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PHYSIOLOGICAL RESPONSES OF RICE (*Oryza sativa* L.) TO ZINC TREATMENTS UNDER DROUGHT STRESS

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ABSTRACT

Rice, as one of the major cereal crops, is severely affected by water deficit in many regions. To better understand zinc role under drought stress, a hydroponic trial was conducted in the Bogor Agricultural University greenhouse where daytime temperature were 34°C to investigate the effects of zinc on plant growth, antioxidant enzyme activities, and malondialdehyde content in two rice cultivars IR64 and INPAGO5 under drought stress. Rice seedlings were grow on modified Yoshida nutrient solution with three different zinc concentrations, i.e., 0.15, 0.3 and 0.6μM and one control. The results showed that zinc promoted rice growth subjected to drought by increasing leaf length, plant height and root length in both rice cultivars except leaf length of INPAGO5 prior drought stress. Superoxide dismutase and catalase activities were significantly increased in IR64. In INPAGO5, increase of catalase activity was not significant, whereas superoxide dismutase activity was significantly increased. Peroxidase activity was less in INPAGO5 than that of IR64. Malondialdehyde accumulation resulting from lipid peroxidation was significantly reduced in root cell of both cultivars under zinc treatment after 8 days of drought. These results suggest that zinc application increased antioxidant enzyme activities and resistance to drought stress. Among zinc concentrations applied, 0.3μM seems more beneficiary for rice to overcome drought stress. An adequate zinc concentration could be useful in preventing any damage in case of short period of drought.

Keywords: Rice cultivars, antioxidant, drought, zinc, malondialdehyde

INTRODUCTION

Rice (*Oryza sativa* L.) is an important food crop around the world which feeds more than half the world's population (Khush, 2005). It is cultivated under various climatic conditions such as the tropics, subtropics, semi-arid tropics and temperate regions. An adequate water and nutrient supply promote rice growth and increase its grain yields in quantity and quality. However, plants including rice are exposed to several environmental constraints that hinder their growth and development. Among these, drought is one of the most dangerous and causes severely damages at the cellular, tissue, organs level and even the whole plant. It severely limits plant growth and development which lead to crop losses by deregulating plant defense systems, modifying plant physiological, biochemical, (Upadhyaya and Panda, 2004; Upadhyaya *et al*., 2008) and molecular processes during vegetative and reproductive phases. Most environmental stresses including drought increase the production of reactive oxygen species (ROS). Plant cell membranes are damaged due to an accumulation of malondialdehyde (MDA) resulting from lipid peroxidation (Dhindsa *et al*., 1981). Among many plant defense systems against environmental stresses, antioxidative defenses, osmotic adjustment (Mahajan and Tuteja, 2005) and gene expression are major mechanisms that help plant to tolerate drought stress. Antioxidant enzymes such as catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) act through detoxification of ROS from plant cells and maintain the balance between plant antioxidative defense and the oxidative generation (Gill and Tuteja, 2010).

Zinc plays a crucial role in many functions of plants, animals and human. Zinc-deficiency in plants reduces crop yields, and the quality of crop products is severely affected (Alloway, 2008). As one of the essential microelements in plant, zinc is a component of carbonic anhydrase and a stimulator of aldolase (Satcher *et al.*, 2011; Tsonev and Lidon, 2012), nitrogen metabolism, cell multiplication, pollen formation, photosynthesis, auxin and carbohydrate synthesis, maintenance of the integrity of biological membranes, resistance to infection by certain pathogens, nucleic acid and lipid metabolism in plants (Marschner, 1986; Pahlsson, 1989; Alloway 2008). Some previous

investigations on various plant species such as cotton (Wu *et al*., 2015), sunflower (Eslami and Dehghanzadeh, 2014), red cabbage (Hajiboland and Amirazad, 2010), winter wheat (Karim *et al.*, 2012), safflower (Movahhedy-Dehnavy *et al.*, 2009), tea (Upadhyaya *et al*., 2013) showed the relevant role that zinc plays in drought tolerance. Although in plant zinc is important, it is highly toxic and impairs plant growth and development at high concentration. An adequate zinc supply is necessary in order to maintain its function in plants. However, several factors such as neutrality to alkaline soils, high phosphate status, low organic matter content, acidic soil of low total zinc status, calcareous soils, permanently wet (waterlogged) under paddy rice, high bicarbonate and/or magnesium in soil or in irrigation water remain the main causes of its deficiency in crops and soil (Alloway, 2008).

The objective of this research was to investigate the effectiveness of zinc in alleviating the adverse impacts of drought in rice. For this reason, we hypothesized that zinc could reduce any detrimental effects of drought in rice by increasing the enzymatic activities and reducing lipid peroxidation production. This research specifically determined zinc impacts on growth, some antioxidant enzymatic activities, and malondialdehyde content in two rice cultivars under drought stress.

