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Vol. 12(41), pp. 3037-3044, 12 October, 2017
DOI: 10.5897/AJAR2017.12709
Article Number: 8CAA5FA66323
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Induction of antioxidant system in niger (*Guizotia abyssinica* Cass.) under drought stress

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Received 3 September, 2017; Accepted 27 September, 2017

Drought stress is a highly pervasive and economically damaging abiotic stress that affects plant yield and productivity worldwide. Physiologically, drought stress in plants is associated with oxidative stress leading to tissue damage. Drought stress imposed over 72 h in 10 days old seedlings of *Guizotia abyssinica* Cass, niger (cv; RCR-18) under greenhouse conditions resulted in elevated levels of oxidative stress markers such as H_2O_2, malondialdehyde, proline, reduced glutathione and ascorbate in a time-dependent manner. Levels of antioxidant enzymes: peroxidases and glutathione reductase, and metabolic enzyme: amylase and acid phosphatase were moderately enhanced. The levels of stress markers, antioxidants, and recovery upon re-watering suggested that the antioxidant system in niger could withstand the drought stress for up to 48 h under greenhouse conditions.

Key words: Drought stress, antioxidants, antioxidant enzymes, malondialdehyde, abiotic stress.

INTRODUCTION

As an important factor that determines geographical distribution of plant species, drought has major yield limiting ability (Nezhadahmadi et al., 2013). One of the earliest biochemical responses of eukaryotic cell to environmental stresses such as drought, salinity, high temperature, chilling etc., is generation of reactive oxygen species (ROS). Water deficit due to drought induces closing of stomata, limiting CO_2 assimilation, which in turn leads to NADPH accumulation and subsequent leakage of electrons. The leakage of electrons causes partial reduction of atmospheric O_2, generating enhanced levels of ROS (Fujita and Alam, 2015). There are four general forms of cellular ROS, singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^•$), each having a characteristic half-life and oxidizing potential. Plants are endowed with an array of versatile and cooperative antioxidant systems comprising enzymatic and non-enzymatic components. Enzymatic components include antioxidant enzymes; guaiacol peroxidase (GPOX), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and metabolic enzymes; amylase (AMY) and acid phosphatase (AP). While, non-enzymatic components include antioxidants like ascorbic acid (ASC) and reduced glutathione (GSH), and osmoregulants like proline to cope with ROS generated in response to stress. ROS generation due to prolonged exposure to drought stress overwhelms the antioxidant

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defense system, thereby causing extensive damage to cellular components like proteins, lipids, DNA and RNA, leading to cell death (Silva and Santos, 2015). Increasing population and growing soil aridity in future are expected to make water a scarce commodity. The profound impact of drought on agriculture and ecosystem thus makes the ability of plant to withstand stress of great economic importance (Rybka and Nita, 2015). Hence, it is important to look for alternative strategies to improve the abiotic stress tolerance of various crop plants.

Guizotia abyssinica (Niger) belonging to family *Asteraceae* is cultivated throughout India, West Indies and East Africa. Niger accounts for 3% of Indian oil seed production and contains 40% oil and 20% protein. Niger seed earns precious foreign exchange as it is exported as bird feed. That apart, it is mainly used for culinary purposes, manufacture of soaps, cosmetics, lighting and lubrication. Various attempts have been made to explore the potential of biodiesel production from Niger, which is cost effective, less corrosive, user and environmentally friendly green catalyst (Yerranguntla et al., 2012). Effects of polyethylene glycol 8000 and drought on *G. abyssinica Cass* cultivars (IGP 76, GA 10, No. 71 and IGPN 2004) at seedling and maturity stages of the plant had been reported (Nikam and Ghane, 2011). The plant-water relationship during drought stress is an essential component for modulating the antioxidant defense mechanism. With a basic physiological and biochemical knowledge, the present study was an effort to determine the role of antioxidant defense system, in response to applied drought stress.

MATERIALS AND METHODS

Plant material and growth conditions

Niger seeds (RCR-18 variety) were procured from University of Agricultural Sciences, Dharward, India. All chemicals used were of analytical grade. Seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 30 s, rinsed immediately with large volume of distilled water. The seeds were sown in plastic trays containing vermiculite and acid-washed sand (1:1 w/w) and irrigated twice a day with distilled water. The germination was carried out under natural greenhouse conditions; day/night temperature and relative humidity were 30/25°C and 75/70%, respectively. The average photoperiod was 12 h light/12 h dark.

