 10 and 50 cm respectively. Seeds were treated with Bavistin and then were sown by hand on June 12, 2011 in 4 cm depth of soil. At the same time plots were fertilized with 100 kg ha^{-1} ammonium phosphate. All plots were irrigated immediately after sowing, but subsequent irrigations were carried out according to the treatments. Crop management practices such as hand weeding and thinning were done as required. At the flowering stage, the youngest fully expanded leaf of randomly selected 10 plants per plot were collected and immediately frozen in liquid nitrogen and stored at -80˚C for biochemical analysis.

Assay of malondialdehyde (MDA)

Lipid peroxidation was estimated in terms of MDA content according to the method of Heath and Packer (1968). Leaf samples (1 g) were homogenized in 10 ml of trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 rpm for 5 min. 4 ml (0.5%) of thiobarbituric acid in 20% trichloroacetic acid was added to 1 ml aliquot of the supernatant. Mixture was heated at 95 ˚C for 30 min and then cooled rapidly in an ice bath. After centrifugation at 10,000 rpm for 10 min, the absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 mM^{-1} cm^{-1} and expressed as µmol MDA g^{-1} fresh weight.

Assay of antioxidant enzymes activities

All operations were performed at 4˚ C. For enzyme extractions, leaf samples (0.5 g) were homogenized with 0.05 M sodium phosphate buffer (pH 7.8) containing 1 Mm EDTA·Na_2 and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Homogenates were centrifuged at 14,000 rpm for 40 min at 4˚ C. The supernatants were used for the determination of protein content and activities of SOD, CAT and APX enzymes.

Total soluble protein contents were determined according to Bradford (1976). SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the methods of Beyer and Fridovich (1987). 5 ml reaction mixture containing 5 mM hydroxyethyl piperazine ethane sulfonic acid (HEPES) (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO3 (pH 10.0) 13 mM methionine, 0.025% (v/v) Triton X-100, 63 µmol (NBT) 1.3 µmol riboflavin and an enzyme extract was illuminated for 15 min (360 µmol m^{-2} s^{-1}) and a control set was not illuminated to correct for background absorbance. A unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reaction of NBT at 560 nm. CAT activity was assayed

by monitoring the decomposition of H_2O_2 at 240 nm by the procedure of Aebi (1984). The reaction mixture contained 50 mM phosphate buffer (pH 7.0.), 0.1% (v/v) Triton X-100, 10.5 mM H_2O_2 and 0.05 ml leaf extract. The reaction carried out at 25 ˚C for 3 min was started with the H_2O_2 addition. APX activity was determined according to Nakano and Asada (1981) by the decrease in absorbance of ascorbate at 290 nm. The assay mixture contained phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and enzyme extract. APX activity was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate per min at 25 ˚C.

Assay of proline

For proline estimation following the method of Bates *et al.,* (1973) 0.5 g of dried powdered leaves was homogenized in 10 ml 3% aqueous sulfosalicylic acid and the homogenate filtered. 2 ml acid ninhydrin (prepared by warming 1.2 g of ninhydrin in 30 ml glacial acetic acid) was added to 2 ml filtrate in a digestion tube and placed in a boiling water bath for 90 min. The reaction was terminated in an ice bath. 4 ml toluene was added to the reaction mixture and agitated vigorously for 30 min. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance read at 520 nm. Finally all data were analyzed by MSTAT-C statistical software and the means were compared by Duncan's Multiple Range Test (DMRT) at the 5% probability level.

Results and discussion

Lipid peroxidation

In the present study, malondialdehyde (MDA) level was increased significantly under water stress conditions (48.3% as compared to control) (Fig. 1). Functions of biological membranes are adversely affected by environmental stresses such as drought that can be measured as level of membrane lipid peroxidation. MDA a decomposition product of polyunsaturated fatty acids, has been utilized as a biomarker for lipid peroxidation that may occur in the presence of ROS (Mittler, 2002). In our research, water stress increased membrane lipid peroxidation through increase of MDA level, but SA application (especially 0.5 mM) reduced lipid peroxidation because MDA content significantly decreased. Application of 0.5 mM SA as compared to no application decreased MDA content by 22% and 14% under drought and control conditions, respectively (Fig. 1). Our results supported that the decrease of membrane damage may be related to the induction of antioxidant responses by SA, which protects the cell from oxidative damage. Senaratna *et al.,* (2000) suggested that a similar mechanism was responsible for SA induced multiple stress tolerance in bean and tomato plants. Kadioglu *et al.,* (2011) also reported that SA treatment prevented lipid peroxidation in *Ctenanthe setosa* while the peroxidation increased in control plants. Saruhan *et al.,* (2011) found that lipid peroxidation increased during drought in both cultivars of maize and to a higher degree in the sensitive cultivar but pretreatment with SA almost totally prevented this increase.