MATERIALS AND METHODS

Plant growth conditions and treatments: Two cultivars of rice IR64 and INPAGO5 seeds were sterilized with 0.5% NaClO solution for 20 minutes, rinsed four times with deionized water, soaked in distilled water at 27ºC for 3 days for seed imbibition, and germinated in petri dish in dark condition at 27ºC for 3 days. Rice seedlings of uniform size were selected and transplanted to 2 liter container containing modified Yoshida solution in the greenhouse where daytime temperature was 34ºC . Each container contained 20 seedlings and the experiment was repeated three times.

The composition of Yoshida nutrient solution and the concentration of each element were as follows: 40 ppm NH_4NO_3, 10 ppm $NaH_2PO_4 \cdot 2H_2O$, 40 ppm K_2SO_4, 40 ppm $CaCl_2$, 40 ppm $MgSO_4 \cdot 7H_2O$, 0.5 ppm $MnCl_2 \cdot 4H_2O$, 0.05 ppm $(NH_4)_6 \cdot MO_7\ O_{24} \cdot 4H_4O$, 0.2 ppm H_3BO_3, 0.01 ppm $CuSO_4 \cdot 5H_2O$, 2 ppm $FeCl_3 \cdot 6H_2O$, H_2SO_4 and Citric acid (monohydrate) (Yoshida *et al.,* 1976). The concentrations of zinc in the nutrient solution varied according to the treatments. The seedlings were grown in three different concentrations of zinc i.e., 0.15, 0.3 and 0.6 µM and one control. The pH of the solution was set to 5.0 until the seedlings were 21 days old. The culture solution was changed two times per week. After 21 days, the seedlings were transplanted to the nutrient solution containing 10 % PEG-6000 to simulate drought stress. The longest duration of stress was 8 days. Fresh leaf and root samples of both cultivars were collected at 0, 3 and 8 days of drought stress and stored at -80ºC for physiological and biochemical analysis.

Leaf length, plant height and root length measurement: Leaf length, plant height and root length were measured the first day of drought and 8 days after drought stress treatment by using a ruler. Plant height was measured from the base of shoot to the tip of longest leaf.

Superoxide dismutase enzyme activity: Superoxide Dismutase (SOD; EC 1.15.1.1) activity was determined by following the method described by Wu *et al*. (2014) with slight modification. Briefly, 0.125 g fresh leaf was ground on ice in 5 ml of chilled extraction buffer containing 50 mM sodium phosphate (pH 7.8), and the homogenate was centrifuged at 3000 g for 15 min at 4ºC. A reaction mixture containing 1.5 ml 50 mM sodium phosphate (pH 7.8), 300 µl 130 mM methionine, 300 µl 750 µM nitro-blue tetrazolium (NBT), 300 µl 100 µM EDTA-Na_2, 300 µl 20 µM riboflavin, 100 µl enzyme extract and 100 µl distilled water was illuminated for 15 min under fluorescent lamp and the sample absorbance was read at 560 nm at 30 second interval for 3 min. One unit of SOD activity was defined as the amount of enzyme corresponding to 50% inhibition of the NBT reduction.

Catalase enzyme activity: Catalase (CAT; EC 1.11.1.6) activity assay was determined by following the method used by Wu *et al*., (2014) with little modification. 0.125 g fresh leaf was ground on ice in 5 ml of chilled extraction buffer containing 50 mM sodium phosphate (pH 7.8) using mortar and pestle. The homogenate was centrifuged at 3000 g for 15 min at 4ºC. The absorbance of the reaction mixture containing 1.5 ml 50 mM sodium phosphate (pH 7.8), 300 µl 0.1 M H_2O_2, 200 µl enzyme extract and 1.0 ml distilled water was read at 240 nm at 1 minute interval for 2 minutes.

Peroxidase enzyme activity: Peroxidase (POX; EC 1.11.1.7) enzyme activity determination followed the method as described by Kar and Mishra, (1976) with slight modification. The leaf (0.125 g) was homogenized with 5 ml of chilled phosphate buffer pH 6.8 (0.1 M) on ice by using mortar and pestle. The homogenate was centrifuged at 4ºC for 15 min at 3000 g. 100 µl of enzyme extract (supernatant) was added to 2.5 ml of 0.2 M pyrogallol, and 250 µl of 1 % H_2O_2 was added to the mixture. The absorbance was then read at 420 nm at 30

second interval for 2 minutes after 4 minutes of reaction at 25°C.

