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Biochemical and Photosynthetic Evaluation of Responses in *Zea mays* L. Under Drought Stress

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ABSTRACT

Antioxidant defense system(s), pigments content and photosynthetic activity as well as some biochemical changes under drought stress were analyzed in maize (*Zea mays* L. cv. Giza 21) leaves to determine the response of plant to drought stress and to elucidate the role of various protective mechanisms against oxidative stress. It was found that the application of drought stress led to changes in the carbohydrates and protein contents. Total soluble sugars, accumulated in the leaves of water-stressed plants, whereas, starch and protein contents were dropped to a small amounts compared to the control. Furthermore, plants have well-developed defense systems against reactive oxygen species (ROS), involving both limiting the formation of ROS as well as instituting its removal. Within a cell, the activities of a range of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) involved in scavenging ROS were investigated. During dehydration the SOD, APX and CAT increased significantly up to 4 days, then declined in their activities but still maintained higher than the control levels this indicates that the defense systems involved are efficient in the protection of plant cells against oxidation. In addition, there was consistent increase in the lipid peroxidation and accumulation of malondialdehyde (MDA). The levels of hydrogen peroxide were also elevated during stressing periods. In this study we are reporting the negative response of maize plants toward drought stress especially on the antioxidant enzymatic activity for the prolonged drought effect.

Key words: Drought, Oxidative stress, Reactive oxygen species (ROS), Antioxidants.

INTRODUCTION

Drought stress is considered as one of the most important environmental factors that causes osmotic stress, limiting plant growth and development. Different pathways can also be affected differently. At the whole plant level, the effect of drought stress is usually perceived as a decrease in photosynthesis and growth (Asada, 1997), and is associated with alterations in C and N metabolism. Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess concentrations of reactive oxygen species, resulting in oxidative damage at the cellular level. Therefore, a consequence of drought stress is the limitation of photosynthesis and usually accompanied by the formation of reactive oxygen species (ROS) in the chloroplasts (Smirnoff, 1993) such as the superoxide radical, H_2O_2, and the hydroxyl radical (Foyer et al., 1994). Hydrogen peroxide is especially toxic in the chloroplasts because even at low concentrations it inhibits the Calvin- cycle enzymes, hence reducing the photosynthetic carbon dioxide assimilation (Takeda *et al.*, 1995).

Plants are equipped with complex and a highly efficient antioxidative defense system composed of protective non-enzymatic and enzymatic protection mechanisms function to interrupt the cascades of uncontrolled oxidation in some organelles (Noctor and Foyer, 1998) and serve to maintain the antioxidants in their reduced functional state (Schwanz *et al.*, 1996) that efficiently scavenge AOS and prevent damaging effects of free radicals (Shalata and Tal 1998).

Enzymatic protection is partly performed by superoxide dismutase (SOD, EC 1.15.1.1) that eliminates superoxide radicals $O_2^{\cdot-}$ and by catalase (CAT, EC 1.11.1.6) and ascorbic peroxidases (APX, EC 1.11.1.11) that degrade H_2O_2 influencing the level of lipid peroxidation (Dat *et al.*, 2000 and Mittler, 2002) which is commonly taken as an indicator of oxidative stress, because it is induced by reactive oxygen species (ROS).Our study aimed to investigate the effect of drought stress by withholding water on some biochemical and physiological parameters in maize plant (*Zea mays* L.Giza 21), moreover clarifying the antioxidant enzymes activity of maize plants under drought stress.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of maize (*Zea mays* L. cv. Giza 21) were surface sterilized by immersion for two min in 0.1 % $HgCl_2$, thereafter they were washed with five changes of sterile distilled water. Seeds were soaked in continuously aerated distilled water for 24 h in darkness. Seeds were sown in plastic pots (15 cm diameter x 20cm height), filled with washed pure quartz sand. All pots were placed in a growth chamber under 70-80% relative humidity with 16/8h day/night cycle and controlled temperature of 28/26°C. Light intensity was 420µmol m^{-2} s^{-1}. Each pot was irrigated with 250 ml of distilled water at first, then occasionally with a certain amount of water in order to keep the soil water content constant. After seven days, all plants were watered on alternate days with half strength of Hoagland solution. After 15 days from sowing one-half of the plants were subjected to drought stress by withholding water for 8 days and sampled in regular intervals for analyses. Just after harvest, the whole plants or dissected organs were blotted dry and weighed carefully for fresh weight determination, then dried in a hot-air oven at 70°C until a constant weight to obtain dry weight. For biochemical analyses, the second leaves were harvested and used either immediately for extractions or were stored at -20°C until analysis. Each experiment was repeated twice, with a total of 20 plants in each case.

