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Increased drought tolerance in maize plants induced by H_2O_2 is closely related to an enhanced enzymatic antioxidant system and higher soluble protein and organic solutes contents

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Abstract Drought stress is one of the most important agricultural problems limiting development and growth in plants. Therefore, mechanisms to alleviate drought stress have been one of the major limiting factors in production. H_2O_2 pretreatment has emerged as a method to induce stress acclimation in plants. In this study, the effects of H_2O_2 leaf pretreatment on plant growth, antioxidative enzymes, soluble protein, and organic solute content in maize plants under conditions of drought stress were analyzed. Results demonstrated that drought stress reduced shoot and root mass compared with the control, and H_2O_2 leaf spraying significantly improved the growth of drought-stressed plants. In general, in drought-stressed plants, CAT, APX, GPX, and SOD activities in roots and leaves were increased by H_2O_2 leaf spraying relative to water spraying. GPX was the main H_2O_2-scavenging enzyme in leaves and roots, and CAT activity was not detected in the leaves of maize plants. Increased organic solute contents (proteins, carbohydrates, soluble proline, and amino acids) were found in the leaves and mainly in the roots of H_2O_2-stressed plants relative to water-stressed plants. In conclusion, it was found that H_2O_2 leaf spraying pretreatment reduced the deleterious effects of drought stress on maize plant growth. This treatment proved to be a beneficial health strategy in plants. This effect could be attributed to the ability of H_2O_2 to induce antioxidant defense system activity, particularly GPX, and to increase organic solute (protein, carbohydrate, proline, and free amino acid) content in roots and leaves.

Keywords Antioxidative enzymes · Drought stress · Hydrogen peroxide · ROS · *Zea mays*

Abbreviations

ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
GPX	Guaiacol peroxidase
APX	Ascorbate peroxidase
H_2O_2	Hydrogen peroxide
FM	Fresh mass
NBT	Nitrobluetetrazolium
SDM	Shoot dry mass
RDM	Root dry mass
TDM	Total dry mass

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1 Introduction

Drought stress is one of the most important agricultural problems limiting development and growth in plants. Water is an important liquid compound which, when scarce during agricultural development, can result in decreased crop yields, especially in arid and semiarid regions of the world (Wang et al. 2003). Moreover, regions with adequate, but nonuniform precipitation also challenge plants with limited water (Lisar et al. 2012). Thus, plants deal with drought stress either when the water supply becomes limiting to their roots or when the transpiration rate becomes intense. Drought stress is mainly caused by a water deficit such as drought or high soil salinity. Hence, mechanisms to alleviate drought stress have been one of the major production-limiting factors. Therefore, the ability of plants to withstand such stress is of immense economic importance (Lisar et al. 2012).

Additionally, drought stress in plants leads to the overproduction of reactive oxygen species (ROS), which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates, and DNA, possibly resulting in oxidative stress. ROS are free radicals ($\cdot O_2-$ superoxide radicals; ·OH hydroxyl radical; $\cdot HO_2$ perhydroxyl radical, and ·RO, alkoxy radicals) or nonradicals (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) (Gill and Tuteja 2010). The harmful effects of ROS in plants can be reduced or eliminated by nonenzymatic and enzymatic defense systems (Azevedo Neto et al. 2008). The nonenzymatic system includes hydrophilic and lipophilic compounds. The enzymatic defense system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and the enzymes of the ascorbate and glutathione cycle: glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase (Azevedo Neto et al. 2008; Munns and Tester 2008).

Plants accumulate compatible solutes, such as proline, carbohydrates and amino acids in response to drought and salinity to facilitate water uptake (Ashraf and Foolad 2013a, b). These osmolytes were suggested to be important for protecting cells against increases in ROS accumulation under stress conditions (Miller et al. 2010). High contents of protective solutes such as proline and soluble sugars in the leaf are a unique plant response to environmental stresses, specifically to drought stress (Sakamoto and Murata 2002). Proline may act as an antioxidant (Heuer and Nadler 1998) and along with soluble sugars (Yuanyuan et al. 2009) act as an osmoprotectants during stress (Ahmed et al. 2010). Organic solutes not only contribute to osmoregulation in stressed plants, but also provide protection to many enzymes, which are active in the cytosol (Ashraf and Foolad 2013a, b). Moreover, soluble sugars are also involved in the metabolism and protection of both ROS-producing and ROS-scavenging pathways, such as mitochondrial respiration, photosynthesis, and the oxidative pentose phosphate pathway (Couée et al. 2006).