Malondialdehyde determination: Malondialdehyde content was determined by applying the method of Quan *et al*., (2004) with slight modification. Malondialdehyde (MDA) concentration was determined as follows: 0.1 g of fresh rice root was homogenized in 5ml of 10% trichloroacetic acid (TCA), centrifuged at 3600 *g* for 34 min at 25°C. Four milliliters of 0.6% thiobarbituric acid (TBA) in 10% TCA were added to 2 ml of the supernatant. The mixture was heated in boiling water at 80°C for 40 min, and then cooled in an ice bath. After centrifugation at 3600 *g* for 10 min, the absorbance at 450, 532 and 600 nm was determined. The MDA content (μmol g^{-1} FW) was calculated by the following formula:

$$[MDA] = 6.45(A_{532} - A_{600}) - 0.56A_{450}$$

Where A_{450}, A_{532} and A_{600} represent the absorbance of the mixture at 450, 532 and 600 nm respectively.

Statistical analysis: Analysis of variance (ANOVA) was performed using SPSS software 23.0 edition. The means comparison was analyzed using Duncan Multiple Range Test with $\alpha = 0.05$.

RESULTS

Leaf length: Before onset of drought (3 weeks after germination without drought stress), leaf length significantly increased in IR64 ($P<0.05$) as the concentration of Zn increase. However, there was no difference in leaf length between control and 0.15μM Zn treatment, whereas the leaf length at 0.30 and 0.60μM Zn was significantly higher than that of the control. In INPAGO5, the leaf length was not significantly different among treatments (Figure 1a). After 8 days of drought (Figure 2a), leaf length significantly increased in all zinc treatments in both cultivars ($P<0.05$) compared to the control. The maximum length was reached for treatments 0.3 and 0.6μM Zn in IR64, whereas in INPAGO5 the maximum length was reached for treatment 0.6μM Zn.

Plant height: Prior to the drought stimulation, plant height insignificantly increased in IR64 as compared to the control, whereas statistically, a significant increase in plant height was recorded in INPAGO5 cultivar. Plant height maximum reached for treatment 0.6 μM in both cultivars (Figure 1b). As can be seen in Figure 2b, the increase in plant height after 8 days of drought was observed in all levels of zinc treatments in both cultivars compared to the control. The maximum height reached at 0.3 μM in both cultivars INPAGO5 and IR64.

Root length: Root length significantly increased ($P< 0.05$) in both cultivars at all treatment levels in which the maximum length was in 0.3 μM (Figure 1c) before drought stress treatment started (3 weeks after germination). A particular attention of root length measurement after 8 days of drought (Figure 2c) indicated that in both cultivars, there was an increase in root length in all treatments as compared to the control. However, the difference in root length was only significant in INPAGO5 cultivar.

Superoxide dismutase enzyme activity: SOD activity before drought stress was higher at the control level as compared to other treatments in INPAGO5, whereas it was low in IR64 (Figure 3a). After 3 days of drought, SOD activity insignificantly increased in IR64 in all treatment levels dropped in both cultivars; but in IR64 there was an insignificant increase with 0.6μM Zn compared to the control. In INPAGO5, there was no difference of SOD activity among the treatments (Figure 3b). According to the statistical analysis, SOD activity significantly enhanced in both cultivars after 8 days of water stress as compared to the control ($P< 0.05$). The SOD activity increased by 25, 59, and 50% in INPAG05, whereas in IR64 it increased by 60, 60 and 73% at 0.15, 0.3 and 0.6μM as compared to the control. The maximum activity was reached in 0.6μM of zinc in IR64 and 0.3μM in INPAGO5 (Figure 3c) which indicate that zinc significantly increased SOD activity in both cultivars after 8 days drought stress.