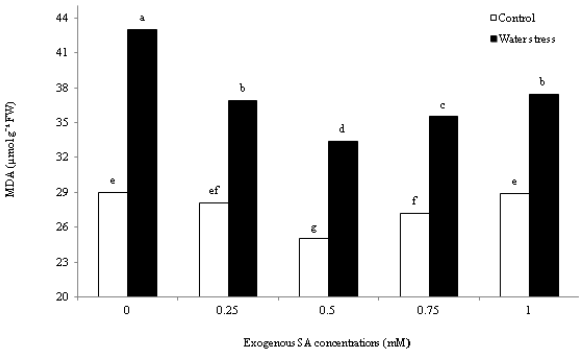

Fig. 1. Effect of seeds soaking in salicylic acid (SA) on malondialdehyde (MDA) content of common bean under water stress and control conditions. Means with the same letter(s) are not significantly different ($P \leq 0.05$).

Antioxidant enzymes activities

We found that drought increased antioxidant enzymes activities. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were

increased 19.5%, 16.4% and 17.9% in comparison with control, respectively (Fig. 2, 3, 4). Drought stress invariably leads to oxidative stress in the plant cell due to higher leakage of electrons towards O_2 during photosynthesis and respiratory processes leading to enhancement in activated oxygen species generation (Stepien and Klobus, 2005). To minimize the affections of oxidative stress, plants have evolved a complex enzymatic and non-enzymatic antioxidant system, such as low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS scavenging enzymes such as SOD, CAT, APX and peroxidase (POX) (Apel and Hirt, 2004). SOD acts as the first line of defense converting $O_2^{\cdot-}$ into H_2O_2. APX, GPX and CAT then detoxify H_2O_2 (Mittler, 2002). We observed that seeds soaking with SA induced further activity of all antioxidant enzymes in the water stress conditions as well as control. The rate of increase was different among the treatments. Maximum antioxidants activities were recorded from 0.5 mM SA. Application of 0.5 mM SA as compared to no application, increased SOD, CAT and APX activities by 50% and 17%, 23% and 13%, 29% and 9% under drought and optimum conditions, respectively (Fig. 2, 3, 4).

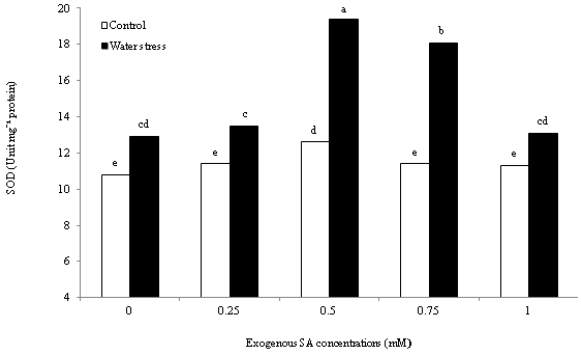

Fig. 2. Effect of seeds soaking in salicylic acid (SA) on activity of superoxide dismutase (SOD) of common bean under water stress and control conditions. Means with the same letter(s) are not significantly different ($P \leq 0.05$).

Antioxidant defense system plays very important roles in tolerance of plants to stressful conditions. Our results showed that exogenous SA application can decrease oxidative damage via the increasing of SOD, CAT and APX activities. Knorzer *et al.,* (1999) showed that when applied exogenously at suitable concentrations, SA enhanced the efficiency of antioxidant system in plants. SA also was found to enhance the activities of antioxidant enzymes, CAT, SOD and POX, when sprayed exogenously to the drought stressed plants of *Lycopersicon esculentum* (Hayat *et al*., 2008) or to the salinity stressed plants of *Brassica juncea* (Yusuf *et al.,* 2008). Panda and Patra (2007) found that the priming of seeds with lower concentrations of SA, before sowing, lowered the elevated levels of ROS due to cadmium exposure and also enhanced the activities of various antioxidant enzymes (CAT, guaiacol peroxidase, glutathione reductase and SOD) in *Oryza sativa*, thereby protecting the plants from oxidative burst. Kadioglu *et al.* (2011) also observed that exogenous SA induced all antioxidant enzyme activities in *Ctenanthe setosa* more than control leaves during the drought. Singh and Usha (2003) revealed that SOD activity in the leaves of water stressed wheat plants was higher than that of water sufficient plants. SA application at 1 and 2 mM induced SOD activity to levels much higher than those for the rest of the treatments under investigation. Krantev *et al.,* (2008) reported the exogenous application of SA enhanced the activities of antioxidant enzymes APX and SOD with a concomitant decline in the activity of CAT in maize plants**.** Further, the treatment with SA resulted in temporary reduction of CAT activity and increased H_2O_2 level (Janda *et al.,* 2003) which possibly played a key role in providing the systemic acquired resistance (SAR) (Chen *et al.,* 1993) and tolerance against the oxidative stress (Gechev *et al.,* 2002) in plants. Farooq *et al.,* (2009) reported that CAT inhibition by SA can't be validated in all plants. In the present study we also observed that, increasing of CAT activity due to SA application was less than the other antioxidant enzymes. It seems that effect of exogenous SA application on CAT activity is related to plant species, kind of stress, stress intensity, SA concentration and application method.

