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Selenium Pretreatment Regulates the Antioxidant Defense System and Reduces Oxidative Stress on Drought-Stressed Wheat (*Triticum aestivum* L.) Plants

Heba M. Ibrahim
Department of Botany, Faculty of Agriculture, Mansoura University, El-Mansoura, 35516, Egypt

Abstract: Drought stress is a worldwide problem that constraint plant productivity. The aim of this study was to assess the alleviative effect of two levels of selenium (Se), 5 and 10 mg L^{-1}, on drought oxidative stress of wheat cv. Giza 168 through monitoring the changes of the plant's antioxidant system and certain metabolites indicative for oxidative stress. Drought stress increased the accumulation of H_2O_2 and the lipid peroxidation product MDA whereas decreased the activities of the antioxidant enzymes CAT and SOD and the content of Ascorbic Acid (AsA). The activity of guaiacol peroxidase (POD) as well as the contents of proline and the non-enzymatic antioxidants, reduced glutathione (GSH) and alpha tocopherol (α-TQ) were increased in response to drought. In drought-stressed, Se-pretreated plants, H_2O_2 and MDA contents were decreased whereas the activities of the antioxidant enzymes CAT and SOD and the content of the non-enzymatic antioxidants AsA and GSH were increased resulting in elevated membrane stability index and root viability. On the other hand, the activity of POD as well as the contents of proline and the non-enzymatic antioxidant α-TQ were decreased in drought-stressed, Se-pretreated plants which may reflect the attenuation of oxidative stress due to Se pretreatment. The lower dose of Se was, generally, more favorable in this respect. Results indicate that Se pretreatment enhanced tolerance of wheat plants against drought oxidative stress through modulation of the plant's antioxidant system.

Key words: Antioxidants, drought stress, selenium, *Triticum aestivum* L.

INTRODUCTION

Drought is one of the major environmental factors that inhibits many metabolic processes and eventually constraints plant growth and crop productivity (Chaves and Olivera, 2004). Exposure of plants to drought is often associated with increased levels of Reactive Oxygen Species (ROS), such as superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Chaves *et al*., 2003). Accumulation of ROS leads to protein degradation, lipid oxidation and pigment bleaching (Nikolaeva *et al*., 2010). Plants have evolved specific protective mechanisms involving antioxidant molecules and enzymes in order to defend themselves against oxidants (Gholami *et al*., 2012). Detoxification of drought-induced ROS is accomplished by the antioxidant defense system comprising non-enzymatic and enzymatic components. Studies indicated a correlation between plant stress tolerance and antioxidant defense capacity (Reddy *et al*., 2004). In addition, plants cope with drought stress by metabolic adjustment involving overproduction of certain osmoprotectants. Se is not an essential element for plants (Terry *et al*., 2000). Nevertheless, it was reported to enhance plant tolerance to various stresses such as photoxidative stress (Seppanen *et al*., 2003), high temperature (Djanaguiraman *et al*., 2010), low temperature (Akladious, 2012), salt (Kong *et al*., 2005; Hawrylak-Nowak, 2009), heavy metals (Filek *et al*., 2008; Malik *et al*., 2011) and drought (Kuznetsov *et al*., 2003; Germ *et al*., 2007; Yao *et al*., 2009; Hasanuzzaman and Fujita, 2011; Nawaz *et al*., 2013; Yao *et al*., 2012; Zanjani *et al*., 2012). The Se induced plant stress tolerance was attributed to its ability to enhance the plant's antioxidative potential (Seppanen *et al*., 2003; Yao *et al*., 2009; Djanaguiraman *et al*., 2010; Malik *et al*., 2011). Se induced drought stress tolerance in wheat was almost tested in plants growing in pot cultures (Nawaz *et al*., 2013) and this ability remain to be affirmed in normal soil cultivations. In addition, plants subjected to water stress was treated with Se either as a spray solution or as a soil additive (Kuznetsov *et al*., 2003; Yao *et al*., 2009, 2012), whereas its application as seed soaking which facilitates adoption in large scale cultivations has received little consideration and the only available report about Se application as seed soaking (Nawaz *et al*., 2013) was conducted in pot culture. Therefore, the current investigation was conducted to evaluate the ameliorative

effect of seed priming with Se on drought stress of wheat growing in normal soil conditions and the underlying physiological and biochemical bases of this effect. I hypothesize that Se would attenuate impact of drought stress on wheat plants through modulating the plant's antioxidant defense system.

