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Biochemical Response Trends of *Macrotyloma uniflorum*, *Lam*. Subjected to Dehydration Stress in Different Growth Conditions

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Abstract: The mechanismof plant toleranceagainst dehydration is differs according to the growth status of the plant. To understand the similarities and differences in the drought responses of two different growth situations of horse gram, seedlings of normal plants along with regenerated tissue culture were subjected to dehydration stresshave been investigated. Enzymatic and non-enzymatic antioxidants of both seedlings behaved similar albeit minor variations. Activity of antioxidant enzymes of both seedlings compared to their controls showed a similar trend. Peroxidase (POX; EC 1.11.1.7), Superoxide dismutase (SOD; EC 1.15.1.1) and Glutathione reductase (GR; EC 1.6.4.2) were elevated. However, Catalase (CAT; EC 1.11.1.6) was reduced in both stressed seedlings. Also the antioxidants levels in both stressed plantlets exhibited 2 folds elevation of ascorbic acid and ~3 folds of GSH, but the elevation of proline was different where it was 6 folds in callus, while normal seedlings increased by 10 times compared to control. Stress markers hydrogen peroxide and thiobarbeturic acid reactive substances (TBARS) 2 folds increased, these coincides with higher levels of Peroxidase, reflecting a great damage of membrane in both stressed status. These suggest that horse gram seedlings behave in similar manner even if grown in different conditions and circumstances which confirm their similar biochemical resistance mechanism when exposed to water deficiency stress.

Abbreviations: PMSF: phenyl methyl sulphonyl fluoride; SGH: reduced glutathione; ROS: Reactive Oxygen Species; ABA: Abscisic Acid

Key words: Antioxidant systems · Oxidative stress · Growth conditions · Tissue culture

INTRODUCTION

Drought is one of the most important factors contributing to crop yield loss [1]. Drying is generally considered as the removal of water and the process is associated with oxidative stress. Drought stress induces a range of physiological and biochemical responses in plants. These responses include stomatal closure, repression of cell growth, photosynthesis and activation of respiration. Dehydration stress also increases the formation of ROS resulting in lipid peroxidation, denaturation of proteins and nucleic acid damage with severe consequences on overall metabolism [2]. Plants adapt to water deficit at both cellular and molecular levels by accumulation of osmolytes and proteins specifically involved in stress tolerance. When a plant is subjected to abiotic stress, a number of genes are turned on, resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection against these stresses. To protect against oxidative damage, cells possess defense mechanisms that include enzymes, such as peroxidases, catalases and superoxide dismutase and non- enzymatic antioxidants, such as glutathione (GSH), vitamin C and E. [3, 4].

All species, from bacteria to higher eukaryotes, possess sensors, transducers and regulators that allow them to respond and adapt to changes in water availability. Genes with diverse functions are induced or repressed by these stresses. In the direction of enhancement of stress tolerance, a number of stress responsive genes and their products have been identified

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and classified into two groups from *Arabidopsis*, rice and other species.The first group is Functional proteins includes chaperones, late embryogenesis abundant proteins (LEA), osmotin, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxificationenzymes and various proteases.The second group is regulatory proteins which includes various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism and other signaling molecules such as calmodulin-binding protein which are involved in further regulation of signal transduction and stress-responsive gene expression[5]. Genetic engineering of the metabolic pathways for production of osmolytes such as proline, glycinebetaine, mannitol, galactinol, fructans, trehalose, raffinose and ononitol have been shown to increase the resistance to drought [6],[7],[8]. Proteins like LEA which is believed to function as chaperone and number of transcription factors and protein kinases have been found useful in engineering drought resistance. 9-*cis*-epoxycarotenoid dioxygenase (NCED), key enzyme in ABA biosynthesis and ABA 8'-hydroxylase, an enzyme that degrades ABA have been implicated in drought resistance by transgenic methods[10][9],[10].Analysis of ESTs generated under salt stress, drought and ABA application have indicated closer match between ABA and drought (100% of genes induced by drought were induced by ABA) signaling pathways and cross-talk between salt, drought and cold stress [11]. Responseof drought stressinABA mutant of tomato confirmed the relationship between ABA elevation and free amino acids accumulation [12].

Plant celltissue cultures are very important in the field of plant biotechnology for the development of transgenic plants, for mass propagation and for the biosynthesis of useful components. These plantlets regenerated in tissue culture environment experience a very different environment as opposed to normal plants. Shoots that have grown *in vitro* are continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets are developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow heterotrophic growth in an atmosphere with high level of humidity.