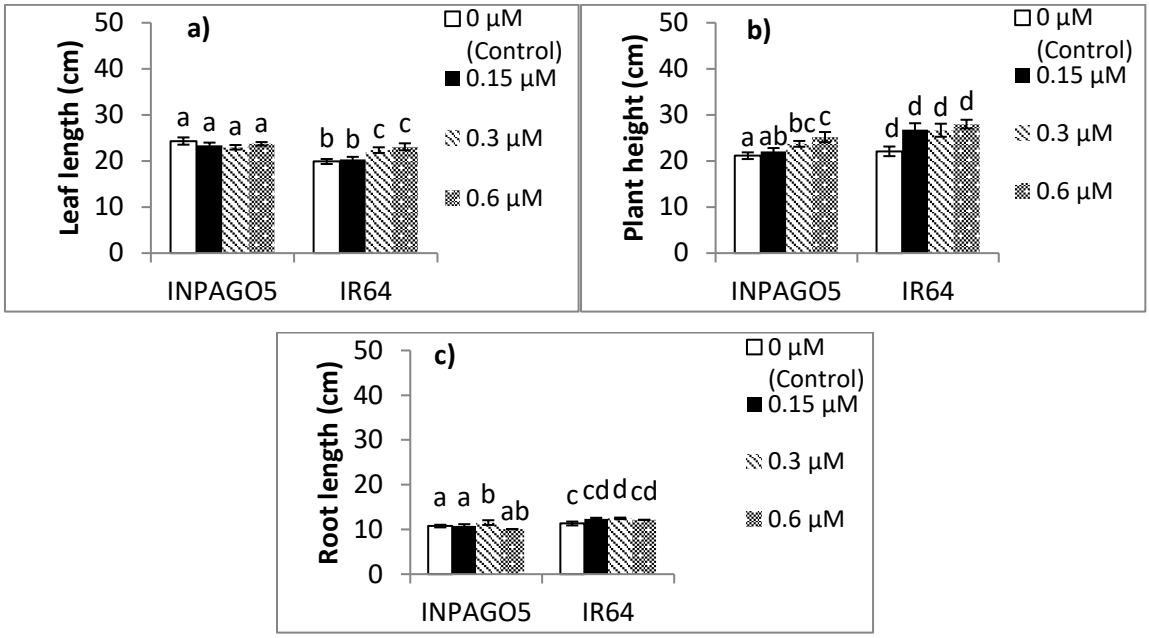


Figure-1: Zinc application effect on leaf length (a), plant height (b) and root length (c) in two rice cultivars INPAGO5 and IR64 before drought in modified Yoshida solution (0, 0.15, 0.3 and 0.6 µM Zn). Bars represent standard error of mean. Dissimilar letters indicate difference between treatments.

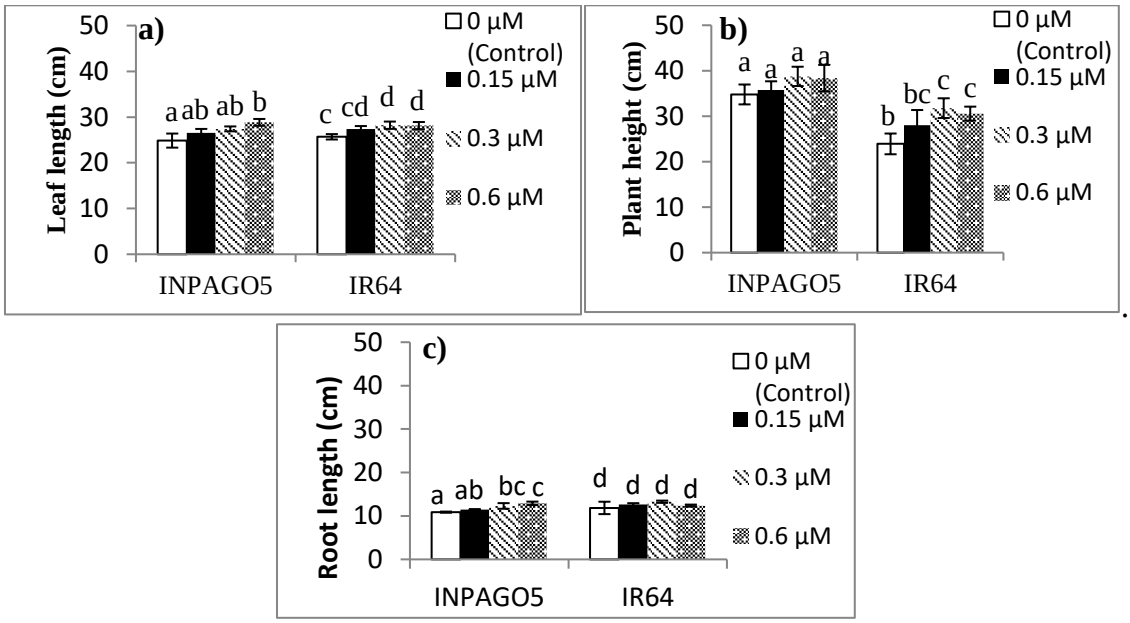
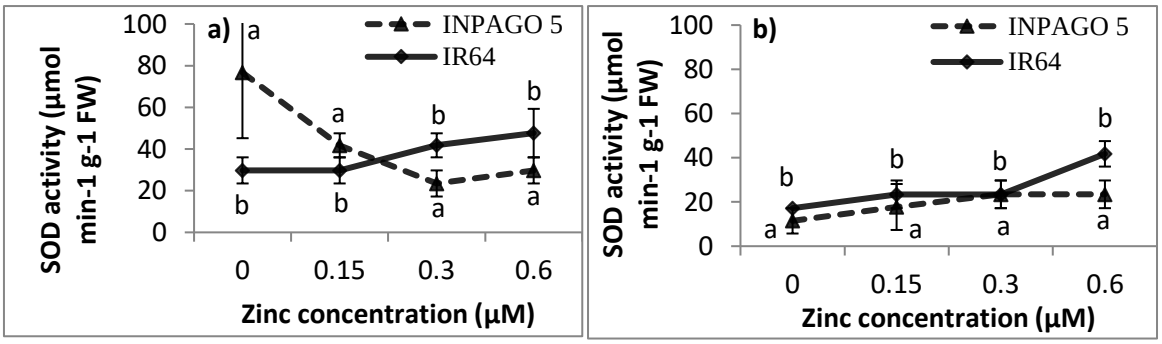