Drought stress and experimental design

Drought stress was applied by withholding water for 10 days after germination (DAG) of seedlings. Leaf samples were collected at 24, 48 and 72 h and assayed for various parameters. The experimental design was carried out employing random factorial scheme, with 3 evaluation points (24, 48 and 72 h). Each experiment comprised 6 experimental units (leaf samples of control and stressed plants) and in triplicate. Seedlings watered twice a day were used as control.

Determination of relative water content (RWC)

The relative water content was determined by following the method of Turner and Kramer (1980), using the equation: RWC = (FW-DW) × 100/ (TW-DW). Leaf discs of 6 mm diameter were weighed to determine the fresh weight (FW); they were soaked in distilled water at 25°C for 4 h to determine the turgid weight (TW), and then oven dried at 80°C for 24 h to determine the dry weight (DW).

Determination of antioxidants and stress markers

Estimation of ascorbate (ASC) and glutathione (GSH) in control and drought stressed leaves was carried out according to Sadasivam and Manickam (1997) and Beutler et al. (1963), respectively. Further, H_2O_2 levels in control and stressed samples were determined according to Velikova et al. (2000). Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 g fresh tissue according to Heath and Packer (1968). The MDA content was calculated using extinction coefficient of 155 mM^{-1} cm^{-1}. Free proline was extracted from 0.5 g of fresh tissue and estimated according to the method of Bates et al. (1973).

Enzyme extraction

Fresh leaf plants were homogenized with pre-chilled 50 mM Na_2PO_4 buffer (pH 7.0), containing 5 mM β-mercaptoethanol and 1 mM EDTA. Homogenate was centrifuged at 4°C for 15 min at 12,000 ×g. Supernatant was used to determine the enzyme activity and protein content. Total soluble protein content was determined according to Lowry et al. (1951), using BSA as standard.

Assay of antioxidant enzymes

Guaiacol peroxidase (POX) activity was determined according to Chance and Maehly (1955) by measuring increase in absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) due to formation of tetraguaiacol. The reaction mixture contained 3 ml of 50 mM phosphate buffer (pH 7), 20 mM guaiacol, 10 mM H_2O_2 and 100 µl enzyme extract. One unit of enzyme activity is defined as the quantity of enzyme required to convert 1 µmol of H_2O_2 min^{-1} at 25°C.

Catalase activity was measured according to Aebi (1984) by following decline in absorbance at 240 nm (ε = 39.4 $M^{-1}cm^{-1}$). The reaction mixture consisted of 50 mM Na_2PO_4 buffer (pH 7.0) containing 50 µl of enzyme extract and 10 mM H_2O_2. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of H_2O_2 min^{-1} under the assay conditions.

Ascorbate peroxidase activity assay was based on the method of Allen (1968) which measures an increase in the absorption at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). One unit of APX is defined as the amount of enzyme required to convert µmol of ascorbate min^{-1} at 25°C. The reaction mixture contained 50 mM HEPES buffer (pH 7), 1 mM EDTA, 1 mM H_2O_2, 0.5 mM sodium ascorbate and 50 µl of enzyme extract.

GR activity was measured according to Carlberg and Mannervik (1985) by measuring oxidation of NADPH at 340 nm (ε = 6220 $M^{-1}cm^{-1}$). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.2 mM NADPH and 37 µl of enzyme extract. The reaction was initiated by the addition of 0.5 mM GSSG. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH min^{-1} under the assay conditions.

Assay of metabolic enzymes

The activity of β-amylase was determined by the method of Bernfeld (1955). Reaction mixture contained 500 µl of 2% starch solution in 50 mM phosphate buffer (pH 7.0) and 500 µl of enzyme extract. The number of µmoles of maltose released min^{-1} is defined

as one unit of β-amylase. Acid phosphatase (AP) activity was determined by measuring the release of *p*-nitrophenol at 410 nm according to the method of Hoerling and Svensmark (1976). Each unit of activity is defined as 1 µmole of *p*-nitro phenol released min^{-1}.