This studyaims to investigatethebiochemical responseof horse gram seedlings, presented in two growth conditions by assessments the levels of antioxidant defense systems including enzymatic and non-enzymatic antioxidant defense parametersin addition to some metabolites and stress markersafter subjecting the normal seedlings and tissue plants to dehydration stress.

MATERIAL AND METHODS

Plant and Stress Treatment: Drought stress in normal plant; Five days oldhorse gram seedlings were watered every 12 h. They were subjected to drought stress by withholding water continuously for four days until ~70% of the plant dehydrated. Shoots were collected and deep frozen for 1h.

Drought Stress in Tissue Culture: Explants of shoot tips were aseptically inoculated on Murashige and Skoog's media [13] with L2 vitamins and leguminous media [14] on solid agar. After the development of sufficient growth, the calli weresub cultured into media containing 10% PEG as a dehydration stress inducer. Well-developed regenerates of (30-35 day old) were used for extraction of enzymes and antioxidants. The regenerates grown in the normal media without PEG served as controls.

Enzyme Extraction and Antioxidants: Frozen shoots were homogenized with 50 mM sodium phosphate buffer, pH 7.5 containing 1 mM of PMSF and 5 mM â-mercaptoethanol. The homogenate was centrifuged at 1000 rpm for 20 min. The supernatant was used as source of enzymes, antioxidants and other components. All the steps in the preparation of the enzyme extract were carried out between 0 to 4°C. Soluble protein content was determined by Lowry method [15] and Bovine Serum Albuminwas used as standard.

Determination Of, Ascorbic Acid and Proline: Ascorbic acid and dehydroascorbic acid contents were estimated according to [16]. Free proline was extracted from 0.5 g of fresh tissue in 3% aqueous sulphosalicylic acid and estimated by ninhydrine method according to[17].

Estimation of Lipid peroxidation and (H_2O_2): Malondialdehyde (MDA) is the product of Lipid peroxidation and indicates damage of the membrane. MDA was measured by reacts with thiobarbituric acid (TBA) to form MDA–TBA complex called (TBARS) which calculated by reading Absorbance at 532nm correction was done by subtracting the absorbance at 600 nm using Extinction coefficient of 156 mM^{-1} cm^{-1}[18].Hydrogen peroxide content was determined according to the method described in [19].

Measurements of GSH: 0.5 g of frozen plant tissue was ground to fine powder and homogenized in 1.0 ml 3% Trichloroacetic acid, (TCA) and centrifuged at 12, 000 rpm for 15 min at 4 °C. Reduced glutathione were determined according to [19].

Assay of Enzymes: Catalase (CAT; EC 1.11.1.6): activity was measured by following the decline in A_{240} nm ofH_2O_2 (ϵ =36 $M^{-1}cm^{-1}$) [21]. In a reaction mixture containing 20 ìl of enzyme extract in 50 mM sodium phosphate buffer (pH 7.0).The reaction was started by adding 15 mM of H_2O_2 as a final concentration and its consumption was measured every 15 seconds for 1-2 min at 240 nm.

Glutathione reductase was assayed by monitoring the GSSG dependent NADPH oxidation according tomethod described in[22].

*Guaiacol peroxidase*activity was measured by monitoring the formation of tetra guaiacol at 470 nm (ϵ=26.6 mM-1cm-1) using H_2O_2 as substrate, One unit of peroxidase is defined as the amount of enzyme that caused the formation of 1 mM of tetra-guaiacol per minute, according to [23]. POX isozymes separated on 9% native acrylamide gels were incubated for 30 minutes in a mixture of *o*-dianisidine HCl with acetate buffer (pH 5.5) at room temperature. Then gels were transferred to 100 mM H_2O_2 until visible bands developed.

*Super oxide dismutase*activity was determined using the photochemical method described in [24] and [25]with little modificationsbased on measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium. 3.0 ml reaction mixture contained 50 mM potassium Phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA and 10 - 150 µl enzyme extract. Riboflavin was added at the end. The tubes were shaken and placed 15 cm below 40 -W lamps. The reaction initiated by exposure to light and was arrested after 15 min by removal of light. The absorbance of thereaction mixture at 560 nm was read against a reactionmixture lacking enzyme, which developed maximum color(control value). One unit of the enzyme is defined as the amount of enzyme which can inhibit the reaction (color development) by 50%.