Maize is a cereal produced on almost every continent, and its economic importance is characterized by various forms of use, ranging from animal feed to the high-tech industry as well as the production of films and biodegradable packaging (Paes 2006). Acclimation is a process by which previous exposure of an individual to a particular type of stress causes metabolic changes, which are responsible for its increased tolerance to a new exposure to stress (Neill et al. 2002; Petrov and Van Breusegem 2012). Among the processes of acclimation to stress, the pretreatment of plants with small amounts of H_2O_2 (hydrogen peroxide) by spraying or in nutrient solution induced acclimation of plants to salt stress (Uchida et al. 2002; Azevedo Neto et al. 2005; Gondim et al. 2012).

There is no information on whether the pretreatment of maize plants by H_2O_2 leaf spraying is able to induce acclimation of plants to drought stress. It is believed that the use of drought-tolerant genotypes or the use of strategies, such as H_2O_2 leaf spraying, to improve production in areas under drought challenge could be an effective option for reducing the risks of grain production in drought areas.

H_2O_2 can act as a signal molecule in regulation of plant growth, morphogenesis and development and has also been considered an essential molecule of signal transduction in abiotic stresses (Sofo et al. 2015). For this purpose, we tested the hypothesis that H_2O_2 leaf spraying changes the H_2O_2 level and can act as a signal molecule. This fact may impact metabolism (enhance in soluble protein, organic solute contents and antioxidantive enzyme activities) in favor of plant growth and development and inducing drought tolerance in maize plants. Therefore, this study investigated the enzymatic defense system, soluble protein contents and organic solute contents and their relationship with plant growth.

2 Materials and methods

2.1 Plant material and experimental conditions

The experiment was conducted in a greenhouse at Instituto Federal de Educação Ciência e Tecnologia do Ceará—IFCE, Maracanaú, Ceará, Brazil. Maize seeds (*Zea mays* L.), cultivar AG1051 were cleaned with sodium hypochlorite solution (0.7 %) and sown in plastic pots (6 L) containing vermiculite and earthworm castings in a proportion of 1:1, and subjected to field capacity daily watering in a greenhouse. After 6 days of sowing, seedlings were sprayed with distilled water (control) or 15 mM H_2O_2 aqueous solution, which also contained the detergent Tween 20 at 0.025 %, in order to break the surface tension and facilitate penetration. Preliminary experiments using different H_2O_2 concentrations and volumes were carried out in order to find optimal conditions. Then, it was observed that the better conditions were: plants sprayed with 15 ml H_2O_2 or distilled water per plant at 6:00 am and again after 24 h. After spraying, half of the seedlings were subjected to watering suspension.

Four treatments were applied to the plants: 1. Plants were sprayed with distilled water and irrigated daily (water/control); 2. Plants were sprayed with a solution of H_2O_2 and irrigated daily (H_2O_2/control); 3. Plants were sprayed with distilled water and not irrigated for 8 days (water/drought stressed); and 4. Plants were sprayed with a solution of H_2O_2 and not irrigated for 8 days (H_2O_2/drought stressed).

After withholding of water for 8 days, the plants were harvested and separated in two groups: the first was used for the determination of fresh and dry mass and the second, for biochemical analysis. Initially, the fresh mass of shoots (leaves+stems) and roots was determined. Afterwards, the material was placed in an oven at 60 °C for 3 days to determine the dry mass of shoots (leaves+stems) and roots.

To carry out biochemical analysis (enzymatic activities and organic solute contents), the fully expanded leaf (counting from the apex) and the final third of the root end of the first group of plants were used. This material was frozen in liquid nitrogen and stored at −25 °C until use.

The experimental design was completely randomized in a 2 × 2 factorial design (sprayed with distilled water or H_2O_2 and irrigated or non-irrigated) with five replications. Data were subjected to analysis of variance (ANOVA) and means were compared by Tukey's test ($P \leq 0.05$). The graphics were created using the Sigma Plot 11.0 program.

2.2 Extract preparation and enzyme assays

For the determination of antioxidative enzyme activities and the contents of soluble proteins, soluble carbohydrates, proline, and free amino acids, extracts of leaves and roots were obtained by maceration in a mortar of 1 g of fresh leaf or root in 4.0 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The macerate was filtered through a muslin cloth and centrifuged at 12,000×*g* for 15 min. The supernatant was used for analysis. All operations were performed at 4 °C.

