IMPACT OF DROUGHT STRESS ON ENZYMATIC BASED ANTIOXIDANT MECHANISM IN ROOTS OF *ZEA MAYS* L.

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ABSTRACT

Drought is one of the major threats to potential yield of crop as it strongly impair growth both at vegetative and reproductive stage leading to decrease in yield and productivity. Thus, identification of the key system in drought tolerance is essential for better crop yield. Roots of two maize varieties i.e. Azam and Baber were evaluated in this study based on the enzymatic defence system. The withdrawal of water for 7 days, the roots of the drought stressed plants not only exhibited reduction in fresh weight but also total soluble proteins were deceased by more than 50 % in comparison to well watered. Cations such as Na^+, K^+ and Ca^+ were also strongly accumulated. Moreover, the huge accumulation of more than 2-fold in H_2O_2 and MDA contents in both varieties upon water deficiency indicating that roots experienced severe oxidative stress. In response to drastic increase in Reactive Oxygen Species (ROS), the enzymatic based defense system actively enhanced to minimize the detrimental effect of ROS. Almost all the enzymes including APX, CAT, SOD and POD were increased 1.4- to 2-fold in roots of drought-stressed plants. Thus, plants with strong scavenging mechanism can be used as an important indicator for drought tolerance.

Keywords: *Zea mays*, drought, roots, reactive oxygen species, antioxidant enzymes

Abbreviations: ROS, reactive oxygen species, H_2O_2, hydrogen peroxide, MDA, Malondialdehyde, APX, ascorbate peroxidase, CAT, catalase, SOD, superoxide dismutase, GR, glutathione reductase, GSH, reduced glutathione, GSSG, oxidized glutathione, RWC, relative water content, ABA, abscisic acid.

INTRODUCTION

Crop yield is vastly prone to abiotic stresses which include drought, extreme temperatures and salinity that are mainly responsible for reduction in growth and development of crop plants. Drought is contributing more in terms of yield loss (Sallah *et al*., 2002) and limiting about 15% or even more in the production of major crop worldwide (Wang *et al*., 2003). It is the deficiency of sufficient moisture essential for a plant growth and development (Zhu, 2002). In coming years, abiotic stresses will cause an elevated impact on crop productivity, due to climate change and the increased competition for land, water and energy (Hu and Xiong, 2014), thus posing a threat to global food security (Boyer *et al*., 2007; Ji *et al*., 2011). Plants replenishing with water leads to a decrease in transpiration and uptake of water by roots (Duursma *et al.*, 2008) and eventually limits the contact between roots and soil under prolong drought (Nobel and Cui, 1992; North and Nobel, 1997). Roots, thus act as the first sensor during water shortage and other rhizosphere (Jackson, 1997; Davies *et al.*, 2000). In roots, the initial signal is recognized by small molecules such as abscisic acid (ABA) that plays a significant role from root to shoot signalling to avoid water loss through transpiration due to stomata closure (Jiang and Hartung, 2007). Also upon water shortage, roots growth is promoted in order to increase water uptake (Sharp, 2002).

Most of the abiotic stresses including drought is considered as a major contributor towards the enhanced production of ROS leading to an increase in the oxidative load in plants (Noctor *et al.*, 2014, Ahmad *et al*., 2016). ROS levels are considerably increased in plants because of environmental stresses, distressing the normal balance of O_2•−, •OH, and H_2O_2 in the intracellular environment (You and Chan, 2015; Khan *et al.*, 2016). Superoxide radical and its reduction product H_2O_2 are potentially toxic compounds (Hasanuzzaman *et al.*, 2012). ROS at high level also damage lipids, membrane and proteins (Gill and Tuteja, 2010).