MATERIALS AND METHODS

Plant materials, growth conditions and application of treatments: The experiment was conducted in the soil of the Experimental Farm and Labs. of the Department of Agricultural Botany, Faculty of Agriculture, Mansoura University, Egypt. Physical and chemical characteristics of the experimental soil are shown in Table 1. Grains of wheat (*Triticum aestivum* L.), cv. Giza 168 [(chosen because it is classified as drought-sensitive cultivar (Almaghrabi, 2012)] were disinfested by immersion in a 2.5% solution of sodium hypochlorite for 10 min and washed thoroughly with distilled water and sown on November 5, 8 during the two successive seasons 2009-2010 and 2010-2011, respectively. Experiment was laid out in a split plot design with four replications. Irrigation schemes were assigned in main plots and Se treatments in subplots. The experimental unit dimensions were 2×3 m and contained 12 rows, 25 cm apart. Drought stress was imposed by withholding irrigation from 50-70 Days after Sowing (DAS) (late tillering to early flowering stages), while control plants were irrigated whenever necessary. At the end of dewatering period, soil water content was 23.2% in droughted plots vs. 50.6% in control plots. Except difference in irrigation scheme between control and drought-challenged plants, all other agricultural practices were applied to both similarly according to the normal recommended tillage practices for wheat. The Se treatments were applied by pre-sowing soaking of grains in the respective Se (Na_2SeO_4) solution (10, 20 mg L^{-1}).

At the 71th DAS, flag leaf samples were collected to determine membrane stability index (MSI) as well as some stress-related biochemical attributes as follows.

Table 1: Mechanical and chemical analysis of the used soil

Parameters	Values
CS (%)	11.20
FS (%)	27.60
S (%)	26.00
C (%)	32.70
$CaCO_3$ (%)	2.70
OM (%)	2.00
TN (%)	0.11
AP (ppm)	14.00
EK (ppm)	213.00
TSS (%)	0.20
ASe (ppm)	0.48

CS: Coarse sand, FS: Fine sand, S: Silt, C: Clay, OM: Organic matter, TN: Total N, AP: Available P, EK: Exchangeable K, TSS: Total soluble solutes, ASe: Available selenium

MSI was evaluated according to Premachandra *et al.* (1990). Leaf discs (0.1 g) were thoroughly washed in distilled water and then placed in 10 mL of distilled water at 40°C for 30 min. At the end of this period, their electrical conductivity was recorded (C_1). Subsequently, the same sample was placed in a boiling water bath (100°C) for 10 min and their electrical conductivity was also recorded (C_2). The MSI was calculated as:

$$MSI = \left[1 - \left(\frac{C_1}{C_2}\right) \times 100\right]$$

Root cell's viability was assessed by the reduction of triphenyltetrazolium chloride (TTC) by tissue to the red-colored insoluble Triphenylformazan (TF) according to Ruf and Brunner (2003).

Determination of metabolites indicative of oxidative stress: The H_2O_2 concentration in leaves was estimated by following the procedure of Velikova *et al.* (2000). Leaf tissues, 0.5 g were homogenized in an ice bath with 5 mL of trichloroacetic acid (TCA) 0.1% (w/v). The homogenate was centrifuged at 12,000×g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM K-P buffer (pH 7.0) and 1 mL of 1M KI. The absorbance of the mixture was measured at 390 nm. The MDA content which reflects the level of lipid peroxidation, was determined by grinding 0.5 g of leaves in 5 mL 1% trichloroacetic acid and centrifugation at 12.000×g for 12 min. The supernatant was incubated at 95°C for 30 min, cooled for 2 min in an ice bath and centrifuged at 15000×g for 10 min. The MDA content was then determined spectrophotometrically according to Meng *et al.* (2009).