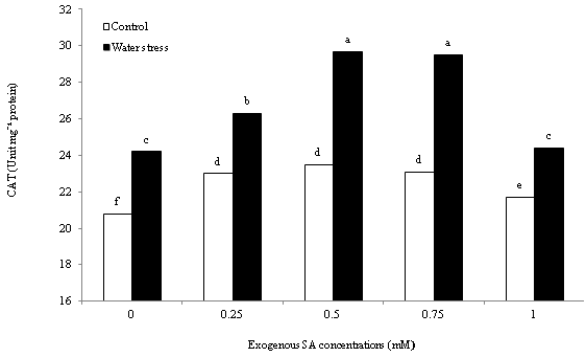

Fig. 3. Effect of seeds soaking in salicylic acid (SA) on activity of catalase (CAT) of common bean under water stress and control conditions. Means with the same letter are not significantly different (P ≤ 0.05).

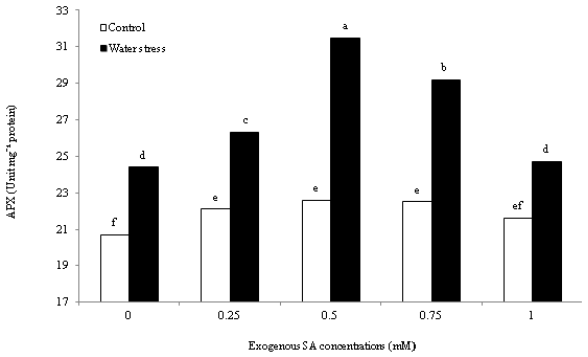

Fig. 4. Effect of seeds soaking in salicylic acid (SA) on activity of ascorbate peroxidase (APX) of common bean under water stress and control conditions. Means with the same letter(s) are not significantly different (P ≤ 0.05).

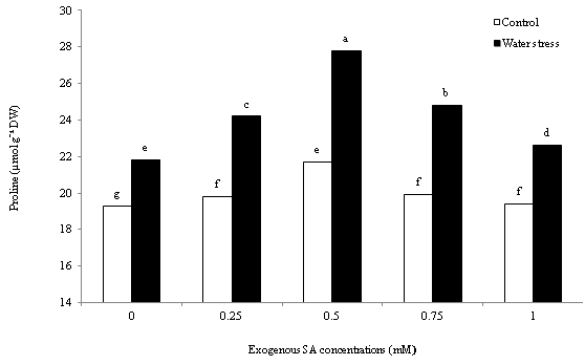

Fig. 5. Effect of seeds soaking in salicylic acid (SA) on proline content of common bean under water stress and control conditions. Means with the same letter are not significantly different (P ≤ 0.05).

Proline content

According to our study leaf free proline content was increased significantly under drought stress by 13% as compared to control (Fig. 5). Plants adapt to water deficit by changes in morphology, altered patterns of development and cellular metabolism. A number of these adaptive responses are associated with the accumulation of osmolytes like sugars and proline (Umebese *et al.,* 2009). Amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses. In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing subcellular structures (e.g. membranes and proteins), scavenging free radicals and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007). On the other hand we observed that SA treatment induced more accumulation of the free proline not only in the water stressed plants but also in the well watered plants. Application of 0.5 mM SA as compared to no application, increased leaf proline content by 28% and 12% under drought and control conditions, respectively (Fig. 5). In this regard, Hussain *et al.,* (2008) also found that drought stress increased the free leaf proline and glycinebetaine (GB) of sunflower and were further increased by exogenous application of GB and SA. Umebese *et al.,* (2009) showed that proline content was only slightly increased at all stages of growth in water stressed tomato and amaranth plants, but 3 mM SA induced a two-fold increase in proline content at the vegetative stage of tomato and significant increases at almost all stages of growth of amaranth. Bandursca and Stroinski (2005) also revealed that SA treatment increased proline level in *Hordeum spontaneum*. These results show that SA treatment may stimulate proline biosynthesis and provide a pool of proline, which has important role in drought tolerance. Proline can thus be considered as an important component in the spectra of SA-induced protective reactions of common bean plants in response to water stress.

Conclusions

The present study indicated that drought stress increased oxidative damage, membrane lipid peroxidation and MDA content as well as antioxidant enzymes (SOD, CAT and APX) activities and proline level in common bean leaves, nevertheless seeds soaking in SA (especially 0.5 mM) induced protection against drought stress via maintenance of membrane integrity by decline in MDA content and more increase in antioxidant enzymes activities (especially SOD) as well as prolin accumulation. Our results showed that although common bean is a sensitive plant to water stress, it was confirmed that exogenous SA application can help to drought tolerance of this crop.

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