Plant possesses complex defense system to scavenge ROS. Nonenzymatic components of the antioxidative defense system include ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, carotenoids, and phenolic compounds (You and Chan, 2015). Such factors (antioxidants) manipulate plant growth and development by influence the vital processes from mitosis and cell elongation to senescence and cell death (De Pinto and DeGara, 2004). They interact with numerous cellular components and helps in ROS removal. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidise (APX) and glutathione

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reductase (GR) (Hasanuzzaman *et al.*, 2017). SODs remove O_2 by catalyzing its dismutation, one O_2 being reduced to H_2O_2 and another oxidized to O_2 (Noctor *et al.*, 2014). CAT is localised in peroxisomes and helps in the removal H_2O_2 that are produced by several mechanisms such as by oxidases concerned in b-oxidation of fatty acids, purine catabolism and photorespiration (Polidoros and Scandalios, 1999). GR primarily catalyzes the reduction of GSH. It is a molecule which plays a pivotal role in a number of metabolic regulation and antioxidation in plants. GR catalyses the NADPH reliant reaction of disulphide bond of GSSG and is therefore, important for maintaining the glutathione (GSH) provision (Chalapathi and Reddy, 2008; Noctor *et al.*, 2014).

Keeping in view the importance of the antioxidant system in drought tolerance, we intend to carry out this study to explore the response of roots to drought stress in the important agronomic maize crop.

MATERIALS AND METHODS

Seed collection and growth conditions

Present study was carried to evaluate the roots of two maize varieties (Azam and Baber) obtained from Cereal Crop Research Institute (CCRI) Pirsabak, Nowshera, Pakistan and grown in the Green House of University of Science and Technology Bannu for drought stressed experiments. Germination and growth conditions were similar as described by Khan *et al.*, (2016).

Determination of relative water content (RWC)

RWC was determined as described by Khan *et al.*, (2016) by taking the fresh weight (FW) of leaves of both varieties. Afterwards, the leaves were kept in water at 4^oC for 4 h to record the turgid weight (TW). To further determine the dry weight (DW), the rehydrated leaves were kept in oven for 2 days at 80^o C.

Determination of total soluble proteins

Total soluble proteins were quantified by the procedure of Bradford, (1976). By using pestle and mortar in 1 mL phosphate buffer (pH 7.0), root of (100 mg) were homogenized. After that for 15 min the crude homogenate was centrifuged at 4000 round per minutes. In reaction mixture 2mL distilled water, 20µl enzyme extract and 0.5mL Bradford reagent was added. Spectrophotometer (UV-2600) was used for absorbance at 595nm.

Determination of Calcium (Ca^{+2}), Sodium (Na^+) and Potassium (K^+)

Approximately, 25mg powdered root material was taken in 50 ml for digestion using mixture of hydrogen peroxide (H_2O_2) and sulphuric acid (H_2SO_4) @ 2:1(v/v). The mixture was then heated followed by addition of distilled water (20ml) to each sample with continuous shaking. Whatman filter paper was used for filtration followed by determination of different ions using flame photometer according to Rudge *et al*., (2009).

Assays for ROS scavenging enzymes

Various antioxidant activities were determined by first grinding of 0.5g of roots with mortar and pestle followed by addition of ice cold 50mM phosphate buffer (pH7.8). After mixing well, the crude enzyme extract was centrifuged twice at 4C, 12000 rpm for 15 minutes. The enzyme extract was further used for various antioxidant enzymes.

Determination of oxidative stress markers (H_2O_2 and MDA)

Method of Velikova *et al*. (2000) was used to quantify H_2O_2. Reaction mixture contained 1ml of enzyme extract, 2mL of 1MKI. H_2O_2 and 1mL of 10mM potassium phosphate buffer (pH7.0). At 390nm, absorbance was determined.

MDA level was estimated by the procedure of Daud *et al*., (2013). Briefly, 2ml of reaction mixture containing 0.5 % thiobarbituric and 10 % trichloroacetic acid was added to 1mL enzyme extract. Following the mixture was then placed at 95^oC in water bath for 30 minutes and then immediately kept on ice for 15 minutes. Afterwards, samples were centrifuged at 4000rpm for 15 minutes followed by the measurement of absorbance at 532 and 600 nm.