Determination of the non-enzymatic antioxidants: Ascorbic acid (AsA) concentration in the leaves was determined according to the method of Cakmak and Marschner (1992). 0.2 mL of the plant extract was mixed with 0.5 mL phosphate buffer (150 mM, pH 7.4) containing 5 mM EDTA. For colour development, the following reagents were added: The 0.4 mL TCA (10%), 0.4 mL orthophosphoric acid (44%), 0.4 mL 1,2 bipyridine in 70% ethyl alcohol and 0.2 mL $FeCl_3$ (3%). The mixture was incubated at 40°C for 40 min. The absorbance was read at 525 nm. Glutathione (GSH) content was determined according to the Ellmann (1959) procedure as described by Coskun and Zihnioglu (2002). The 0.1 mL of the plant extract was mixed with 2 mL of Tris-HCl buffer (100 mM, pH 8.4) and 0.1 mL Ellmann reagent [60 mg DTNB (5,5`-dithiobis (2-nitrobenzoic acid)/100 mL Tris-HCl buffer (0.1M , pH 7.0)] and the absorbance of the reaction mixture was recorded at 412 nm. For extraction of α-TQ,

200 mg sample was homogenized with 1 mL acetone in a prechilled mortar and pestle at 4°C, then 0.5 mL hexane was added and the homogenate was fortexed for 30 sec then centrifuged at 1000×g for 10 min. The upper hexane layer was removed while the acetone layer containing vitamin E remained in the vial. The extraction process was repeated by adding a second 0.5 mL aliquot of hexane. The method of Kanno and Yamauchi (1997) was adopted to estimate α-TQ content. To 0.2 mL extract, 0.4 mL of 1% (w/v) 3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine was added and the volume was made up to 3 mL with absolute ethanol and 0.4 mL of 0.1% (w/v) $FeCl_3$. The $6H_2O$ was added, then the content was gently mixed under dim light in a dark room to avoid photochemical reduction. After 4 min at room temperature, 0.2 mL of 0.2 M orthophosphoric acid was added and the mixture was left for more 30 min. Absorbance was determined at 554 nm.

Determination of the activity of the antioxidant enzymes: Leaf tissues were homogenized (1:5 w/v) in an ice cold mortar and pestle using 50 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl, 1% polyvinylpyrrolidone and 1 mM EDTA. After centrifugation (20,000×g, 15 min), the supernatant (crude extract of leaves) was used to determine enzyme activities and will be denoted henceforth as enzyme extract, EE. All procedures were done at 4°C. CAT activity (EC 1.11.1.6) was assayed by the degradation of H_2O_2 according to Aebi (1984). Two hundred microlitter EE was added to 1.8 mL Reaction Mixture (RM) containing 50 mM K-P-buffer (pH 7.0) and 30 mM H_2O_2. The decrease in H_2O_2 was followed as a decline in optical density at 240 nm. One unit of CAT activity is defined as the amount of enzyme that decomposes 1 M of H_2O_2 in one min. POD activity (EC 1.11.1.7) was assayed by the method of Urbanek *et al.* (1991). Twenty five microlitter of the EE was added to 2 mL of a solution containing 50 mM K-P-buffer (pH 6.8), 20 mM guaiacol and 20 mM H_2O_2. After incubation for 10 min, the reaction was stopped by adding 0.5 mL 5% (v/v) H_2SO_4 and the absorbance was read at 480 nm. One unit of POD activity is defined as the amount of substrate transformed by the enzyme in 1 min. SOD activity (EC 1.15.1.1) was assayed according to the method of Van Rossun *et al.* (1997). Fifty microlitter of the EE was added to a solution containing 13 mM L-methionine, 75 μM nitroblue tetrazolium chloride (NBT), 100 μM EDTA and 2 μM riboflavin in a 50 mM K-P-buffer (pH 7.8). The reaction took place in a chamber under illumination of a 30 W-fluorescent lamp, started by turning the lamp on and stopped 5 min later by turning it off. The blue formazane produced through NBT's photoreduction was measured as increase in absorbance at 560 nm. Control RM had no EE. The blank solution had the same components included in the complete RM but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction in comparison with tubes lacking the EE.