Figure-2: Zinc application effect on leaf length (a), plant height (b) and root length (c) in two rice cultivars INPAGO5 and IR64 after 8 days of drought in modified Yoshida solution (0, 0.15, 0.3 and 0.6 µM Zn). Bars represent standard error of mean. Dissimilar letters indicate difference between treatments.



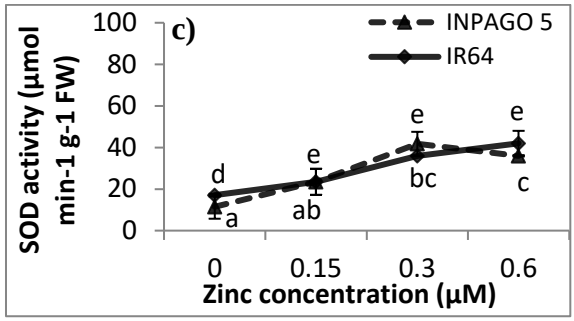


Figure-3: Zinc application effect on SOD activity in two rice cultivars INPAGO5 and IR64 before drought (a), 3 days (b) and 8 days (c) of drought in modified Yoshida solution (0, 0.15, 0.3 and 0Zn). Bars represent standard error of the mean. Dissimilar letters indicate difference between treatments.

Catalase enzyme activity: Prior to the drought stimulation, CAT activity was higher in 0.3µM Zn treatment in IR64, whereas in INPAGO5, 0.6µM Zn showed a higher activity of CAT (Figure 4a). In IR64, there was a significant increase of CAT activity in 0.3 and 0.6µM Zn treatments ($P < 0.05$) after 3 days of water stress, whereas in INPAGO5, the activity of CAT was slightly increased as compared to the control (Figure 4b). As shown in Figure 4c, catalase activity significantly increased in IR64 ($P < 0.05$) in all treatments after 8 days of drought stress, whereas in INPAGO5, the increase in cata-lase activity was not significant. It increased by 19, 29%, in INPAGO5 at 0.3 and 0.6µM Zn decreased by 5% at 0.15µM Zn, and in IR64, it increased by 42, 65 and 55% at the treatments of 0.15, 0.3 and 0.6µM Zn respectively compared to the control. The highest activity was obtained at 0.3 and 0.6µM in IR64 and INPAGO5 respectively. Zinc increased catalase activity in both rice cultivars.

Peroxidase enzyme activity: An increase in POX activity in both cultivars was noted before the drought stimulation started (Figure 5a). This increase was only significant in IR64. The greater POX activity was reached at 0.6µM Zn treatment in both cultivars. As showed in Figure 5b, after 3 days of drought stress, there was a significant difference in POX activity in INPAGO5 cultivar. The highest POX activity reached with 0.3µM Zn treatment in IR64 and INPAGO5 compared to the control. At the end of drought stress (Figure 5c), the increase of POX activity was not significant in both cultivars. The activity of POX was decreased by 23 and 6% at 0.15 and 0.6µM Zn respectively, and increased by 10% at 0.3µM Zn in INPAGO5 compared to the control. However, in IR64, POX activity was increased by 36, 56 and 48% at 0.15, 0.3 and 0.6µM Zn respectively. Zinc only increased POX activity in IR64 cultivar.