Quantification of antioxidant related enzymatic assays

Determination of Ascorbate peroxidase (APX) activity

Nakano and Asada, (1981) method was followed for APX activity. First of all 3mL of a reaction mixture was prepared containing 0.1 mL of 2 % H_2O_2, 0.1 mL of 0.5 mM ascorbic acid, 2.7 mL of 50 mM potassium phosphate

(pH7.0) and enzyme extract (0.1 mL). Afterwards, absorbance was recorded at 290 nm for 1 minute. Extinction coefficient (ε=2.8 mmol cm^{-1}) were used for the oxidizing amount of ascorbate.

Determination of catalase (CAT) activity

Daud *et al.,* (2013) method was followed to determine catalase activity. At 240 nm the disappearance of H_2O_2 were measured (E=0.036 mmol cm^{-1}) by taking a reaction mixture of 25 uL of 10 mM H_2O_2, potassium phosphate buffer of 25 mM in 2.7 mL and 100 uL enzyme extract. Activity was determined in terms of umol/mg min protein.

Determination of superoxide dismutase (SOD) activity

Zhou *et al*., (1997) method was followed to determine SOD activity by using spectrophotometer assay. First of all, beaker containing 3 ml of reaction mixture was prepared, 2.725ml reaction substrate (containing NBT 15.5mg, Riboflavin 0.02mg, NaEDTA 10mg, and methionine 485mg in 250 mL), 25µl H_20_2 and 25µL enzyme extract. The reaction mixture was kept at 4000lux light intensity for 20minutes; In addition, both conditions i.e. dark and light were used for control sample. Distilled water (25 µl) was used in control sample instead of enzyme extract. At 560 nm absorbance were measured.

Determination of peroxidise (POD) activity

POD activity was determined as described by Zhou *et al*., (1997). The reaction mixture contained 100 µL enzyme extract, 100 µL of 0.4 % H_2O_2, 100 µL of 1.5% guaiacol (used as a substrate) and 2.7 mL of 50mM potassium phosphate buffer (pH 6.1). At 470 nm increase in the absorbance were measured. Activity of enzyme were calculated and determined in terms of umol/mg min protein.

Statistical analyses

Using SigmaPlot 12.0 for unpaired t-test, statistical analysis was performed for constant variance and normal distribution of data. Significance level was used at p 0.05*; 0.01**; 0.001***, in all figures.

RESULTS AND DISCUSSION

Viable and an impeccable agriculture system for increasing world population are vital to improve and tolerate human health, benefit producer and consumers that produce enough food and also protect the environment. Water inadequacy has a great influence on crop yield. It is not a negligible question nowadays, as the weather has become more irregular and unreliable. Therefore, adaptation of non-tolerant plant to drought is a great challenge. On the basis of morpho-physiological and biochemical characteristics, the selection of tolerant genotypes is the main goal of present research work.

Drought influence on growth of maize roots

Two maize varieties i.e., Azam and Baber were grown in green house for drought stress experiment under long day condition to evaluate the response of roots to water shortage in terms of growth and enzymatic activities of antioxidant system. The response of antioxidant system in leaves upon water shortage was already published (Khan *et al.,* 2016). In current study, roots were evaluated during drought using antioxidant system. The withdrawal of water for 7 days, the RWC in drought stressed plants decreased to approximately 56 and 51% in leaves of Azam and Baber, respectively whereas the control plants remained at full water capacity (90 and 95 %) (Khan *et al*., 2016). This almost 50 % reduction in the RWC is suggesting the proper drought stress condition (Fig. 1A). In addition, root fresh weight was also reduced by 38 and 30 % in Azam and Baber, respectively upon the water stress.

This decrease in root growth rate is common phenomena under drought stress in the crop plants (Nayyar and Gupta, 2006). The result of the effect of drought stress on total soluble proteins content is somehow different. It revealed that protein contents in roots of maize plant decreased under drought stress as compared to control. In roots upon water stress, this decrease was approximately 50% when compared with the control (Fig. 1C). Similar reduction in total proteins was also reported by Jha and Dubey, (2004) in rice seedling, when exposed to drought stress.