Proline content was estimated spectrophotometrically at 520 nm according to the method of Bates *et al.* (1973).

Statistical analysis: Data of the two growing seasons were subjected to combined analysis of variance using MSTAT-C software. Significance of differences between treatments means were compared with Duncan's multiple range test at the 0.05 probability level.

RESULTS

Oxidative stress indicators: Root viability in control plants was enhanced due to Se application. Drought stress increased H_2O_2 and MDA content whereas decreased membrane stability index and root viability evidenced by the tetrazolium test (Table 2). Treatment of drought-stressed plants with Se decreased the accumulation of H_2O_2 and MDA thereby increased MSI and root viability. The H_2O_2 and MDA-scavenging activity of Se was more pronounced at the lower level, though there was no significant difference between the two levels on MSI and root viability.

Enzymatic antioxidants: In drought-stressed plants not treated with Se, the activities of CAT and SOD were decreased whereas that of POD was increased (Fig. 1). Imposition of drought stress decreased CAT and SOD activity by 46 and 42%, respectively whereas increased POD by 178%. When drought-stressed plants were

Table 2: Selenium reduced H_2O_2 accumulation and MDA content whereas increased MSI and root viability in drought-stressed wheat plants

Variables	H_2O_2 (μM g^{-1} FW)	MDA (nM g^{-1} FW)	MSI (%)	Root viability (mM TF g^{-1} DW)
Cont	3.0±0.35^{de}	14.2±1.5^{d}	84.0±11.0^{ab}	12.0±0.6^{b}
Se 5 mg L^{-1}	2.7±0.40^{e}	14.6±0.9^{d}	86.2±8.3^{a}	16.2±0.8^{a}
Se 10 mg L^{-1}	3.3±0.28^{cd}	16.0±1.2^{cd}	85.0±9.1^{abc}	15.3±0.7^{a}
DS	5.8±0.56^{a}	41.7±3.4^{a}	50.3±4.2^{f}	5.0±0.4^{d}
DS+Se 5 mg L^{-1}	3.6±0.23^{c}	19.0±1.1^{c}	78.0±5.7^{d}	9.4±0.5^{c}
DS+Se 10 mg L^{-1}	4.6±0.33^{b}	24.2±2.0^{b}	72.8±6.0^{de}	8.6±0.7^{c}

Values are Means±SD of four replicates. Data of each column indicated by the same letters are not significantly different ($p<0.05$)