The activities of catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), and superoxide dismutase (SOD, EC 1.15.1.1) were determined. CAT activity was determined according Beers and Sizer (1952), by the decrease in absorbance at 240 nm due to the consumption of H_2O_2; GPX by the method of Urbanek et al. (1991), and the reaction monitored by increase in absorbance at 470 nm due to the formation of tetraguaiacol. APX was determined by the method of Nakano and Asada (1981), the oxidation of ascorbate measured by the decrease in absorbance at 290 nm. For APX estimation, 2 mM ascorbic acid was added to the extraction buffer. The activities of CAT, APX, and GPX enzymes were expressed in μmol H_2O_2 min^{-1} g^{-1} FM, where FM is fresh material.

SOD was determined by the method of Giannopolitis and Ries (1977), and the reaction measured by the increase in absorbance 560 nm due to the production of blue formazan, the photoreduction product of the p-nitrobluetetrazolium (NBT). SOD decreases photoreduction and one SOD activity unit (U) was defined as the amount of enzyme required to cause 50 % inhibition of the NBT photoreduction rate, and the results were expressed as U g^{-1} of fresh mass (FM). Each extract was assayed in duplicate.

2.3 Organic solutes contents

Soluble carbohydrate contents were determined by the method of Dubois et al. (1956), using the phenol–sulfuric acid method followed by absorbance readings

at 490 nm using as standard D-glucose reagent and proline by the method of Bates et al. (1973), using acidic ninhydrin reagent, followed by reading absorbance at 520 nm using proline as a standard. Free amino acids were determined by the method of Yemm and Cocking (1955), using ninhydrin, followed by absorbance readings at 570 nm using glycine as a standard.

Soluble protein contents were determined by the method of Bradford (1976) using the reagent Coomassie Brilliant Blue G-250, followed by absorbance readings at 595 nm using bovine serum albumin as standard. Each extract used for determination of organic solutes and protein contents was assayed in duplicate. Organic solute concentrations were expressed as μmol g^{-1} FM and the protein as μg protein g^{-1} FM.

3 Results

The suspension of irrigation caused reductions in the shoot fresh mass of maize plants (Fig. 1a). Average values in treatments subjected to water stress (water/drought-stressed and H_2O_2/drought-stressed) were 27 % lower than in irrigated plants. However, the shoot fresh mass in the H_2O_2/drought stress treatment group was 33 % higher than in the water/drought stressed group. There were no significant differences in root fresh mass between the two groups of stressed plants (Fig. 2a). The results for total fresh mass (Fig. 1c) were similar to those seen in shoot fresh mass. The total fresh mass of H_2O_2/drought-stressed plants was 39 % higher than that of water/drought-stressed plants.

It was observed that drought stress reduced the shoot dry mass (SDM) of water/drought-stressed plants in relation to water/control plants. However, in the H_2O_2/drought-stressed plants, the values did not differ from those of H_2O_2/control plants (Fig. 2a). The root dry mass (RDM) was reduced by 16 % in water/stressed plants relative to water/control plants (Fig. 2b). However, similar to SDM, the RDM values of H_2O_2/drought-stressed plants did not differ from those of control plants. In total dry mass (TDM), the behavior was similar to that of SDM. The TDM of water/drought-stressed plants was reduced in 16 % relative to water/control plants. Additionally, this effect was not observed in H_2O_2/drought-stress plants,