Impact of drought on cations (Na^+, Ca^{++} and K^+)

In most plants osmotic adjustment along with ion balance is considered key factors for plant growth and development (Zeng *et al*., 2003). In roots of Azam and Baber, increase in Na^+, Ca^{++} and K^+ was noted with increasing in drought stress. Both the varieties showed an accumulation of these ions in roots when subjected to

drought. Taking together, this increase was between 1.5 to 2.3-fold under drought compared to control (Fig. 2A, B & C).

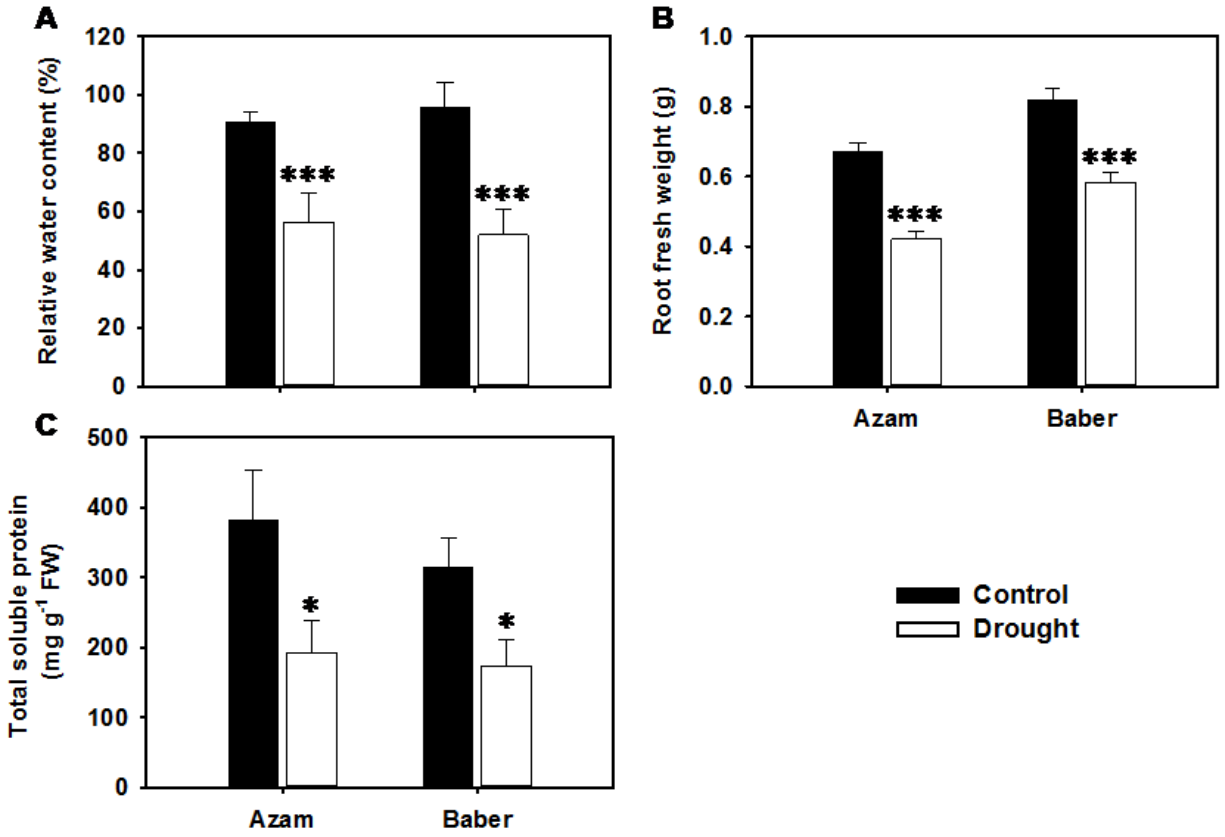

Fig. 1. Response of root growth to drought stress. Maize plants grown on soil for 2 weeks and then subjected to drought stress for 7 days, (A) Relative water content, (B) Root fresh weight (C) Total soluble proteins. Asterisks in all figures indicate significance level at p 0.05*; 0.01**; 0.001***

The effect of water stress on the level of stress markers

Hydrogen peroxide (H_2O_2) is an important stress marker and accumulated when plants experiences water shortage (Ahmad *et al.,* 2016). H_2O_2 contents were determined in roots of control and drought stressed plants in order to know severity of oxidative stress. In roots, 2.2-fold increase was recorded in Azam variety in the level of H_2O_2 upon drought in comparison to unstressed roots. Similarly, this accumulation was 1.3-fold in roots of Baber variety relative to control (Fig. 3A). This result showed that ROS formation occurred upon the exposure of roots to water stress.