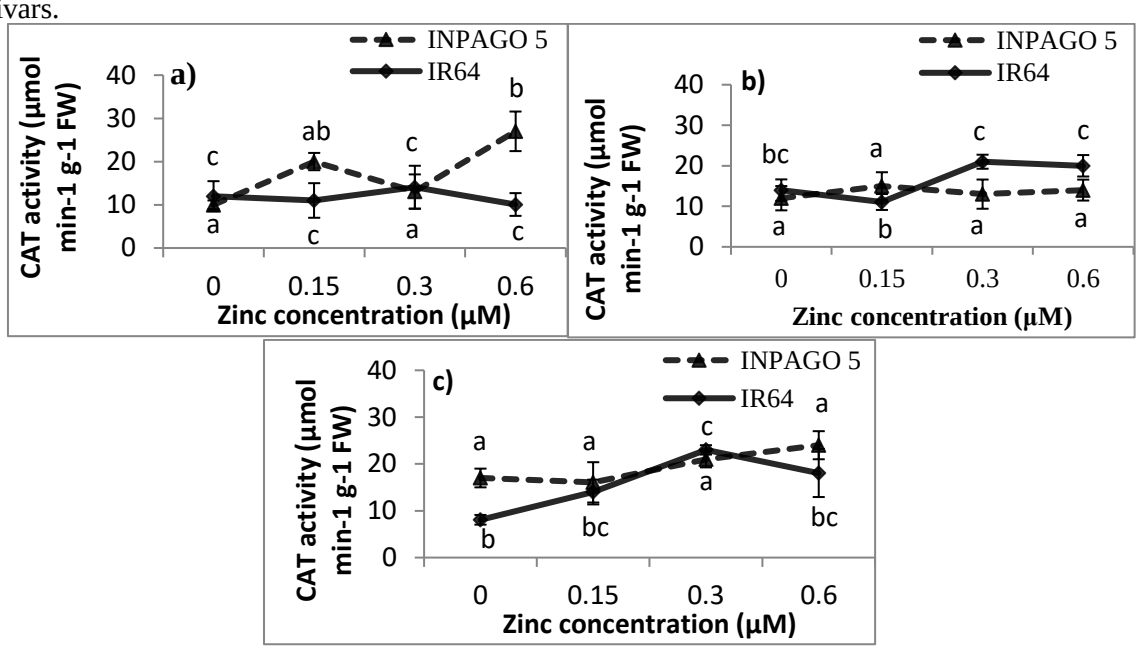


Figure-4: Zinc application effect on CAT activity in two rice cultivars INPAGO5 and IR64 before drought (a), 3 days (b) and 8 days (c) of drought in modified Yoshida solution (0, 0.15, 0.3 and 0.6µM Zn). Bars represent standard error of the mean. Dissimilar letters indicate difference between treatments.

Malondialdehyde content: The results showed that before drought treatment (Figure 6a), MDA content was lower in INPAGO5 in all treatments as compared to the control. After 3 days of drought (Figure 6b), a reduction in MDA content was observed in all treatments in IR64, whereas in INPAGO5, it was observed an increase in MDA content in all treatments except 0.6µM Zn treatment. As can be seen in Figure 6c, MDA content was significantly influenced in both cultivars by drought and the use of zinc. It was recorded a significant decrease in MDA accumulation after 8 days of drought in both cultivars in all zinc treatments compared to the control ($P< 0.05$). MDA content decreased by 19, 23 and 36% in INPAGO5 and by 13, 32 and 32% in IR64 at 0.15, 0.3 and 0.6µM Zn respectively. The minimum MDA Zn treatment in both cultivars. Zinc considerably reduced MDA content in both rice cultivars after 8 days of drought stress.