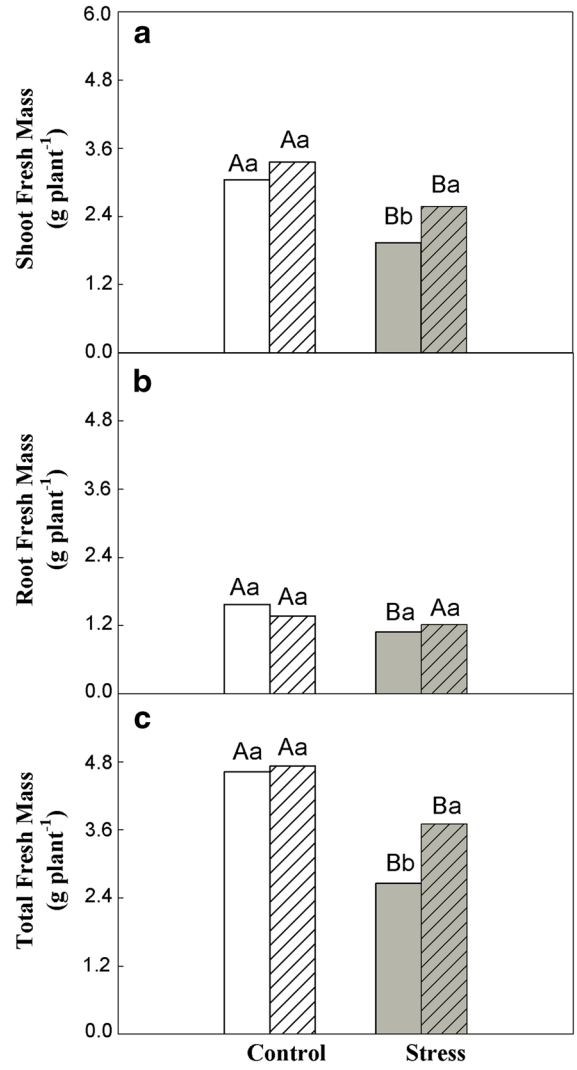

Fig. 1 Shoot (**a**), root (**b**), and total (**c**) fresh mass of maize seedlings under control (*white bars*) or water stress (*grey bars*) conditions, sprayed with distilled water (*not hatched*) or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. different capital letters indicate significant differences due to water stress (comparisons between water/control × water/drought stressed or H_2O_2/control × H_2O_2/drought stressed), while different lowercase letters indicate differences due to the H_2O_2 leaf spraying (water/control × H_2O_2/control or water/drought stressed × H_2O_2/drought stressed), according to Tukey's test ($P \leq 0.05$)

which did not differ from control plants in this respect (Fig. 3c).

CAT activity was not detected in the leaves of maize plants in the present study. Therefore, GPX was the most elevated H_2O_2 scavenging enzyme in the leaves. GPX activity was found to be higher in the

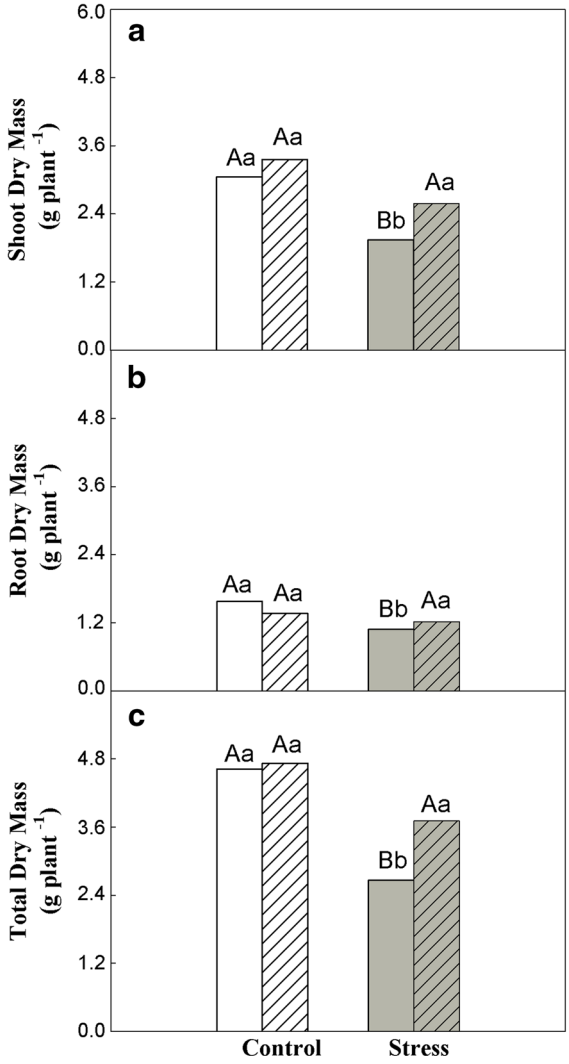

Fig. 2 Shoot (**a**), roots (**b**) and total (**c**) dry masses of maize plants under control (*white bars*) or water stress (*grey bars*) conditions sprayed with distilled water (*no hatched*) or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. Additional details in the legend of Fig. 1