Determination of carbohydrates constituents and protein content

This was done by alcoholic extraction method. Reducing sugars were analyzed according to Irigoyen *et al.*,(1992), Three ml of the modified Nelson's reagent were added to 5 ml of the sugar extract. The whole was mixed thoroughly in a boiling tube immersed in a vigorously boiling water bath for 15 min. The tubes were then cooled rapidly. Three ml of arsenomolybdate reagent were run into each tube with gentle shaking till effervescence stopped. The colored solution was diluted to known volume and then measured at 700 nm using spectrophotometer (JENWAY, 6305, UK). Protein fractions were determined according to the method described by Breadford (1976) in which 5 ml of the protein reagent* were added to 0.1 ml of the extract and the contents mixed by vortexing. The absorbance was measured at 595 nm during one hour. The concentration of protein was calculated from a previously constructed standard curve using bovine serum albumin (Fluka, analytical grade).

Pigments analyses

The photosynthetic pigments chlorophyll a, b (Chl. a, Chl. b) and carotenoids (Carot.) were determined following N, N-dimethyl formamide (DMF) method described by Inskeep and Bloom (1985). A known weight of the dissected plant leaves (50 mg) were incubated in 10 ml of DMF reagent and kept in dark at 4ºC for 24 hours. The extract-containing pigments was decanted and the absorbance was measured at three wavelengths 647, 665 and 470 nm using spectrophotometer (JENWAY, 6305, UK) Formula and extinction coefficients used for determination of photosynthetic pigments were:

$$\text{Chl. a} = 12.70\ A_{665} - 2.79\ A_{647}$$

$$\text{Chl. b} = 20.70\ A_{647} - 4.62\ A_{665}$$

$$\text{Carotenoids} = 4.2\ A_{453} - (0.0264\ \text{Chl. a} + 0.426\ \text{Chl. b})$$

*One hundred mg of Coomassie Brilliant Blue G-250 was dissolved in 95% ethanol. Then 100 ml 85% (w/v) phosphoric acid was added . The resulting solution was diluted to a final volume of one liter and filtered

Determination of lipid peroxidation

Malondialdehyde, (MDA) content was assayed as indicators of the extent of lipid peroxidation in leaf tissue by the method of Hodgson and Raison (1991). MDA concentration was calculated using a molar extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Determination of hydrogen peroxide

The level of H_2O_2 was measured colorimetrically as described by Jana and Choudhuri (1982). H_2O_2 level was calculated using the extinction coefficient 0.28μmol $^{-1}cm^{-1}$.

Extraction of antioxidant enzyme and activity determination

Fresh maize leaves ($\approx$0.5g fresh material) were ground to a fine powder in liquid nitrogen. Frozen powder were transfer into 10 ml of ice-cold extraction buffer containing 100 mM KH_2PO_4/ K_2HPO_4, pH 7.8, 5 mM ascorbate, 400mg of insoluble polyvinylpolypyrro- lidone (PVP), and 2 % Triton X-100 (Schwanz *et al.*,1996), mixed for 1 min, and incubated on ice for 30 min. According to Asada (1997), the elution buffer for APX contained additionally 1 mM ascorbic acid in order to keep APX enzyme in the active state. The purified extracts were used immediately for the determination of super oxidedismutase, SOD; catalase, CAT and ascorbic peroxidase, APX activities.