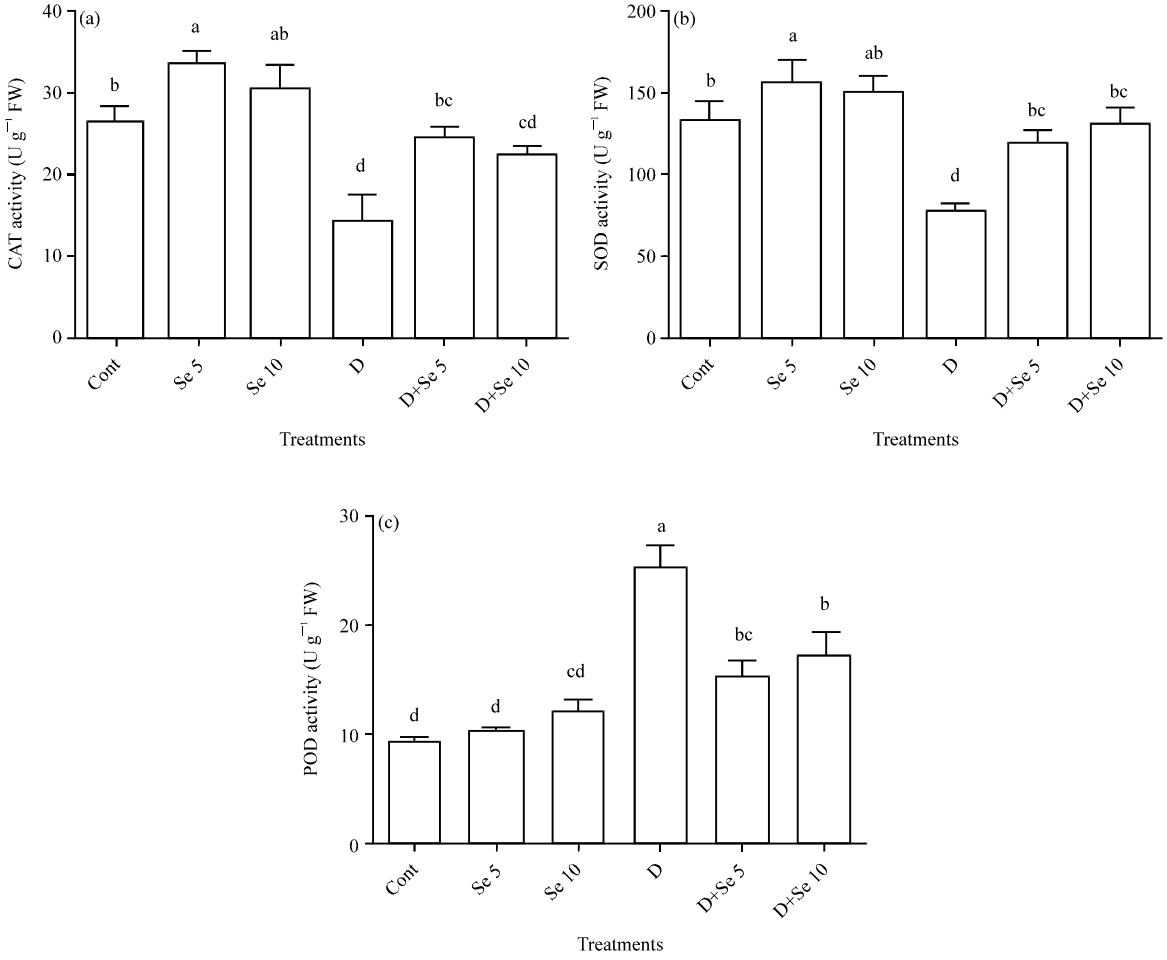

Fig. 1(a-c): Activities of (a) CAT and (b) SOD were induced whereas that of (c) POD was depressed in drought-stressed, Se-treated wheat plants. Mean±SD was calculated from four replicates. Bars headed with different letters are significantly different at $p<0.05$

treated with Se, the activities of CAT and SOD were increased wheres that of POD was decreased. Se at 5, 10 mg L^{-1} increased CAT and SOD by 71, 57% and 55, 70%, respectively, whereas decreased the activity of POD by 40, 32% respectively in drought-stressed plants.

Non-enzymatic antioxidants: Treatment of control plants with Se increased content of AsA, GSH and α-TQ in the leaves though the increase was insignificant at the higher level. In plants challenged with drought, AsA content was decreased whereas those of both GSH and α-TQ were increased (Fig. 2).

Drought-induced increase in α-TQ content was pronounced and approximately tripled compared with control. In drought-stressed plants treated with Se, AsA content that was originally depressed was increased whereas α-TQ content which was originally induced was decreased but still above the control level. On the other hand, Se treatment exacerbated GSH content in drought-stressed plants, so further accumulation was recorded in response to Se. Higher AsA and GSH contents were recorded in drought-stressed plants that treated with Se at 5 mg L^{-1} compared with those received Se at 10 mg L^{-1} though the difference is insignificant (Fig. 2).