An important marker of oxidative stress is the peroxidation of lipids. The greatest detrimental effect of ROS in plants is the lipid peroxidation. To govern level of lipid destruction, membrane damage is sometimes taken as a single important indicator under various stresses that sometime lead to cell death. First target for many stresses are cell membrane thus the conservation of cell membrane under water stress is important in drought tolerance (Bajji *et al.*, 2002).

Here, it has been noted that when the roots of maize plants were exposed to drought stress then they also showed high accumulation of almost 2.8 and 2.3-fold in the MDA level in Azam and Baber, respectively than unstressed roots (Fig. 3B). Both varieties responded in the same way by increasing the level of MDA suggesting that the elevated level of ROS also caused the lipid peroxidation. Excess generation of H_2O_2 and consequently increase in MDA contents showed that roots are the primary target of oxidative stress causing damages to membrane lipids and proteins (Mittler, 2002; Gill and Tuteja, 2010; Hasanuzzaman *et al.,* 2017).

Response of the ROS scavenging enzymes to drought

Plant cells secure themselves against the dangerous oxygen intermediates by increasing the defence mechanisms to keep their level at minimum. Elimination of ROS is necessary for the normal function of cells and enzymes and especially for Calvin cycle in chloroplast. For H_2O_2 detoxification, ascorbate peroxidase plays a key

role in converting H_2O_2 into water. We also observed an increase in the activity of APX between control and drought stressed maize roots. Taking together, almost 1.7 to 1.9- fold accumulations in the activity of APX in roots of both varieties Azam and Baber were observed upon the onset of drought in comparison to non stressed roots (Fig. 4A).

CAT is essential enzyme for ROS detoxification that is localised to peroxisomes. An average increase of 2-fold was observed in the drought stressed plants when compared with the non-stressed in Azam variety. This increase in the activity of catalase was about 1.7-fold in Baber variety (Fig. 4B). Similar increase was observed in CAT activity in *Oryza sativa* by Wang *et al.,* (2005). SOD acts a first line of defence and helps to minimize the superoxide radical (Noctor *et al*., 2014). The activity of SOD was high under drought stress in roots of both varieties in comparison to control (Fig. 4C). This increase was almost 1.4-fold under the stress treatment suggesting that the SOD induction in root helps to minimize the detrimental effect of ROS and converting it to less toxic form. This result is in line with Lee *et al.,* (2013), who also reported increased SOD production resulting from drought in the seedlings of Arabidopsis and tobacco.