Enzymes assay

Enzymatic assays were performed at 25°C. All solutions used for analytical and enzymatic investigations were prepared with double-ionized water.

SOD (EC 1.15.1.1) activity was measured according to the method of Stewart and Bewely (1980). One unit of SOD activity was the amount of enzyme activity that caused 50 % inhibition of the initial rate of the reaction in the absence of enzyme.

APX (EC 1.11.1.11) activity was assayed according to Asada (1997). One unit of APX was the amount of enzyme that oxidized 1mmol of ascorbate per min at room temperature.

CAT (EC 1.11.1.6) activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm (Luck 1965). One unit of enzyme activity is equal to 1 mmol of H_2O_2 decomposed per min.

RESULTS

Effect of drought stress on the carbohydrate and protein content

A study of the changes in the carbohydrate fractions in leaves of maize plant subjected to drought stress shows that these fractions have different patterns. For example, the total carbohydrate content under drought stress was dropped from initial values of 218.2 at the beginning of treatment to 166 mg g^{-1} DW at the end of experiment (Fig 1). The corresponding values for the well-watered plants were 218.2 and 210 mg g^{-1}DW respectively. Whereas, the total soluble sugars content was consistently higher in the leaves of water-stressed plant amounted to 140.5 mg g^{-1} DW at the end of experiment compared to 89.6 mg in control (Fig 1). Non-reducing sugars were generally remained substantially higher than reducing sugars but significantly far more non-reducing sugars were accumulated relative to the control at the end of experiment (Fig1)

Table 1: Changes in chlorophyll a and b, total chlorophyll content, total carotenoids and chlorophyll a/b ratio in leaves of maize plant grown under drought stress for 8 days. Values were expressed as the percent of increase or reduction relative to the control

Time (days)	Chl.*a*	Chl.*b*	Total chl. %	Car.	Chl *a*/*b*
0	100	100	100	100	100
2	81	86	97	96	92
4	70	85	90	94	84.5
8	55	72	79.3	84.8	72

The leaves of water-stressed plant contained significantly lower amount of starch 26.9 mg g^{-1} DW, at the end of experiment, compared to121.1mg g^{-1} DW in those of well-water plants (Fig1).

Furthermore, the results shown in Fig.2 indicate clearly that drought stress had a pronounced effect on the total soluble proteins content in leaves of maize plants. Thus, when leaves were subjected to water stress, protein content declined rapidly as compared to the control (Fig.2). At the end of exposure time, the total soluble protein in leaves of water stressed plant was 76.8 mg g^{-1} dwt compared to 206.5 mg in control.

Chlorophylls and Carotenoid Contents

In maize leaves drought stress caused a general decrease in the pigment contents, including chlorophyll *a*, b, and β-carotene. This pattern of change was not evident in control, in which all pigments did not change statistically (data