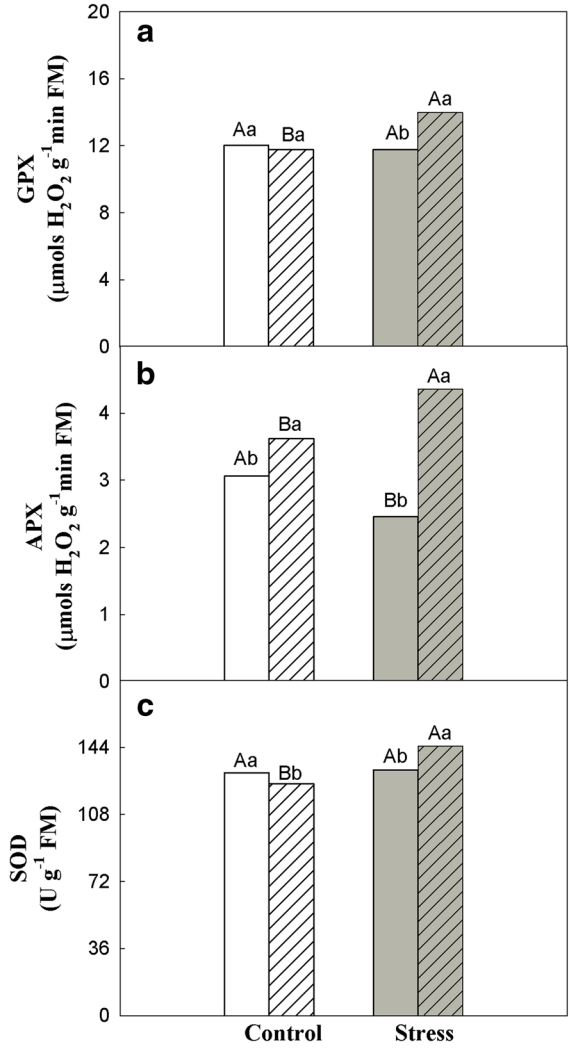

Fig. 3 GPX (**a**), APX (**b**) and SOD (**c**) activities in leaves of maize plants under control (*white bars*) or water stress conditions (*grey bars*) sprayed with distilled (*no hatched*) water or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. Additional details in the legend of Fig. 1

H_2O_2/drought-stressed treatment: 19 % higher than in the water/drought-stressed plants (Fig. 3a). Figure 3b demonstrates the beneficial effect of H_2O_2 leaf spraying, which increased APX activity in the leaves under both control and water stress conditions. APX activity was 18 % higher in the H_2O_2/control compared with water/control plants. Under stress conditions, APX activity in the H_2O_2/drought-stressed treatment proved to be 76 % higher than in the water/drought-stressed treatment. For SOD activity under stress conditions (Fig. 3c), H_2O_2 leaf spraying also proved beneficial in bringing about an incremental change. The SOD activity of H_2O_2/drought-stressed plants was 10 % higher than that of the water/drought-stressed plants. No relevant significant differences were detected between the other treatments.

Figure 4 depicts the activity of antioxidative enzymes in the root system. CAT activity (Fig. 4a), was increased by H_2O_2 leaf spraying under stress conditions. CAT activity in the H_2O_2/drought-stressed