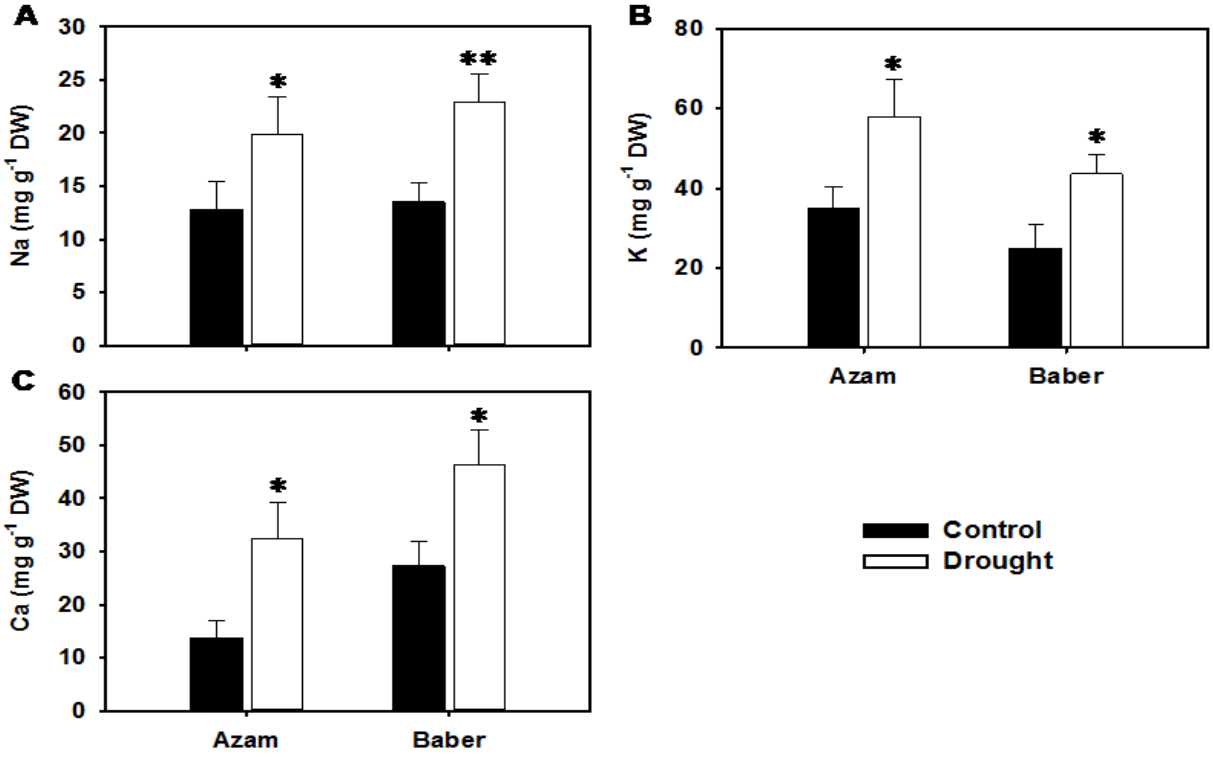

Fig. 2. Response of cation to drought stress in roots. Maize plants grown on soil for 2 weeks and then subjected to drought stress for 7 days, (A) Na level (B) K level (C) Ca level. Asterisks in all figures indicate significance level at p 0.05*; 0.01**; 0.001***

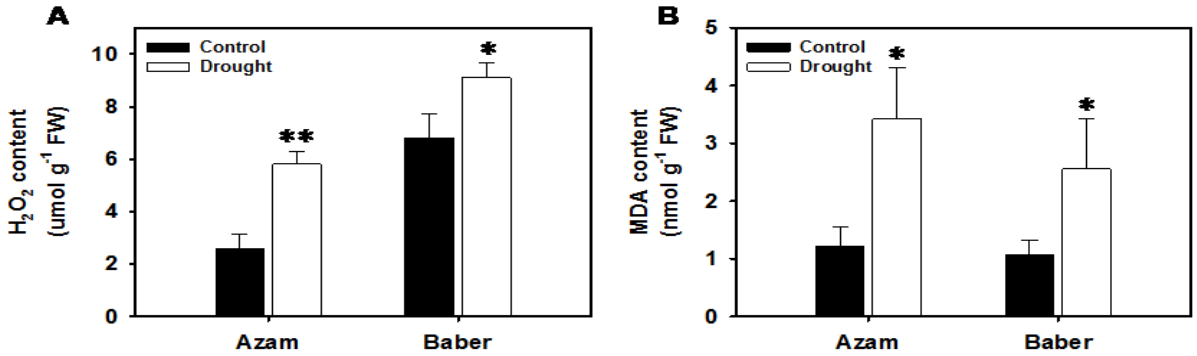

Fig. 3. Response of stress markers to drought stress in roots. Maize plants grown on soil for 2 weeks and then subjected to drought stress for 7 days, (A) Hydrogen peroxide level (B) MDA level. Asterisks in all figures indicate significance level at p 0.05*; 0.01**; 0.001***