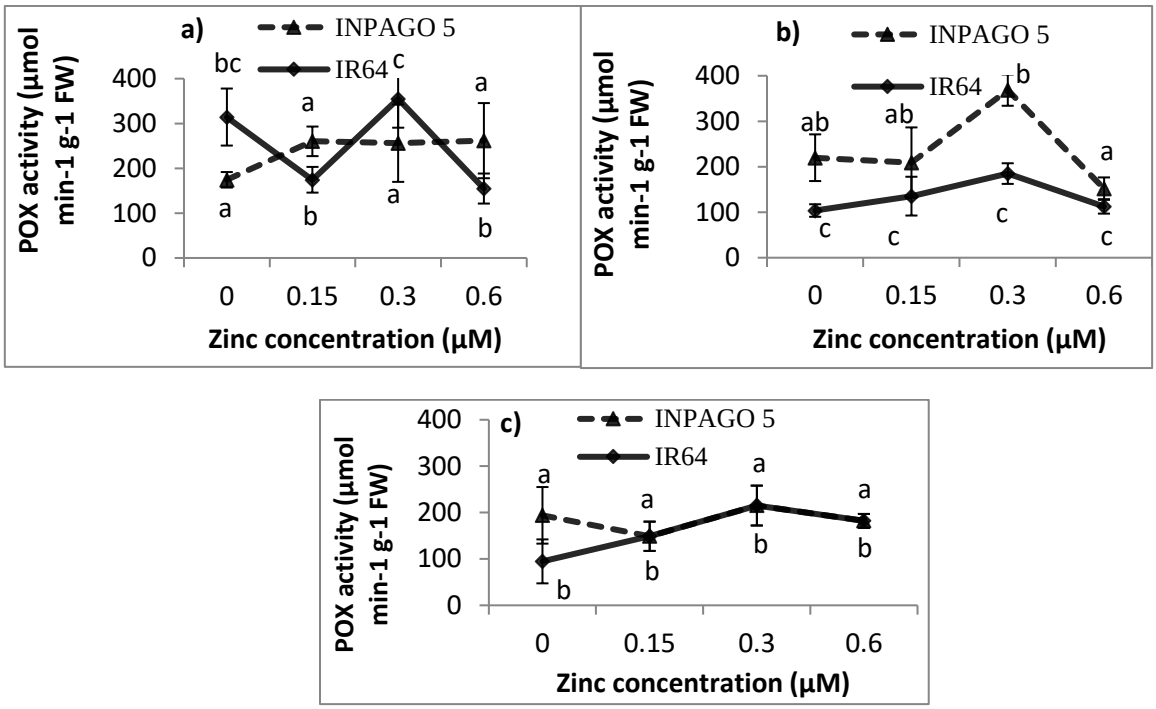


Figure-5: Zinc application effect on POX activity in two rice cultivars INPAGO5 and IR64 before drought (a), 3 days (b) and 8 days (c) of drought in modified Yoshida solution (0, 0.15, 0.3 and 0.6µM Zn). Bars represent standard error of the mean. Dissimilar letters indicate difference between treatments.

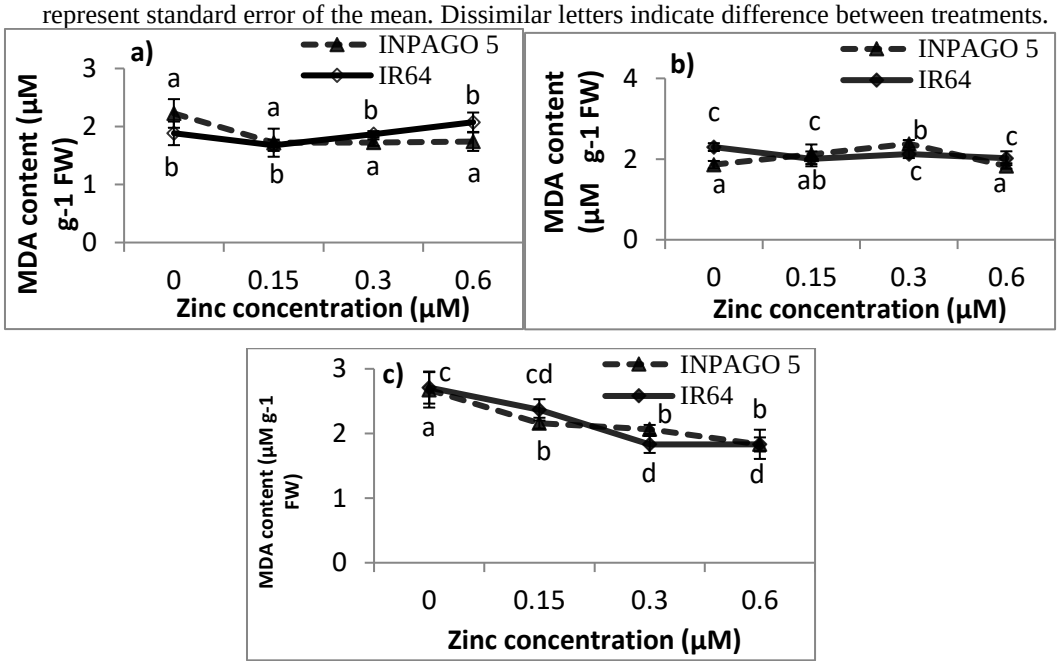


Figure-6: Zinc application effect on MDA content in two rice cultivars INPAGO5 and IR64 before drought (a), 3 days (b) and 8 days (c) of drought in modified Yoshida solution (0, 0.15, 0.3 and 0.6µM Zn). Bars represent standard error of the mean. Dissimilar letters indicate difference between treatments.

DISCUSSION

This study showed that zinc improved rice growth by increasing leaf length, plant height and root length after drought (Fig.2). It seems that zinc up-regulates the synthesis of some plant growth regulators such as auxin, gibberellin and cytokinin which are involved in cell division, expansion and elongation in different part of plant. It has been shown that application of gibberellin (GA3) and kinetin increased rice growth under water stress conditions (Khan *et al*., 2016a). Khan *et al*., (2016b) reported that application of different $ZnSO_4$ concentrations on *Brassica junceae* signify-cantly increased root length, plant height under water deficit. Wu *et al*., (2015) noted that the cotton growth could be improved by zinc under drought. It has also been shown that zinc foliar application increased plant height under drought stress (Thalooth *et al*., 2006).