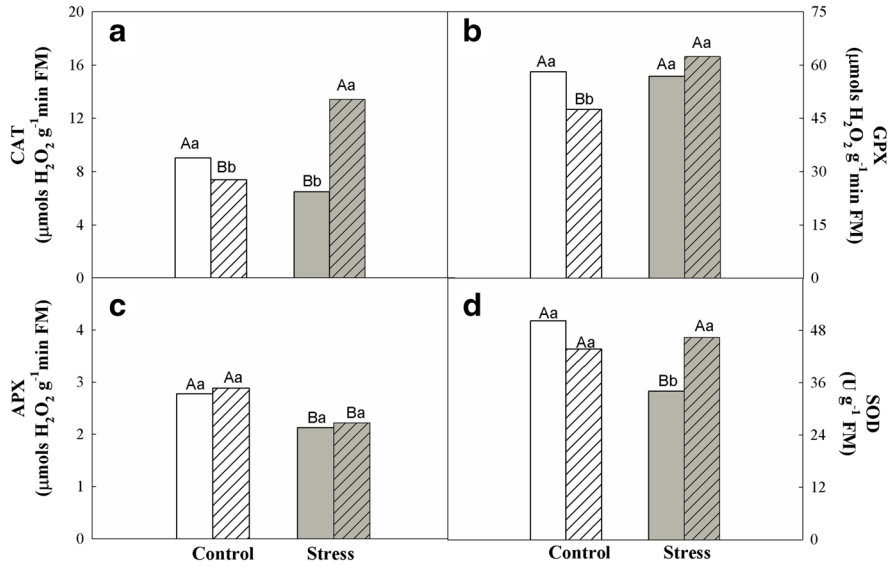

Fig. 4 CAT (**a**), GPX (**b**), APX (**c**) and SOD (**d**) activities in roots of maize plants under control (*white bars*) or water stress (*grey bars*) conditions sprayed with distilled (*no hatched*) water or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. Additional details in the legend of Fig. 1

treatment was 69 % higher than the water/drought-stressed treatment and 49 % higher than in the water/control. Some small differences were observed among treatments for GPX activity in roots (Fig. 4b). The H_2O_2/control treatment showed a small reduction relative to the water/control treatment. In the root system, H_2O_2 leaf spraying did not impact the activity of APX under either control or drought stress conditions (Fig. 4c). However, there was an average 33 % reduction in stress treatments in comparison with their respective controls. In the SOD activity in roots (Fig. 4d), there was a small reduction in plants sprayed with H_2O_2 under control conditions relative to those sprayed with distilled water. However, under water-stress conditions, root SOD activity in the H_2O_2/drought stressed treatment was 36 % higher than in the water/drought-stressed treatment and neither were significantly different from their respective controls.

Soluble protein and organic solute contents were analyzed in the leaves of maize plants and can be observed in Fig. 5. H_2O_2 leaf spraying caused an increase in soluble protein content of leaves under both control and water stress conditions. The leaf soluble protein content in the water/control group was 41 % higher than in the H_2O_2/control, while in the H_2O_2/drought-stressed group it was 21 % higher than in the water/drought-stressed condition (Fig. 5a). For the soluble carbohydrate contents (Fig. 5b), the only difference was between control and stress conditions and was not influenced by H_2O_2 leaf spraying. Therefore, the highest values were found under water stress. The proline content in the leaves (Fig. 5c) of H_2O_2/drought-stressed plants was 19 % higher than that of the water/drought-stressed plants, and did not differ significantly from those of the other treatments. Water stress did not affect free amino acid content (Fig. 5d). However, leaf spraying promoted an increase in this solute. The free amino acid content of the H_2O_2/control was 12 % higher than that of the water/control and in the H_2O_2/drought-stressed condition, it was 9 % higher than in the water/drought-stressed group.

The soluble protein and organic solute contents were analyzed in the roots of maize plants and can be observed in Fig. 6. The soluble protein content of roots (Fig. 6a) was higher in H_2O_2/drought-stressed plants, being 27 % higher than in the water/control and 25 % higher than in the water/drought-stressed plants. The values of soluble carbohydrates in the leaves (Fig. 5b) and roots (Fig. 6b) increased as a result of water stress. This was more apparent in H_2O_2/drought-stressed plants, being 135 % higher than in the water/control and 50 % higher than in water/-drought-stressed plants. The behavior of the proline content of roots (Fig. 6c) was similar to that of protein (Fig. 6b). The root proline content of H_2O_2/drought-stressed plants was 21 % higher than that of the control/water treated plants and 27 % higher than in water/drought-stressed plants. For the free amino acid content of roots (Fig. 6d), the treatments water/control and H_2O_2/control did not differ significantly from