Proline: Treatment with Se didn't significantly affect proline content in control plants. A pronounced increase in proline content was recorded when plants were challenged with drought. Drought stress increased proline content by 193%. When drought-stressed plants were treated with Se, proline content was decreased but still higher than the level in control plants (Fig. 3).

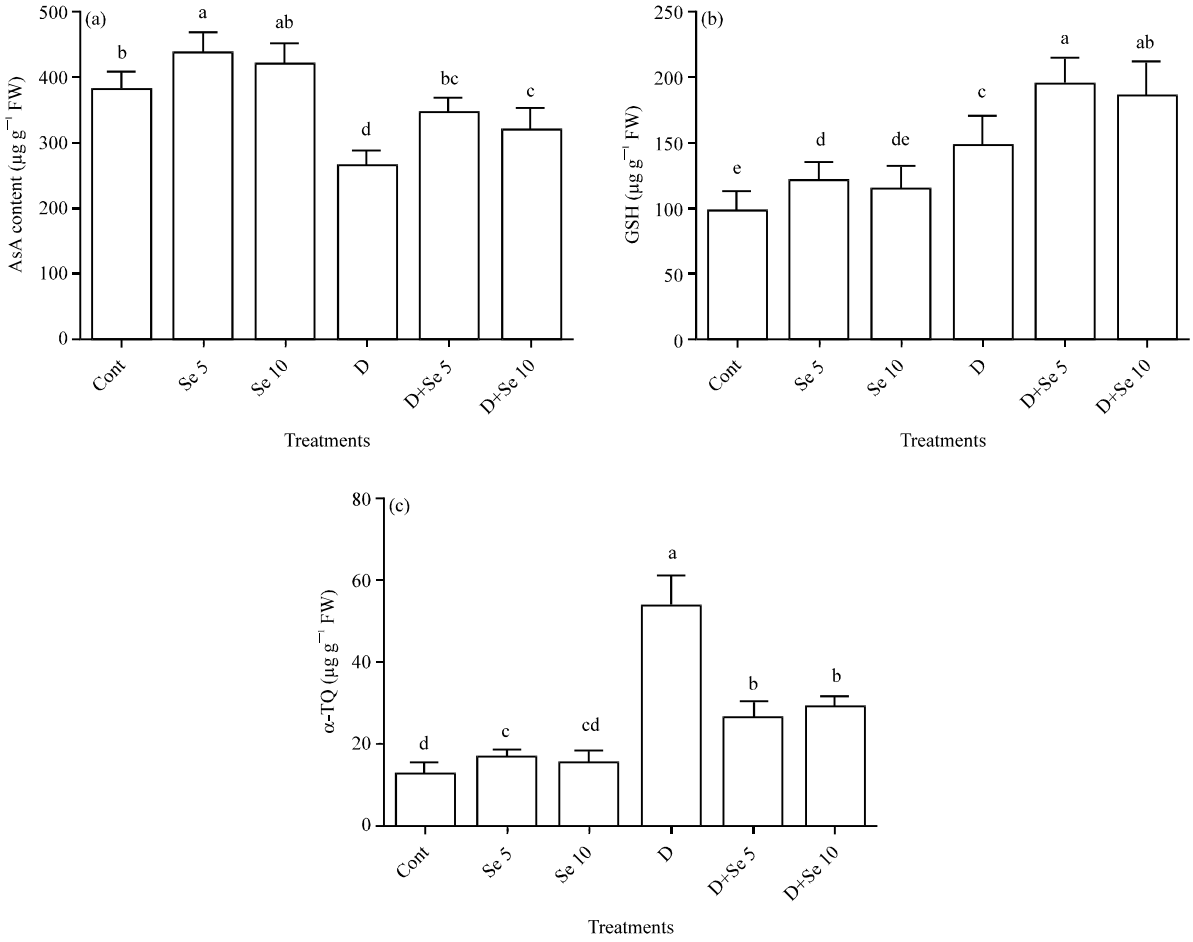

Fig. 2(a-c): (a) AsA, (b) GSH contents were increased whereas that of (c) α-TQ was reduced in drought-stressed, Se-treated wheat plants. Mean±SD was calculated from four replicates. Bars headed with different letters are significantly different at $p<0.05$