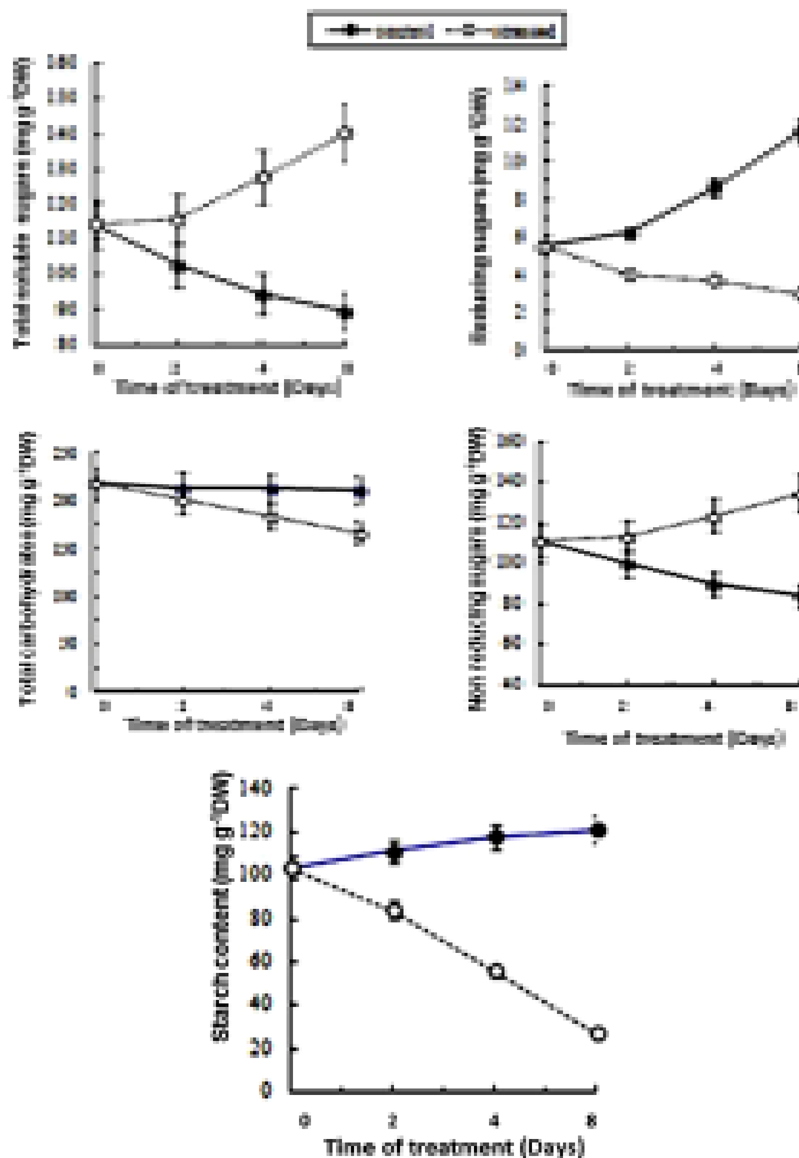

Fig. 1: Effect of drought stress on carbohydrates constituent in leaves of Zea maize plant. Value are means ±SE (n=5)

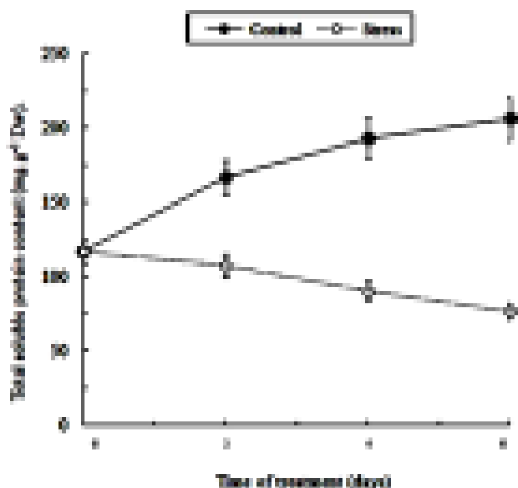

Fig. 2: Effect of drought stress on protein in leaves of *Zea maize* plant. Values are means ±SE. (n=5)