Acid phosphatase (EC 3.1.3.2)activity was estimated using*p*-nitrophenyl phosphate as substrates as mentioned in [26]. Each unit of activity was defined as the number of ìmoles of *p*-nitro phenol released per minute. *β-Amylase* activity was determined according to [27].

Electrophoretic Analysis: Non-denaturing, discontinues slab gel electrophoresis was carried out essentially based on the method described in [28], while SDS-PAGE was performed based on [29], using 10% acrylamide resolving gel and 5 % stacking gel.

Statistical Analysis: All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using graph Pad prism software. Data were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared (P< 0.05).

RESULTS AND DISCUSSION

Plant's ability to responds to different environmental cues and adaptation is due to altered expression of genes. When the plants are grown in tissue culture media, they switch their metabolic programs and adapt to new conditions. Horse gram shoot explants were used to regenerate the tissue. The combination of MS media and L2 vitamin mix yielded good shoot growth (Figure 1) the plantlets obtained from tissue culture in addition to native plants were analyzed for their ability to respond to dehydration stress.

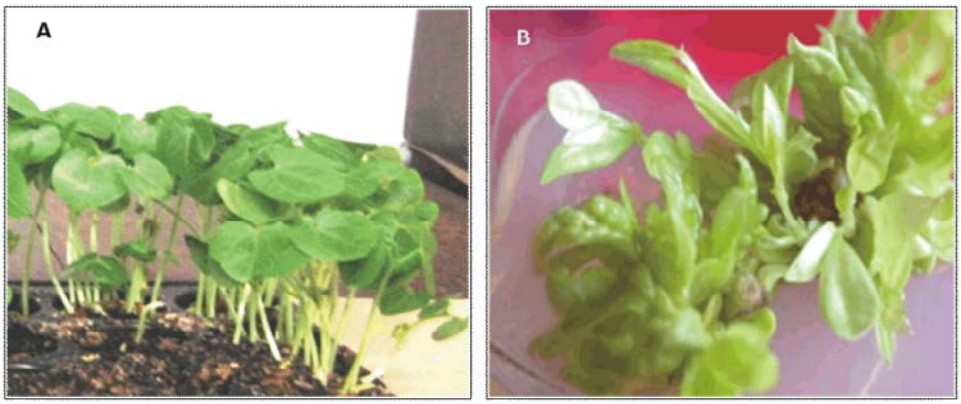

Fig. 1: Seedling of Horse gram (*Macrotyloma uniflorum*);(A) normal germination and (B) regenerated Tissue cultures

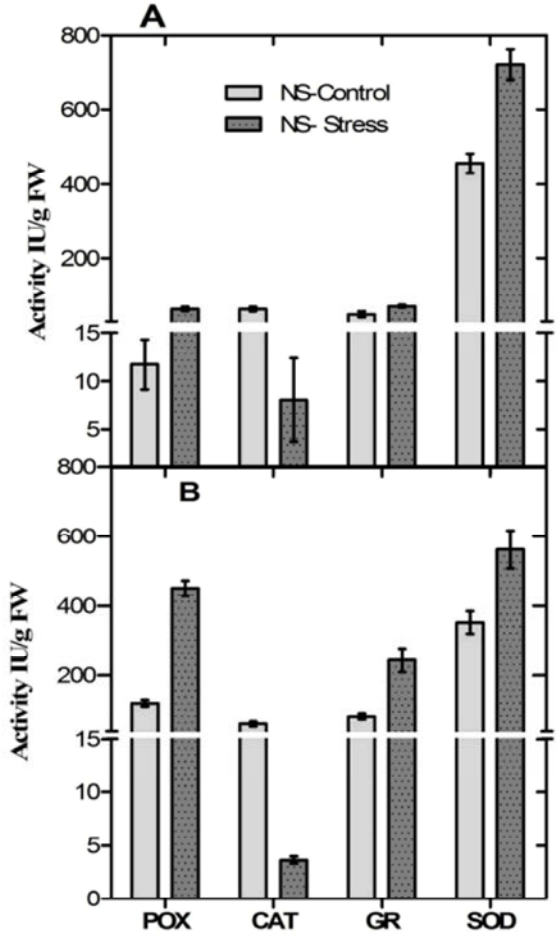

Fig. 2: Levels of antioxidant enzymes activitiesin Horse gram (A) normal seedlings under drought stress (B) tissue culture plants under dehydration stress induced by 10% PEG. Data plotted are mean ± SD of triplicate of two separate replicates. Data analyzed by ANOVA test, (P <0.05 was considered significant)