Statistical analysis

All data are expressed as a means of triplicate experiments unless mentioned otherwise and experiments were performed using randomized design. Data were subjected to analysis of variance (ANOVA) using GraphPad Prism version 6.0 and the mean differences were compared by lowest standard deviations test (LSD). Comparisons with $P \leq 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Growth parameters

Water cycling through the soil-plant-atmosphere continuum can be estimated by water potential, which also suggests the energy status of plant water. Physiological consequences leading to water deficit in cell can be appropriately measured by determining RWC. Plants exhibit waved RWC values in species specific manner and extent of drought. Some drought tolerant species have been shown to possess a range of RWC from 60 to 70%, while severely desiccated and dying leaves in drought sensitive species was about 30 to 40% (Barrs and Weatherley, 1962). Niger exhibited RWC of 75 and 50%, respectively at 48 and 72 h of drought stress (Figure 2), but 72 h stressed plants failed to revive on re-watering. This suggests the detrimental effects of drought beyond 48 h. A similar relationship between RWC and survival was observed in faba bean under salinity (Tavakkoli and Rengasamy, 2010). These effects of reduced RWC were reflected in fresh weight of drought stressed seedlings (Figure 3). However, dry weight of the seedlings remained unchanged relative to control, emphasizing primacy of physiological mechanisms in water balance under drought stress.

Stress markers

Tolerance of plants to an applied stress involves many complex and multifaceted process. Drought in plants induces oxidative stress, and ability of plants to tolerate depends upon unfolding of the genetic plasticity in order to induce specific antioxidant defense mechanism. Quantitative estimation of antioxidant components would indicate the prevalence of an adverse condition, severity of the stress and strength of antioxidant system. Oxidative stress creates imbalance in electron transport and metabolic utilization of reducing power, thereby increasing ROS levels in the cell. Progressive reduction in PS-II and β-oxidation of lipids during drought results in generation of H_2O_2, most stable intermediate circumventing the other ROS species (Wang et al., 2011). A 0.74-fold increase in H_2O_2 after 48 h of applied stress in niger suggested the onset of oxidative stress, as observed in Azolla exposed to different levels of paraquat (Sood et al., 2011). A decrease in H_2O_2 level during extended drought for 72 h suggested the onset of enzymatic and non-enzymatic antioxidative defense system to control the oxidative damage.

Level of MDA is indicative of the extent of lipid peroxidation in plant under oxidative stress induced by various environmental factors. Almost exponential increase in MDA with extended duration of drought indicated the severity of stress leading to suppression of antioxidant defense systems. While similar negative correlation between lipid peroxidation and antioxidant system has been reported in wheat (Selote and Khanna-Chopra, 2010) and maize genotypes subjected to drought stress (Vishal et al., 2013), Li and Zhang (2013) had reported lower MDA content in *Zoysia japonica* pretreated with 5 and 10 mM $CaCl_2$ under drought conditions. This suggests that pretreatment with the appropriate $CaCl_2$ concentration reduces oxidative damage that results from drought.

A number of metabolic pathways are synchronized to alleviate induced oxidative stress. Synchronized operation of ascorbate-glutathione (ASC-GSH) pathway known to establish a balance between generation and metabolism of ROS and its reaction products is an essential plant stress tolerance mechanism (Vivancos et al., 2013). GSH, a multifunctional water soluble tripeptide contributes to cell protection against detrimental effects of free radical by recycling ascorbate in its reduced form. GSH either forms adducts directly with reactive electrophiles, or acts as a proton donor in the presence of ROS, yielding GSSG; thus, providing a mechanism to protect biological macromolecules (Shao and Kang, 2008). A 1.2- fold increase in GSH level during 48 h and further decline at 72 h of drought indicated the operation of GSH-ASC cycle during early drought stress and its inefficiency during extended drought. A similar response was reported in drought stressed Hyacinth bean (Myrene and Devaraj, 2011). ASC acts as a specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate. As a potent reducing power, ASC maintains metalloenzyme activity and chloroplastic α-tocopherol (Pang and Wang, 2010). ASC levels in stressed seedlings of niger showed a 0.48-fold increase over 72 h of exposure time. These results are in conformity with drought stressed almond (Sorkheh et al., 2011). Parallel increase in ASC and GSH levels suggested efficient operation of ASC-GSH cycle during early period of drought stress.