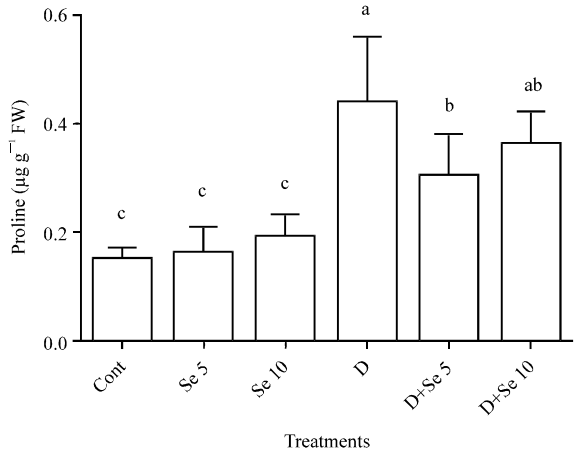

Fig. 3: Proline content was increased in only drought-stressed whereas decreased in drought-stressed, of Se-treated wheat plants. Mean±SD was calculated from four replicates. Bars headed with different letters are significantly different at $p<0.05$

DISCUSSION

Results of the present study show that drought imposed oxidative stress on wheat plants and pretreatment with Se attenuates oxidative stress through modulating the plant's antioxidant system. ROS are accumulated in plants exposed to various stresses which account for plant damage. Under drought stress, ROS production rate exceeds the scavenging activity of the antioxidant system, resulting in extensive cellular damage (Reddy *et al*., 2004). The MDA content is correlated with that of the hydroxyl radical ($OH^{\bullet}$) which is highly active and affects the cell membrane (Gill and Tuteja, 2010). In the present study, drought stress increased H_2O_2 accumulation and MDA content which explain its effect on decreasing membrane stability index and root viability. Increased levels of H_2O_2 accumulation and MDA content in response to drought stress may be due to enhanced generation of ROS (Gill and Tuteja, 2010). On the other hand, treatment with Se, especially at the lower dose, decreased levels of H_2O_2 accumulation and MDA content hence, increased membrane stability index and root viability in drought-stressed plants. Se-induced root viability may reflect enhanced drought tolerance as growth and development of plants is directly reflected by root activity (Yao *et al*., 2009). The antioxidant potential of Se in drought-stressed plants was previously elucidated (Yao *et al*., 2009, 2012; Hasanuzzaman and Fujita, 2011; Zanjani *et al*., 2012). So, Se-induced lowering of H_2O_2 and MDA levels in stressed plants was previously reported (Djanaguiraman *et al*., 2005; Hasanuzzaman and Fujita, 2011). Se also stimulated the activity of the antioxidant enzymes CAT and SOD which were depressed in response to drought and increased contents of AsA that was decreased whereas exacerbated GSH which was induced in drought-stressed only plants. These findings substantiate the hypothesis to which the study explores, as Se affected both the enzymatic and non-enzymatic components of the plant's antioxidant system. An enhancing effect of Se on CAT activity in drought-stressed plants was previously recorded (Yao *et al*., 2009; Hasanuzzaman and Fujita, 2011; Soleimanzadeh, 2012; Zanjani *et al*., 2012; Proietti *et al*., 2013). SOD activity was also reported to be enhanced in drought-stressed Sunflower (Soleimanzadeh, 2012) and in *Trifolium repens* plants (Wang, 2011) as well as in high-temperature stressed grain sorghum (Djanaguiraman *et al*., 2010) in response to Se treatments. Feng *et al*. (2013) hypothesized that high levels of Se enhance the production of SH, resulting in a burst of $O_2^{\bullet-}$ which may thus activate SOD and other antioxidant enzymes to restore the balance of $O_2^{\bullet-}$. Contrary to the results of the present study, Yao *et al*. (2012) reported that CAT and SOD were enhanced in response to drought whereas their activity was decreased in recovered plants treated with Se. This may be due to the nature and magnitude of drought stress. According to Bai *et al*. (2006), antioxidant enzymes activities increased as a defense response to drought stress but this physiological self-regulation mechanism became progressively ineffective with increasing drought stress.