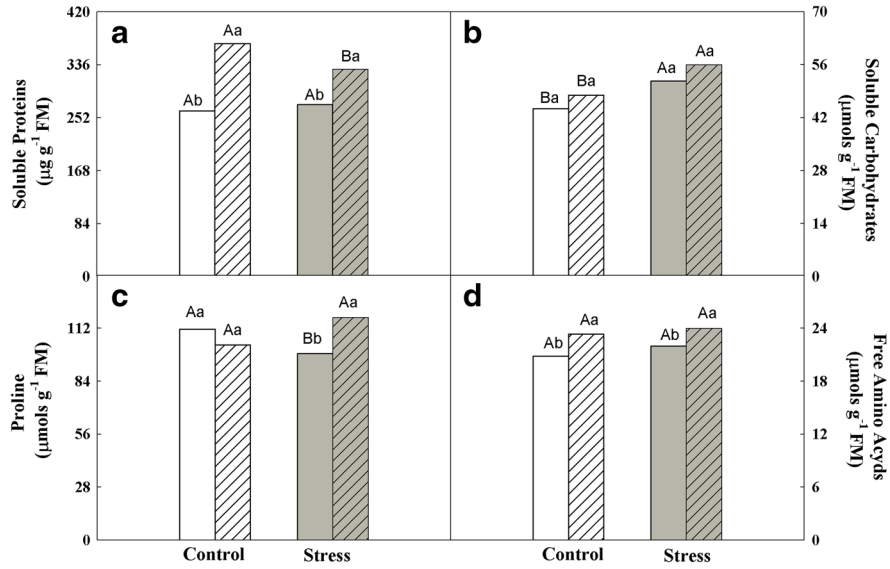

Fig. 5 Soluble proteins (**a**), carbohydrates (**b**), proline (**c**) and free amino acids (**d**) contents in leaves of maize plants under control (*white bars*) or water stress (*grey bars*) conditions sprayed with distilled water (*no hatched*) or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. Additional details in the legend of Fig. 1

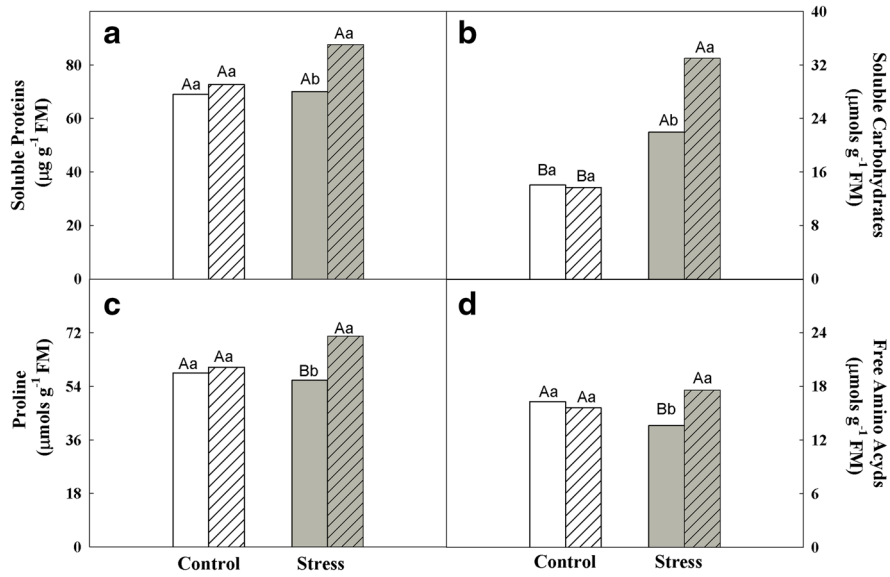

Fig. 6 Soluble proteins (**a**), carbohydrates (**b**), proline (**c**) and free amino acids (**d**) contents in roots of maize plants under control (*white bars*) or water stress (*grey bars*) conditions sprayed with distilled water (*no hatched*) or 15 mM H_2O_2 solution (*hatched*). The plants were harvested 8 days after withholding of water. Additional details in the legend of Fig. 1

each other, while the root free amino acids in H_2O_2/drought-stressed plants were 29 % higher than in water/drought-stressed plants. Additionally, the H_2O_2/drought-stressed plants did not differ from control plants in this parameter.

4 Discussion

The experiment was effective in showing that drought stress is able to reduce the growth of maize plants. However, it may be observed that in plants under drought stress that received H_2O_2 leaf spraying, this effect was less pronounced.

The suspension of irrigation reduced plant growth relative to control growth conditions, mainly in water/stressed plants, while H_2O_2 leaf spraying effectively minimized this effect in H_2O_2/drought-stressed plants (Figs. 1, 2). This effect was more evident in the dry matter of plants (Fig. 2). The data also revealed that the H_2O_2/drought-stressed treatment managed to produce dry matter similar to plants growing under

control conditions. Therefore, the present work suggests that the fresh mass parameter was more sensitive than the dry mass parameter to the suspension of irrigation.