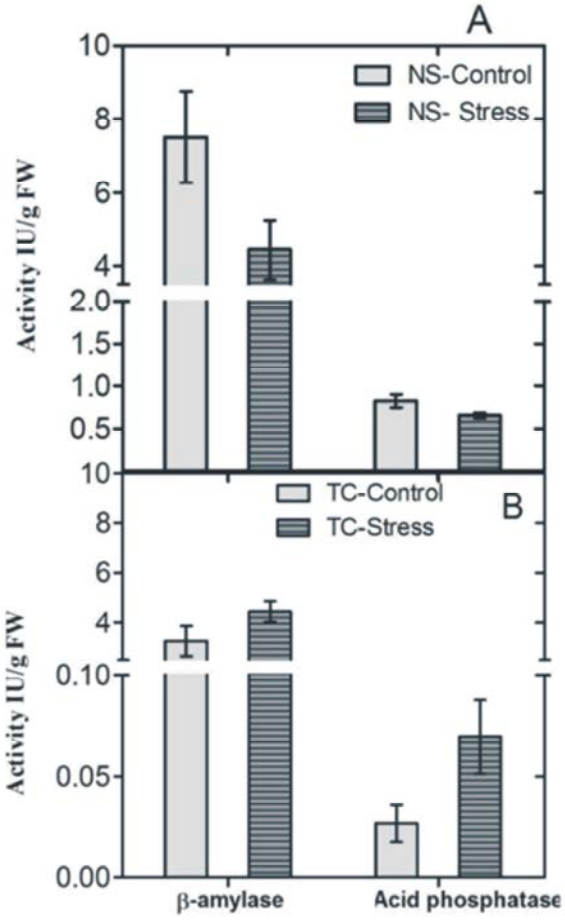

Fig. 3: Activities of metabolite mediated enzymes of Horse gram normal seedling (a) exposed to drought stress and (b) tissue culture exposed to dehydration with 10% PEG. Data were analyzed by one way analysis of variance (ANOVA) test and a P< 0.05 was considered significant.

The ROS produced during applied stress is efficiently processed by antioxidant enzymes. Considerable elevation in the levels of antioxidant enzymes in both tissue culture and native seedlings indicated that greater degree of stress imposed on the plant and presence of an efficient detoxification system in horse gram.

The levels of antioxidant enzymes in tissue culture and normal seedlings relative to their respective controls showed a similar responsetrend (Figure 2). Three of the four antioxidant enzymes tested, POX, SOD, GR were elevated under stress in both normal and tissue culture regenerates. Nevertheless the pattern of on enzyme bands showed different in POX, new expressed isoform in tissue culture was reported [30]. Similar elevation in the antioxidant enzymes have been noted in many plants [31]. However, the CAT levels were reduced in both the tissues under applied stress. The patterns obtained from drought for normal plant and tissue culture regenerates is essentially similar (Figure 2.A). However, the patterns of enzyme levels did not match with those obtained duringsalt stress [32]. This suggested operation of same response mechanism under drought and high temperaturestresses. It also suggested that same kind of stress elicitor (dehydration) and transducer of signal are involved in these stresses. However, the two tissues differed in their response to stress in terms of metabolic enzymes amylase and phosphatase (Figure 3). Both enzymes were reduced under drought stress in normal seedlings, whereas elevated in regenerates.