Accumulations of osmoregulatory molecules such as proline, glycine betaine and sugars in response to oxidative stress had been reported in many plants (Deinlein et al., 2014). In addition to being compatible

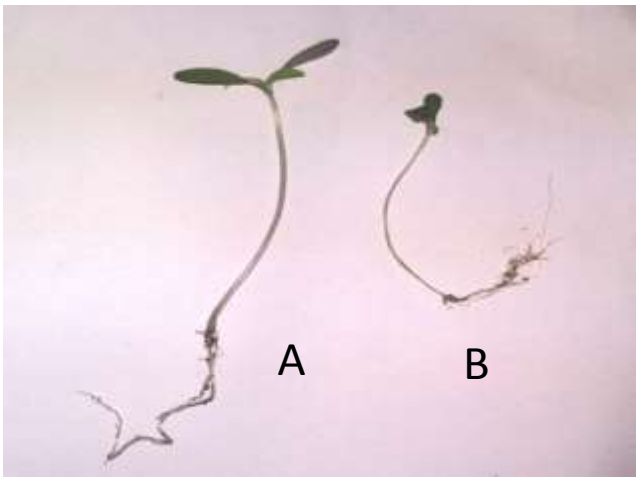

Figure 1. Effect of drought on niger seedlings: A: Control seedlings, B: stressed seedlings after 48 h.

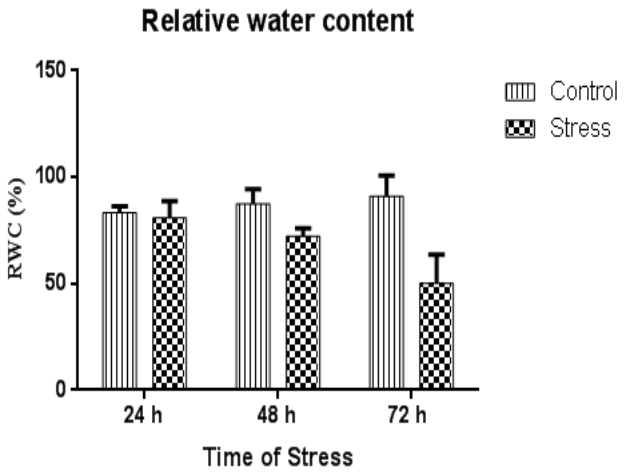

Figure 2. RWC in leaf tissues of niger seedlings at 24, 48, 72 h of drought stress.

solute and enzyme protectant, proline is also known to render structural stability to macromolecules and organelles. Increase in proline levels ~20%, as compared to control during the entire period of applied stress suggested effective osmotic adjustment contributing to osmotic stress tolerance in niger. The observed proline levels in niger were in consonance with those of drought stressed sugar cane (Abbas et al., 2014) and corn cultivars (Sinay and Karuwal, 2014) (Table 1).

Seedlings of niger were drought stressed and individual seedlings were analyzed for determination of H_2O_2, ASC, GSH, PRO and MDA. Results are mean ± SD, obtained from three replicates.

Antioxidant enzymes

In many plant species which exhibit stress tolerance, both enzymatic and non-enzymatic antioxidant systems operate simultaneously. Guaiacol specific POXs are known to play a vital role in various biosynthetic processes like ethylene and auxin metabolism, maintain redox homeostasis in plasma membrane, lignification and suberization of cell wall and several other developmental and defense mechanisms (Lepeduš et al., 2004) thereby, making it an indispensable component of the antioxidant system. Elevated levels of GPOX up to 48 h of applied stress, and its decline beyond 48 h suggested the enzymes potential ROS quenching during 48 h of drought stress, and its insufficiency beyond 48 h of drought (Figure 1), similar to observations made in wheat species under drought stress (Sheoran et al., 2015).