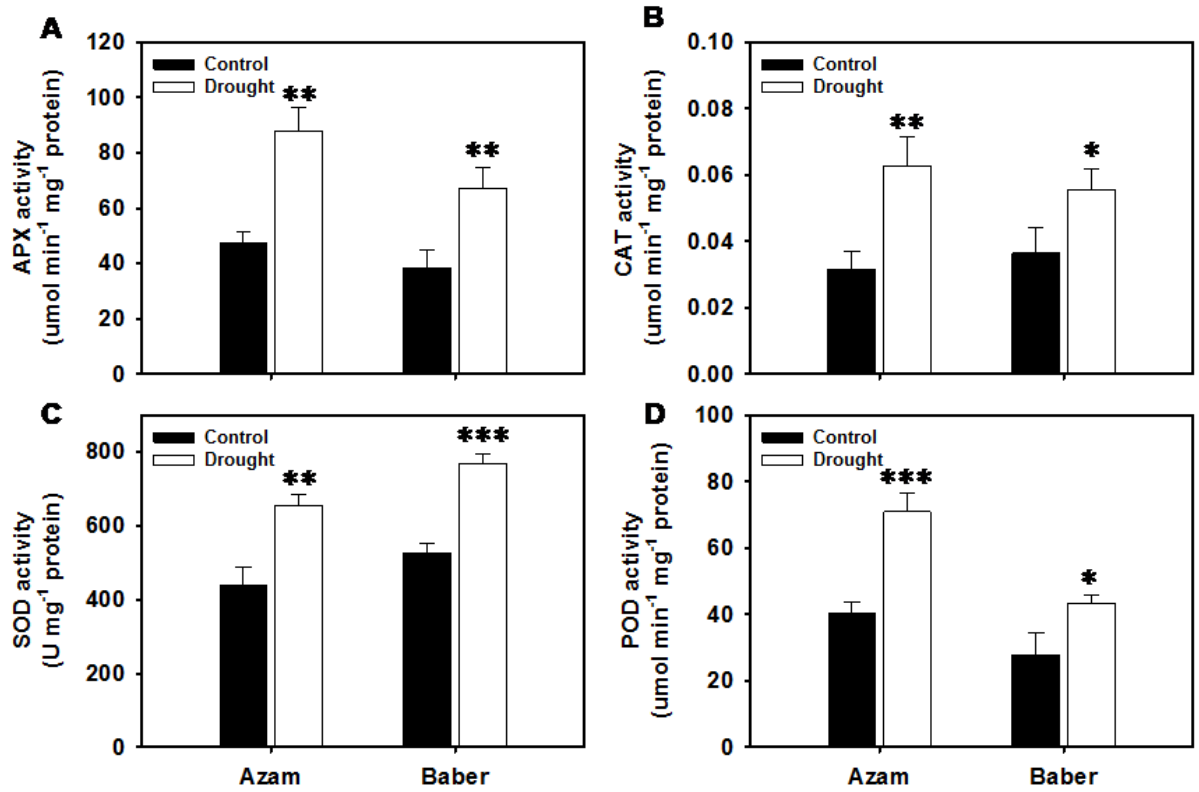

Fig 4. Response of enzymes of the defense mechanism to drought stress in roots. Maize plants grown on soil for 2 weeks and then subjected to drought stress for 7 days, (A) APX (B) Catalase (C) SOD and (D) POD activities. Asterisks in all figures indicate the significance using the unpaired t-test: *, $0.05 \geq p > 0.01$; **, $0.01 \geq p > 0.001$; ***, $p \leq 0.001$. Mean ± standard deviations are shown.

Peroxidase (POD) helps in decreasing H_2O_2 content and lipid peroxidation. POD activity in roots of both genotypes of Azam and Baber increased in the drought stress as compared to the control. Approximately 1.7-fold increase was recorded for Azam variety whereas 1.5-fold for Baber (Fig. 4D). This is in agreement with Zhang *et al.,* (2006), who found that soybean plants have higher peroxidase activity under drought stress.

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