Various environmental stresses affect the physiological, biochemical and molecular processes of plants. Plant exposure to environmental stresses such as drought, heavy metal, salinity, cold, high light, and extreme temperature leads to overproduction of reactive oxygen species in plant cells. ROS compounds are highly toxic and cause oxidative damage at cellular level. To prevent or repair these damages, plant cells increase antioxidant activities. SOD, CAT and POX are some of antioxidant enzymes present in plant that play a crucial role in detoxification of ROS compounds. SOD is the first key enzyme that plays a full role of protecting the plant cell membrane against super-oxide anion O_2^- (Gratao *et al*., 2008). SOD converts superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) which is then converted by CAT to water and oxygen molecule (Bowler *et al*., 1992; Willeken *et al*., 1997) for preventing the cellular damages under any environmental stresses that generate overproduction of ROS. In this study, it was found that SOD and CAT activities increased in both rice cultivars after 8 days of drought stress (Fig.3c, 4c). Zinc effect on POX activity was more effective in IR64 cultivar than in INPAGO5 after 8 days of drought stress (Fig.4c). The increase in SOD activity is related to an increase of free radical production especially superoxide anion. The increase in SOD and CAT activities showed that zinc reinforced antioxidative defense system in both rice cultivars so that they can easily detoxify all excess of free radicals from plant cell to better tolerate drought and reduce its harmful effects on growth and metabolism process. Zinc concentrations of 0.3 and 0.6μM efficiently enhanced the ability of antioxidant enzymes for reducing oxidative damages. It could be deduced from this experiment that among the three antioxidant enzymes assayed, SOD and CAT were more involved in ROS-scavenging. However, in IR64 cultivar, zinc increased POX activity. Zhang *et al*., (2014) found that zinc finger protein 36 gene (ZFP36) increased some enzyme activities such as superoxide dismutase and ascorbate peroxidase for increasing rice plants tolerance against water stress and oxidative stress. It has been showed that SOD, POX and CAT activities were increased under drought stress with zinc application in wheat and common bean (Yavas and Unay, 2016; Sharaf *et al.,* 2016). Zinc foliar application on sunflower (*Helianthus annuus L*.) under water deficit increased SOD activity (Zafar *et al*., 2014). Similarly, in cotton and corn, zinc application significantly increased the activities of SOD and CAT under drought stress (Moghadam *et al.,* 2013; Wu *et al.,* 2015). Weisany *et al*., (2012) reported that zinc application increased CAT and POX activities in soybean (*Glycine max* L.) under salinity stress. Zinc application increased SOD activity in tobacco (Yu *et al.,* 1998).

Malondialdenyde is the product of lipid peroxidation that indicates cell membrane damages during stresses (Moore and Roberts, 1998). A weak plant defense system is the cause of the permanence overproduction of ROS during environmental stresses that lead to high MDA accumulation and then cell membrane destabilization, which loses its properties and functions. In that condition, cells are exposed to any external attack that delays their metabolism, so that cell regeneration becomes a major problem to the plant. This experiment revealed that zinc deficiency in rice nutrition increased MDA accumulation during drought stress (Fig.5c). This accumulation could accelerate plant leaves senescence. Unlike control, MDA content significantly decreased in both cultivars in all zinc treatments. However, it was lower in 0.3 and 0.6μM treatments in both cultivars as compared to 0.15μM treatment and control. This decline in MDA accumulation was related to the enzyme activities which scavenged oxidative stress by reducing its influence on cell membranes. It also shows zinc-efficiency in both rice cultivars that are exposed to drought stress. Elimination of ROS excess from the tissue by enzyme activities avoids MDA accumulation in the plant cells. Zinc plays a relevant role in prevention or inactivation of ROS compounds, protection and stabilization of cell membrane against harmful effects of environmental stresses. This finding is in agreement with that of Wu *et al*., (2015) who found that MDA content was lower in zinc application in cotton than zinc-deficient under PEG-6000 simulated drought

stress. Zinc application reduced lipid peroxidation in soybean under salinity stress (Weisany *et al.*, 2012).

CONCLUSION

This research reveals that zinc reduces the harmful effects of drought stress in rice by increasing the antioxidant enzyme activities. In addition, 0.3μM zinc concentration seems more advantageous for rice under and without drought stress. Zinc may be used in adequate concentration especially in the regions in need for improving the quantity and quality of rice grain yield, and also for preventing any damage that can occur in rice plant during water deficit in short term.

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