In line with the results of the present study, POD activity was reported to be elevated in response to drought stress (Zhang and Kirkhan, 1994; Terzi *et al*., 2010; Yao *et al*., 2012). In addition Se was reported to decrease POD activity in drought-stressed plants (Kuznetsov *et al*., 2003) which was considered (Yao *et al*., 2012) as an indication of the alleviative effect of Se against oxidative stress. The POD has a role in the biosynthesis of lignin and defense against biotic stresses by consuming H_2O_2 (Gill and Tuteja, 2010).

AsA is the most efficient water soluble antioxidant for detoxifying ROS in plants. Its powerful ROS scavenging activity is due to its ability to donate electrons in a number of enzymatic and non-enzymatic reactions (Gill and Tuteja, 2010). Drought-induced decrease in AsA content may be due to its transformation to the oxidized form during the ROS-scavenging process under stressful conditions (Demirevska-Kepova *et al*., 2006). Elevated AsA content by the addition of Se may be due to its stimulating effects on GSH content (Fig. 2) which may chemically reduce DHA to ascorbic acid via the AsA-GSH cycle (Gill and Tuteja, 2010). In addition, Se may play a role in AsA regeneration through enhancing the activity of the enzymes MDHAR and DHAR (Hasanuzzaman and Fujita, 2011). Increased GSH content was associated with subjecting plants to abiotic stresses e.g., drought (Hasanuzzaman and Fujita, 2011) and heavy metals (Sun *et al*., 2007). In this context, Sumithra *et al*. (2006) concluded a better protection against oxidative damage in leaves of salt-stressed *Vigna radiata* cultivars exhibiting higher GSH concentration. The increased GSH provides reducing substrates for the reduction of DHA to AsA by DHAR which is used by APX to directly quench H_2O_2. The Se exacerbation of GSH content may be due to its enhancing effect on GR activity (Wang *et al*., 2011). Induced GSH and α-TQ in response to drought stress may be a plant defense strategy against drought. Increased levels of α-TQ have previously reported in response to water stress (Shao *et al*., 2007). Drought-induced α-TQ accumulation may be due to the activation of the expression of genes responsible for α-TQ synthesis (Wu *et al*., 2007).

Proline accumulation is a common response in plants exposed to abiotic stresses which was the case in the present study. Increased proline content in drought stressed-plants may be due to increased synthesis or decreased degradation (Van Rossun *et al*., 1997). It could function as a hydroxyl radical scavenger to prevent membrane damage and protein denaturation (Yao *et al*., 2012). In addition, it has been proposed to act as an osmoprotectant, a protein stabilizer, an inhibitor of LBO and a scavenger of $OH^{\bullet}$ and $^{1}O_2$ (Trovato *et al*., 2008). Results indicated that Se decreased proline content which was elevated in response to drought. Similar results were reported in earlier reports (Demirevska-Kepova *et al*., 2006; Yao *et al*., 2012). Yao *et al*. (2012) regarded this response as an indirect evidence for the antioxidant action of Se.

CONCLUSION

Se enhanced the plant's antioxidant potential via inducing the activities of the antioxidant enzymes CAT and SOD as well as increasing the content of the non-enzymatic antioxidants AsA and GSH thereby reduced drought-induced oxidative stress manifested by reducing H_2O_2 accumulation and MDA content which resulted in improved membrane stability and cell viability and a general recovery response involved downregulation of POD and decreased α-TQ accumulation which were elevated in response to drought stress. Further studies are needed to further elucidate regulatory mechanism of Se on stress-related metabolites towards application of more efficient Se-dependent stress tolerance protocols.

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