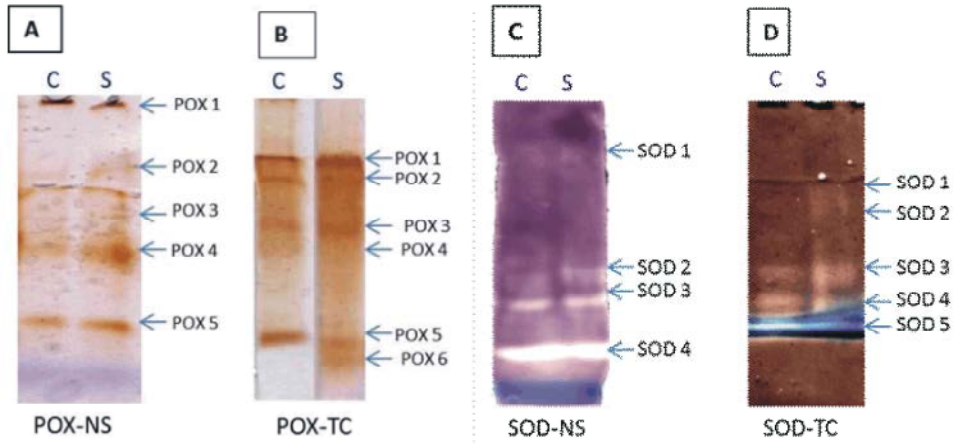

Fig. 4: Zymogram of isoenzyme activity: (A) and (B) forGuaiacol peroxidase; (C) and (D) for SOD. (NS = normal seedling, TC= tissue culture seedling); (C = control,S= stress)

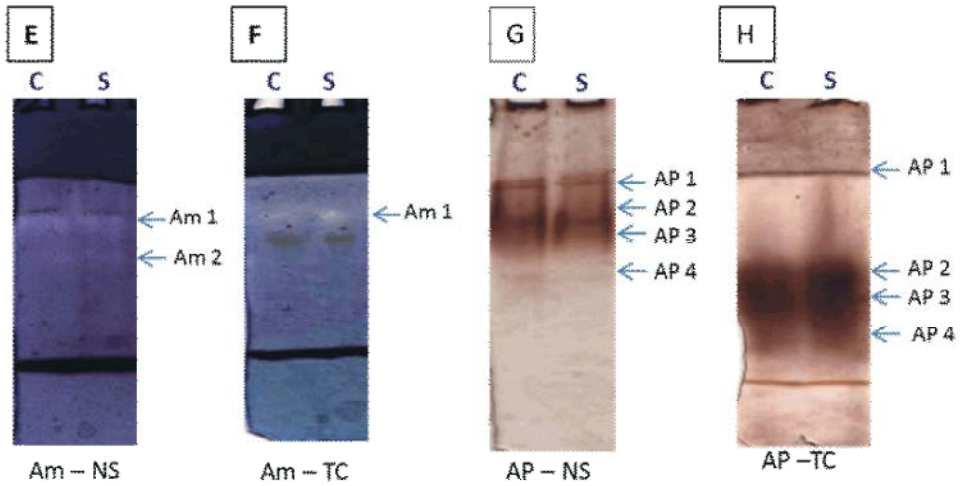

Fig. 5: Zymogram of isoenzyme activity: (E) and (F) forAmylase;(G) and (H) for Acid Phosphatase. (NS = normal seedling, TC= tissue culture seedling); (C = control,S= stress)

The patterns of amylase and phosphatase in drought stress resembled the temperature stress pattern in normal seedling. but The enzyme level pattern in tissue culture regenerates however resembled those obtained for salt stress [32], suggesting the fact that dehydration by PEG and NaCl elicit similar response on metabolic enzymes like amylase and phosphatase [32].

The sequence of events in the plant tissue subjected to drought stress are; increased production of ROS and oxidized target molecules; increase in the expression of genes for antioxidant functions; increases in the levels of antioxidative systems and increased scavenging capacity for ROS, resulting in tolerance against the drought stress [33]. Considering the known roles of each of the antioxidant enzymes it appears that such a sequence is in place in horse gram.

The difference in amylase and phosphatase activities between normal and tissue culture plants could be due to difference in their nutritional/metabolic patterns. The growth of plant under tissue culture condition can also be considered as acclimation to microenvironment. This is also reflected from the SDS-PAGE patterns of the normal and tissue cultured plants, wherein the number of bands in tissue culture regenerates is significantly lower than those in the normal plant tissue (Figure 4B).

Evaluation of stress response in terms of antioxidants and other stress markers indicated greater degree of stress in tissue culture regenerates than normal seedlings. Even though the amount of antioxidants and other parameters were relatively low in tissue culture plantlets than in native seedlings, the difference between the control and stressed tissue culture plantlets was striking.