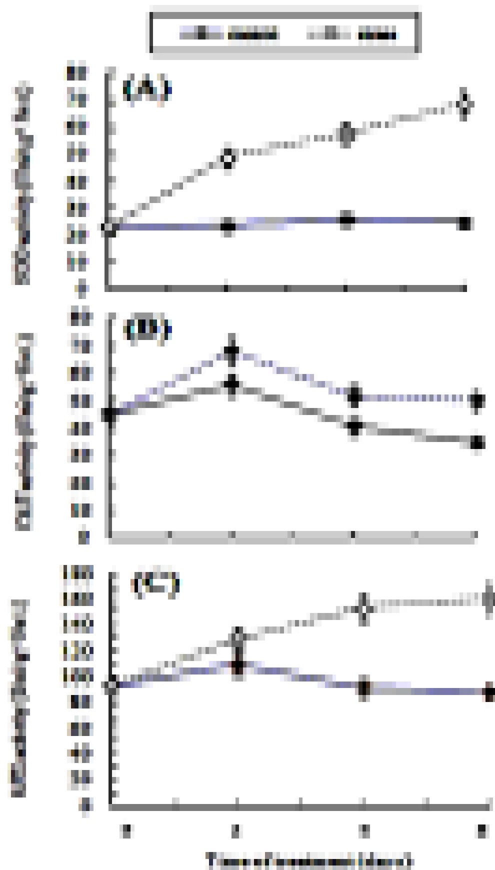

Fig. 4: Effect of drought stress on antioxidant enzymes activities in leaves of *Zea maize* plant. Values are means ±SE. (n=5)

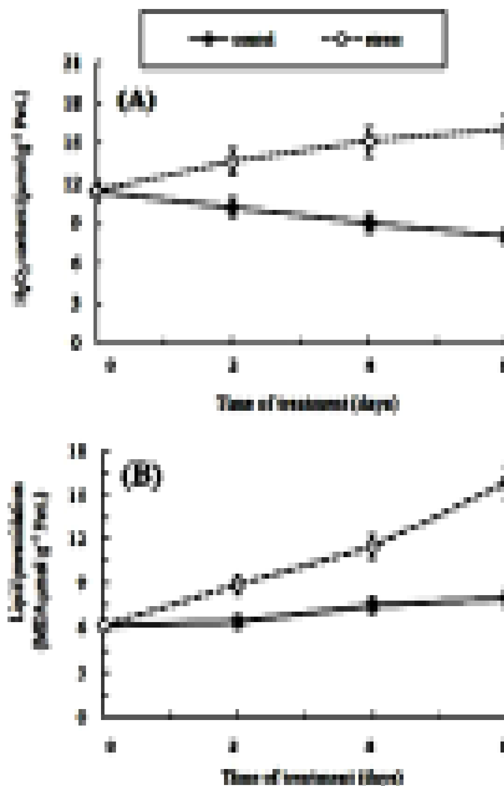

Fig. 3: Effect of drought stress on hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) contents in leaves of *Zea maize* plant. Values are means ±SE. (n=5)

not shown). The content of chlorophyll *a*, b and -carotenes in maize leaves under drought stress particularly at the end of experiment, were decreased by about 45 and 28 and 15.2 % of control respectively. As a consequence, the Chl *a/b* ratio was decreased significantly under drought stress (Table 1).

Hydrogen peroxide content (H_2O_2)

Hydrogen peroxide has a negative effect on various biochemical processes inside the plant cell. According to our results, the level of H_2O_2 did not change significantly in control plants, during the experimental period (Fig.3A). In contrast, drought stress caused a significant increase in the generation H_2O_2 during the drought stress period. After 8 days of treatment, the production of H_2O_2 reached the maximum values, amounted to 46%, compared with control. Despite the accumulation of H_2O_2 during the exposure time of water stress, did not result immediately in cell death.

Lipid peroxidation of plasma membrane

One of the described damages provoked by water-stress is the membrane injury. This is a consequence of an oxidative burst leading to lipid peroxidation. Peroxidation can be measured by quantifying the amount of malondialdehyde, (MDA). As shown in Figure 3B, the MDA production was increased significantly with leaf ageing and was enhanced by water stress. For convenience, at the end of exposure period, control leaves produced only 7.99 ìmol MDA g^{-1} FW., whereas drought stress greatly increased MDA, reaching 15.9 ìmol MDA g^{-1} FW.