The levels of APX and GR in leaves of Niger at 48 h of drought stress showed increase in both control and stressed seedlings. Ascorbate peroxidases are class I heme-peroxidases, which utilize ASC as specific electron donor and catalyze the reduction of H_2O_2 into H_2O. APX activity in response to environmental stress generally increases along with other enzymes activities, such as CAT, POX and GR (Shigeru et al., 2002). APX showed 2.5-fold enhancements after 48 h of stress (Figure 3), which coincided with relatively lower H_2O_2 levels. Such changes had been reported in drought stressed *Solanum melongena* L. and *Piper longum* L. (Caverzan et al., 2012) and suggested to contribute to drought tolerance. GR evokes cell defense by detoxification of ROS and regeneration of GSH from GSSG with the accompanying oxidation of NADPH. Detoxification of ROS and maintenance of redox potential via NADP production by GR had been shown to contribute to abiotic stress tolerance. A 3.5-fold enhancement in GR levels in niger under drought indicated its role in stress tolerance (Figure 4). Similar activation in GR levels had been demonstrated in cowpea under water stress (Torres-Franklin and Zuily-Fodil, 2008) and tobacco under heat stress (Tan et al., 2011). Enhanced levels of APX and GR showed that they are essential components of ASC-GSH cycle. Contrary to the current findings, Chugh et al. (2010) reported inhibition of GR activity in maize (Paras, sensitive genotype) under drought stress, rendering it susceptible to drought.

Catalase showed a progressive decline during drought stress with 2.2-fold decline after 72 h (Figure 2). CATs are ubiquitous enzymes known to contain tetrameric-heme moiety catalyzing dismutation of two molecules of H_2O_2 into oxygen and water. Although, an enzyme with high turnover number CATs lower affinity towards H_2O_2 and photo-inactivation distinguishes it from other alternative H_2O_2 scavenging systems. Consequently, these inhibiting conditions lower the steady state levels of the enzyme as observed in Niger. CATs are known to be efficient tools for gross removal of high H_2O_2 levels, but they are less suited for fine tuning of sensitive redox balances with low H_2O_2 concentrations (Nicholls and

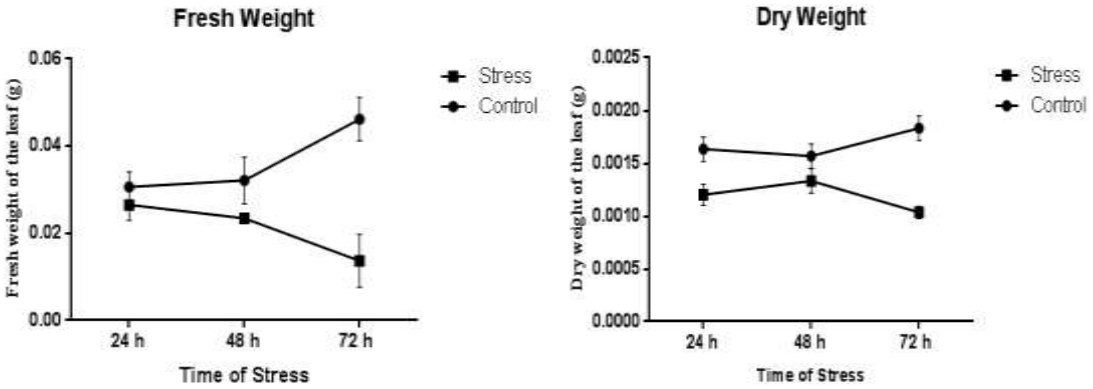

Figure 3. Fresh and dry weight of niger leaves at 24, 48 and 72 h of applied drought stress. Values for FW and DW are ± SE (±0.001).

Table 1. Levels of stress markers in leaves of niger (*Guizotia abyssinica*) subjected to drought stress*.