Table 1: Non-enzymatic antioxidants of normal seedlings and tissue culture of horse gram subjected to drought stress

	Normal seedlings		Tissue cultured seedlings	
	control	stress	control	stress
GSH (µg/g FW *)	166.70 ± 10.60	383.30 ± 16.28	16.54 ± 1.30	58.65 ± 4.14
ASA (mg/g FW)	3.30 ± 0.52	4.78 ± 0.67	0.2987 ± 0.05	0.70 ± 0.09
Proline (µg/g FW)	0.95 ± 0.17	10.81 ± 1.03	0.11 ± 0.01	0.60 ± 0.03

Data are means of three replicates + SE (P < 0.05)

**FW= Fresh weight*

Table 2: Stress markers in horse gram tissue culture and normal seedlings subjected to drought stress

	Normal seedlings		Tissue cultured seedlings	
	control	stress	control	stress
H_2O_2 (mM/g FW)	6.347 ± 0.93	9.130 ± 0.78	0.980 ± 0.10	1.932 ± 0.04
MDA (µg/g FW)	0.911 ± 0.05	1.082 ± 0.07	0.8387 ± 0.06	2.072 ± 0.03
Total Chlorophyll (mg/g FW)	0.642 ± 0.06	1.040 ± 0.05	0.6190 ± 0.05	0.898 ± 0.03
Total soluble sugar (mg/g FW)	27.371 ± 1.51	34.03 ± 1.44	0.6190 ± 0.05	0.898 ± 0.03

Data are means of three replicates + SE (P < 0.05)

**FW= Fresh weight*

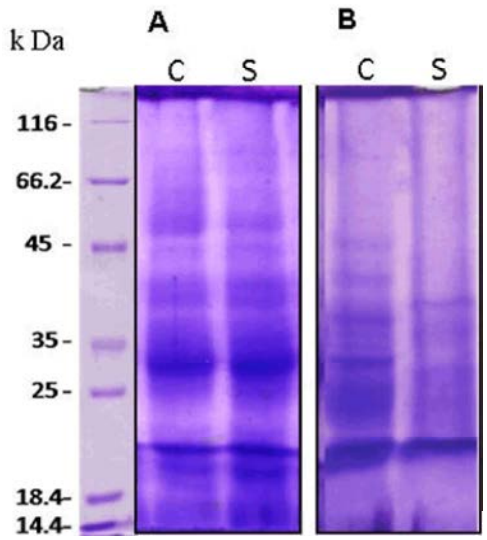

Fig. 6: 10% SDS-PAGE Stained for Proteins with coomassie brilliant Blue R of crude extracts. (A): of Normal seedlings; (B): of tissue culture. (C) = control and (S) = stress

In comparison with the controls tissue culture plantlets, the stressed plantlets had 2-fold increase in H_2O_2, MDA and ascorbic acid, ~3-fold increase in GSH and a 6-fold increase in proline. Except the proline, which increased by 10 fold relative to control in native seedlings, the enhancement in other components was not as high as those in tissue culture plantlets (Table 1). The elevated levels of H_2O_2 in both tissues under stress indicated induction of POX and its levels in regenerates and native seedlings are in agreement with this.The elevated levels of MDA which indicated a greater level of lipid peroxidation in drought and dehydration stress also coincides with higher levels of POX. Increased lipid peroxidation during water loss was reported under drought in wheat seedling [34], in sunflower [35]. These observations are also in consonance with the levels of SOD in both tissues under stress and induction of a new isoform of SOD in native seedlings (Figure 4.3). Proline accumulation may help the plant to survive short periods of drought and recover from stress. The marked increase in the levels of proline in both seedling indicated operation of an efficient osmotic regulation in both native and tissue cultured plantlets. The same was also noticed in temperature stressed seedlings but not in salt stressed horse gram [32]. Plants respond to drought stress by physiological and biochemical strategies. Anti-oxidative enzymes and osmoregulation are usually considered to be critical factors under water deficient conditions.

It is proposed that soluble sugar, in addition to their storage functions have an important role in controlling cellular metabolism. Soluble sugars can be involved in osmoregulation and can decrease osmotic potential as well as improve stability of soluble protein [36]. The observed increase in the soluble sugars of both horse gram seedlings is in agreement with these generalizations (Table 2). If the repertoires of genomic responses are not sufficient or appropriate, then primary metabolism is

impaired and oxidative stress becomes increasingly important and cell death and senescence response are triggered [37, 38].

The tissue culture regenerated plantlets stressed with dehydration also behaved similar to drought stressed seedlings in terms of antioxidant enzymes and antioxidants albeit minor variations. The tissue culture regenerates were more stressed than the native seedlings.The above results show that horse gram has repertoire to respond to the deficiency of water stress in both growth conditions. However, higher intensities of drought and dehydration may invoke the senescence responses.

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