Effects of drought stress on antioxidant enzymes

The effects of drought stress on the activities of several important antioxidant enzymes such as SOD, CAT, and APX, in maize leaves, were investigated and the results are shown in Fig 4. The results clearly demonstrate that drought stress led to a significant enhancement in the activities of SOD, CAT and APX (Fig.4A, B and C) within 4 days of treatment reached almost the maximum values amounted to 123 %, 21%, and 67 %, respectively, relative to the control whereas the initial activities were maintained at control levels.

However, after 4 days of drought stress treatment, the activities of these antioxidant enzymes had a tendency to decrease. No significant changes in the activities of these enzymes in control were observed during experimental period. The positive response of SOD, CAT, and APX activities were, however, maintained over the whole stress treatment.

DISCUSSION

The significance increase in the carbohydrate content seems to be involved in osmotic adjustment. Total soluble sugars concentrations in the leaf blade after 2 days of drought stress increased by 13 % relative to the control plants. As the stress progressed, the increment in total soluble sugars was more evident (Fig.1). Although, non-reducing soluble sugar concentration still remained higher, that of reducing sugars had dropped. Wardlaw and Willenbrink (1994) have reported that the changes in leaf blade reducing sugars are paralleled by the changes in invertase activity also sucrose synthase activity continuously increasing in the blade with drought stress severity that is consistent with findings results obtained by Tabaeizadeh (1998) which described the correlation between the increase in enzyme activity with drought stress, as well as, non reducing sugars accumulation.

When withholding water, the first signs of stress in maize involved pronounced changes in sugar metabolism. According to our results, the observed variation in the soluble sugars concentrations may be the result of growth being more inhibited by drought stress than photosynthesis, as well as an increased partitioning of fixed carbon to sucrose, as shown for wild species under drought stress (Quick *et al*.,1992). This accumulation of soluble sugars may be related to osmoregulation and desiccation tolerance (Hare *et al*., 1998) contributing to plant survival.

The large alterations observed in maize sugar metabolism preceded the drastic decrease of soluble leaf protein. These proteins are typically related to stress responses, such as freezing, osmotic and salt stress and pathogen attack (Chen *et al*., 1994, Yun *et al*., 1996, , Tabaeizadeh 1998 and Trudel *et al*., 1998). Thus the water response of maize seems to have characteristics in common to other adverse conditions in agreement with suggestions made for other species (Tabaeizadeh 1998).

Chlorophyll, carotenoid and photosynthetic rate

Drought stress induced changes in the photosynthetic apparatus and the membrane permeability properties of chloroplasts. This fact may be the result of chlorophyll degradation and/or synthesis deficiency together with a decrease of thylakoid membrane integrity (Tabaeizadeh 1998). In the present study, the decline in the chlorophyll content under drought stress may be explained by the earlier structural loss of the chloroplast stroma lamellae, containing photosystem I and most of the chlorophyll a, (Loggini et al.1999). Photoinhibition and photodestruction of pigments may contribute to such changes (Dean *et al*., 1993). Furthermore, drought stress decreased the capacity to preserve the photosynthetic apparatus. However, it was found in our study that the effect of drought stress is

likely to follow similar processes to those observed during senescence, severely affected these parameters, therefore the drastic effect by drought stress at chloroplast level may be expected (Tabaeizadeh 1998). Also, the inhibition was probably connected with the increase in the rate of chlorophyll degradation (Garty *et al.*, 1992) through the effect of drought stress on the chlorophyll binding protein, leading to the destruction of chlorophyll that may contribute to such changes(Abdel Nasser 2000). Also, the decrease in the chlorophyll content may also be a phytotoxic consequence of lipid peroxidation and is associated with a decrease in photochemical efficiency. Moreover, the ratio of chlorophyll *a/b* was more sensitive to the drought stress treatment, showing that Chl *a* was more susceptible to water stress, being degraded at a higher rate than Chl *b*. This can be explained by the fact that part of the decrease in chlorophyll *a* could be accounted by conversion to chlorophyll *b* by the oxidation of the methyl group on ring II to the aldehyde (Fang *et al.*, 1998). In this connection Ciscato *et al.*(1997) have reported that the reduction in the Chl *a/b* ratio in maize plant might be due to a direct effect of drought stress on the light harvesting complex of photosystem II (LHC II). Typically, decreases in chlorophyll *a/b* ratio are observed during senescence (Dean *et al.*, 1993), suggesting that drought stress treatment induced a lower rate of synthesis and accumulation of chlorophyll *a*.