Tissue	Time	Stress marker	Control	Stress
Leaf	24 h	H_2O_2[a]	29.10664 ± 1.902405	38.78818 ± 0.748218
		Proline[b]	410.9 ± 6.38	505.3 ± 15.39976
		MDA[c]	3.460347 ± 0.37537	4.9717 ± 0.539988
		GSH[a]	80.605 ± 7.230614	164.15 ± 2.76101
		Ascorbate[b]	24.885 ± 0.518965	33.12 ± 0.476235
	48 h	H_2O_2[a]	33.91708 ± 1.301969	59.12682 ± 2.062253
		Proline[b]	488.2667 ± 12.76784	576.3333 ± 12.95411
		MDA[c]	3.347 ± 0.266551	7.6508 ± 0.447347
		GSH[a]	97.755 ± 3.771727	195.51 ± 6.407581
		Ascorbate[b]	29.7 ± 1.091192	44.01 ± 1.169134
	72 h	H_2O_2[a]	20.70311 ± 2.352013	31.29817 ± 2.50181
		Proline[b]	543.2667 ± 9.648201	629.0267 ± 9.16527
		MDA[c]	2.503267 ± 0.109233	13.6808 ± 0.673692
		GSH[a]	104.615 ± 3.65863	171.5 ± 6.095529
		Ascorbate[b]	30.555 ± 0.273724	40.85167 ± 0.837269

*[a]µg/g fresh weight tissue; [b]mg/g fresh weight tissue; [c]m moles/g fresh weight tissue.

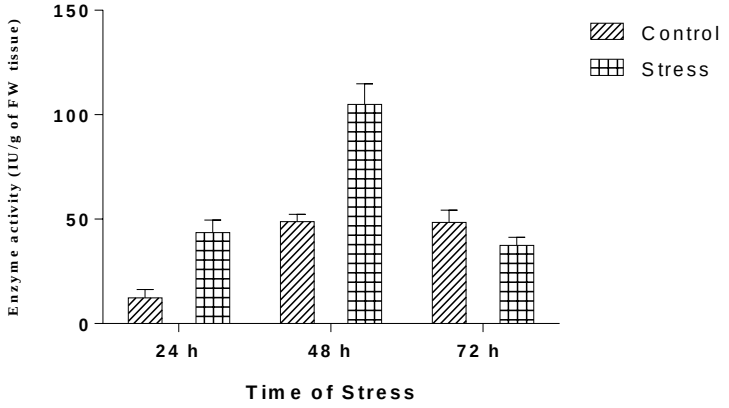

Figure 3. Effect of drought stress on APX activity in leaves of niger. Results are mean ± SE (P≤0.05), obtained from three replicates.

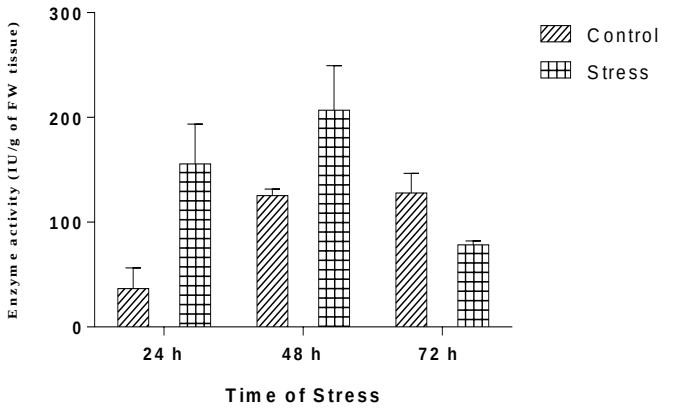

Figure 4. Effect of drought stress on GR activity in leaves of niger. Results are mean ± SE (P≤0.05), obtained from three replicates.

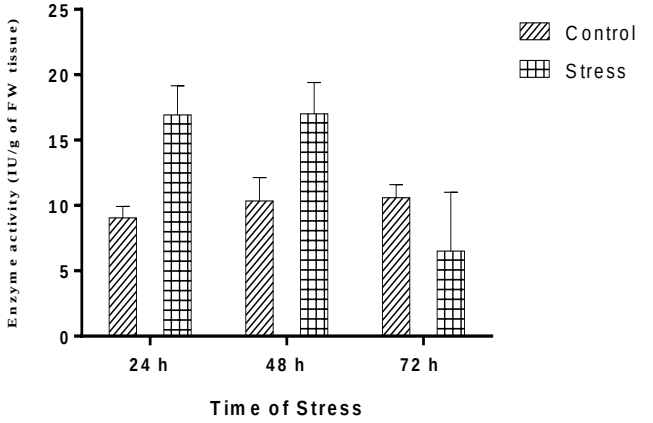

Figure 5. Effect of drought stress on AMY activity in leaves of Niger. Results are mean ± SE (P≤0.05), obtained from three replicates.