The production of ROS are generated during normal cellular metabolism, and its production can be increased by water stress (Møller et al. 2007; Noctor et al. 2014). However, in order to combat oxidative stress, plants have developed an elaborate system to control cellular ROS level (Hossain et al. 2015). In the present study, the enzymes including CAT, APX, GPX, and SOD were likely able to promote a satisfactory elimination of ROS, such as superoxide radicals and H_2O_2, which cause damage to cells, especially in stressed plants receiving H_2O_2 leaf spraying (Figs. 3, 4).

Presently, it has been suggested that, rather than a negative influence that must be overcome, ROS are a major mechanism for acclimation in plants (Foyer and Noctor 2016). Genetic and biochemical studies have confirmed the presence of a H_2O_2 signaling molecule in plants under conditions of biotic and abiotic stresses, which is also involved in stomatal closure, root gravitropism, and tolerance to oxygen deficiency (Neill et al. 2002). According to Niu and Liao (2016), H_2O_2 may be involved in cellular signaling transduction pathways and gene expression modulations in plants. Moreover, studies conducted under other stress conditions found increases in the activities of enzymes and stress tolerance (Uchida et al. 2002; Azevedo Neto et al. 2005; Gondim et al. 2012). Recently, Talbi et al. (2015), in *Oudneya africana* plants, showed that drought tolerance is involved in the ability to activate a complex antioxidative defense regulatory system, likely involving H_2O_2-dependent signals. For this reason, the increase in antioxidative enzyme activities found in this study in H_2O_2/stressed plants when compared with water/stressed plants (Figs. 3, 4) likely contributed to the observed improvement in plant growth (Figs. 1, 2).

Many other species, when exposed to water or salt stress, are able to maintain the water potential gradient between the root environment and the plant due to the absorption of ions and the accumulation of organic solutes of low molecular mass. Inorganic and organic acid ions tend to accumulate in the vacuole, while organic solutes or osmolytes, which include amino acid compounds, carbohydrates and soluble quaternary ammonium compounds, accumulate in the cytosol. This particular phenomenon is referred to as osmotic adjustment (Chinnusamy et al. 2005; Türkan and Demiral 2009). In addition to their osmoprotective roles, compatible solutes may increase antioxidant defense mechanisms against stress damage (Ashraf and Foolad 2013a, b) and improve salt tolerance in plants (Khedr et al. 2003).

Osmotic adjustment not only maintains the water potential gradient in the soil-system plant, but is considered responsible for the maintenance of cell turgor to enable cell growth (Smirnoff 1998). Further, it was found that osmotic adjustment in leaves, but mainly in roots, (Figs. 5, 6) of H_2O_2/drought-stressed plants resulted in increased organic solute contents (proteins, carbohydrates, soluble proline, and amino acids) compared with water/drought-stressed plants. Notably, this buildup may have contributed to osmotic adjustment, which resulted in higher plant growth (Figs. 1, 2) of H_2O_2/drought-stressed plants when compared with water/drought-stressed plants. Ishibashi et al. (2011), found that H_2O_2 leaf spraying enabled soybean plants to avoid drought stress by helping them to maintain leaf water levels, and that leaf water retention was likely due to increased oligosaccharide biosynthesis, rather than to rapid stomatal closure. On the other hand, Zhao et al. (2014) found that the concentrations of soluble sugar and proline in the roots of maize plants under salt stress and manganese deprivation were not high enough to adjust the osmotic potential in maize seedlings under stress conditions.

According to Hossain et al. (2015), these findings demonstrate that H_2O_2 pretreatment can induce tolerance to salinity and drought stresses in plants by modulating physiological and metabolic processes, such as photosynthesis, proline accumulation, and ROS detoxification, and that this ultimately leads to better growth and development. Additionally, ROS metabolism also plays a pivotal role in the development of stress and cross-stress tolerance.

According to our hypothesis, in the present study, we showed that H_2O_2 can act as a signal molecule in regulation of maize plant growth and development under drought stress. It may be that H_2O_2 had changed the activities or expression of the antioxidative enzymes, and/or enzymes involved in organic solutes synthesis. Taken together, it contributed to enhance plant growth.

In conclusion, it was found that H_2O_2 leaf spraying pretreatment reduced the deleterious effects of

drought stress on maize plant growth. This treatment proved to be a beneficial defense strategy in plants. This effect could be attributed to the ability of H_2O_2 to induce antioxidant defense system activity, especially that of GPX, and higher organic solute content (protein, carbohydrate, proline, and free amino acids) in roots and leaves.

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