Lipid peroxidation of plasma membrane and H_2O_2 content

Lipid peroxidation has been shown to be one cause of membrane deterioration and disassembly during senescence and is associated with most membrane disorders of plants (Marengoni *et al.*, 1996 a and b). Drought stress was accompanied by increases in the contents of malondialdehyde (MDA) indicating lipid peroxidation and oxidative stress. Drought stress like other environmental stresses can generate the production of a powerful oxidation, which brings about lipid peroxidation, suggesting that fatty acids in thylakoid membrane were targets for drought stress damage. This could be achieved through the activation of toxic O_2 molecules that can then attack fatty acids chains resulted in an increase of the membrane damage with a corresponding increase in the formation of MDA in maize leaf. Accordingly, drought stress-induced effect could reflect some modifications of the plasma membrane structure such as the changes in the physical properties of the membrane which reflect the changes in its chemical composition as a result of alteration in metabolic processes (Navari-Izzo *et al.*, 1996). Furthermore, in the present study, there is an accumulation of H_2O_2 (Fig. 4A), which acts as a redox signal molecule in plants exposed to drought stress (Mehdy 1994). It has been suggested that H_2O_2 functions as a second messenger in plant cells exposed to environmental stresses such as heat (Dat *et al.*,1998), and pathogens (Levine *et al.,*1994). Although, H_2O_2 inhibits chloroplast sulfhydryl-containing enzymes by readily oxidizing their sulfhydryl groups, it induces an orchestrated sequence of reactions involving the activation of peroxidases. Therefore, it is important for plant cells to keep the levels of H_2O_2 low or to scavenge it efficiently.

Antioxidative defense mechanism

Reactive oxygen species produced under various abiotic stresses are extremely damaging to lipids, proteins, and pigments unless they are rapidly scavenged by antioxidant enzymes such as SOD, CAT and APX (Asada et al., 1998) to maintain the concentration of any active oxygen species formed at relatively low level. Shalata and Tal (1998) suggested that, the resistance of plants toward environmental stress may depend on the inhibition of ROS production or the enhancement of antioxidant levels. Also, the higher tolerance of some genotypes to environmental stresses has been associated with higher activities of antioxidant enzymes. It is possible that the observed changes in the antioxidant systems occurred as a result of unspecific cellular degradation processes. However, another possibility is that drought stress triggers common defense pathways in plant cells like other biotic or abiotic environmental stresses. In fact, electron spin resonance studies have shown that water-stressed plants displayed elevated concentrations and production rates of superoxide radicals (Price and Hendry 1991).

In maize plants, there were already symptoms of oxidative stress, such as an increase in the total activity of SOD under drought stress and

retained most of their antioxidant capacity, which may explain why oxidative damage in control plants was incipient compared with stressed plants. The present investigation showed that the increase in APX activity induced by drought stress and remained at a higher level compared to the control suggesting that the increase in the activity of this enzyme can be ascribed at least in part to substrate accumulation. Therefore, the increased APX activity could be the protection against oxidative damage (Tabaeizadeh, 1998). An additional function of the increase in APX activity under drought stress could be related to changes in the cell wall properties, potentially important for the stem in order to cope with the stress. Since drought stress causes the formation of reactive oxygen species.

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