Ferguson, 2001). On the other hand, APX is known to have greater affinity for H_2O_2 (Sofo et al., 2015), and play a pivotal role in H_2O_2 detoxification. The results are in agreement with previous studies carried out in bentgrass species (Michelle and Bingru 2007) and wheat cultivars (Chakraborty and Pradhan, 2012) subjected to drought stress, exhibiting countervailing CAT activity by APX. However, the extent of stress and susceptibility of plant species may lead to significant increase in the levels of CAT activity as was reported in oats (Islam et al., 2010) and maize (Chugh et al., 2010) under drought stress.

Though antioxidant enzymes play a pivotal role in stress tolerance, there are reports of induction of metabolic enzymes like acid phosphatase and β-amylases (Yang et al., 2007) during abiotic stress. In response to water stress, levels of inorganic phosphate are maintained by acid phosphatase, which is accomplished by co-transporting it with H^+ along the gradient of proton motive force. AP activity in niger showed 1.4-fold increase after 48 h and declined with extended exposure to stress (Figure 6). A similar observation was noted in alfalfa (*Medicago sativa* L.) explants subjected to salt and drought stress (Ehsanpour and Amini, 2003) and pigweed leaves under drought stress (Cunhua et al., 2010). β-Amylase plays a major role in starch degradation and in the daily turnover of transitory starch in photosynthetic organs. β-Amylase catalyzes the breakdown of glucans into maltose, which in cytosol is converted to glucose and ultimately leading to formation of sucrose and fructose. β-Amylase contributes to stress tolerance by increasing maltose and other soluble starch that can act as emergency compatible solutes (Krasensky and Jonal, 2012). Enhanced β-amylase levels in niger during 48 h of applied stress (Figure 5) suggested increased accumulation of sugars, which act as osmoregulant to maintain cell turgidity and maintain membrane integrity in response to the applied stress. Increase in β-amylase

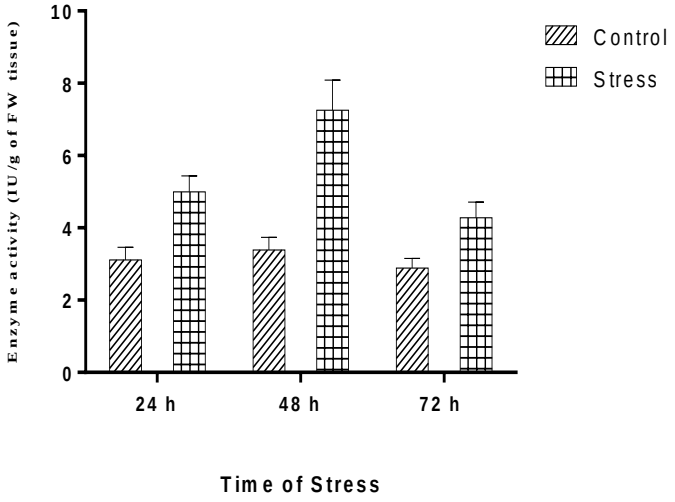

Figure 6. Effect of drought stress on AP activity in leaves of Niger. Results are mean ± SE (P≤0.05), obtained from three replicates.

activity was seen in *Populus nigra* L. clones subjected to drought stress (Regier and Streb, 2009), which is interpreted as a tolerance mechanism.

Conclusion

Effects of different abiotic stresses such as drought on plants involve overproduction of ROS leading to oxidative stress. Plants exert a positive adaptation to drought by strategizing potent antioxidant defense systems and efficiently combat by non-enzymatic components, ASC, GSH and proline and enzymatic components, GPX, APX, GR, AMY and AP. From induction of antioxidant enzymes, enhanced antioxidants and revival of plants

upon re-watering, it is concluded that Niger is tolerant to drought stress of up to 48 h. Thus, the drought response in Niger measured in terms of antioxidant and antioxidant enzymes levels suggested participation of both components in tolerance mechanism. However, the antioxidant system employed by Niger to overcome/tolerate drought appears to be sufficient to protect against short term (up to 48 h) drought stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

H. Kavya Naik acknowledges the Department of Science and Technology, Government of India for financial support with reference No. SR/WOS-A/LS-1167/2014 (G).

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