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Comparative Study on Biochemical Parameters and Antioxidant Enzymes in a Drought Tolerant and a Sensitive Variety of Horsegram (*Macrotyloma uniflorum*) under Drought Stress

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ABSTRACT

Drought is one of the major abiotic stresses affecting the agricultural production worldwide. A generally drought tolerant legume, horsegram was chosen to compare and decipher the biochemical mechanisms of drought stress tolerance. For this, 25 day old plants of a drought tolerant (HPK 4) and a sensitive variety (HPKC 2) of horse-gram (*Macrotyloma uniflorum* L.) were subjected to drought stress (PEG; polyethylene glycol treatment) and control conditions (without PEG) for 48 h. Leaf and root tissues of these horsegram plants were harvested for biochemical and antioxidant enzymatic assays. The Relative Water Content (RWC), proline and phenol content were found to be significantly higher in the tolerant variety under drought stress. While the protein and Malondialdehyde (MDA) content was observed to be significantly higher in the sensitive variety under drought stress. Among the antioxidant enzymes, Peroxidase (POD), Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione-s-transferase (GST) showed significant increase in the tolerant variety than the sensitive one under drought stress. However, Glutathione Reductase (GR) activity was observed to be decreased. The results suggested that higher levels of RWC, phenols and proline accumulation in tolerant variety of horsegram could play an important role in drought stress tolerance. The diverse levels of antioxidant enzymes may be responsible for the differential drought tolerance capacities of the two varieties.

Key words: Antioxidant enzymes, drought stress, horsegram, malondialdehyde, proline

INTRODUCTION

Drought has been considered as one of the most devastating among the abiotic stresses (Vinocur and Altman, 2005). Over the time it leads to scarcity of the water resources for agronomic uses which ultimately makes the lands barren (Wardlaw, 2002; Llorens *et al.*, 2004; Flexas *et al.*, 2006). The need of the hour is to develop and identify drought tolerant crop lines. Understanding the functioning capacity of drought tolerant plants under drought stress is inevitable in this respect. Different parameters like RWC, proline, phenols, MDA content and some antioxidant enzymes activities have been considered as markers of stress. These have been associated with the different tolerance levels of plants towards drought stress (Azooz *et al.*, 2009; Unyayar and Cekic, 2005; Hura *et al.*, 2007; Wang *et al.*, 2009; Gajewska and Sklodowska, 2008).

During cell metabolism Reactive Oxygen Species (ROS) such as singlet oxygen (O_2^1), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH) are generated as natural by products (Misra and Gupta, 2006a). However, the exposure of plants to environmental stresses enhanced the generation of ROS. To avoid the subsequent damage, cells are normally protected against ROS by the cellular antioxidant defence systems (Gajewska and Sklodowska, 2008). The activities of antioxidant enzymes in plants under stress are usually regarded as an indicator of the tolerance of genotypes against stress conditions (Unyayar and Cekic, 2005).

Horsegram (*Macrotyloma uniflorum*) is generally drought tolerant legume. Earlier a study on identification of suitable varieties of horsegram for dryland has been conducted (Prakash *et al.*, 2008). Proximate analysis and effect of heavy metal stress has been reported in horsegram (Yadav *et al.*, 2004; Reddy *et al.*, 2005). However, studies on biochemical factors and antioxidant enzymes during drought stress in a drought tolerant crop like horsegram still remain elusive. Therefore, the present study was framed to compare the biochemical and antioxidative responses in leaf and root tissue of a drought sensitive and a drought tolerant variety of horsegram under drought stress. This study has investigated the influence of drought stress on RWC, proline, phenol, protein and MDA content. Also, the activities of SOD, POD, GR, CAT and GST were estimated.

MATERIALS AND METHODS

Plant material and drought stress treatment: Nine varieties of horsegram (*Macrotyloma uniflorum*) were procured from the Department of Plant Breeding and Genetics, CSK HPKV, Palampur, Himachal Pradesh, India. These were raised during the months of March-June, 2010 and were screened for drought tolerance. After screening a drought tolerant (HPK 4) and a drought sensitive (HPKC 2) variety were chosen for further study. Twenty five days old plants were given drought stress hydroponically with 18% solution of PEG 6000 for 48 h. Plants grown hydroponically without PEG treatment were used as controls. Leaf and root tissues of treated plants were harvested for further analysis.

Relative water content: Relative Water Content (RWC) defined as the percentage of water content in relation to the fully water saturated plant tissue was determined according to Smart and Bingham (1974). RWC was calculated from the following formula:

$$RWC = \frac{(FW-DW)}{(TW-DW)} \times 100$$

Dry Weight (DW) was measured after oven-drying of the plant material for 48 h at 50-60°C. Turgid Weight (TW) was determined after floating of plant material on water/PEG solution overnight at room temperature.

The level of lipid peroxidation was determined by measuring the amount of MDA produced by the Thiobarbituric Acid (TBA) reaction as described by Heath and Packer (1968). The total phenols were measured by Folin-Ciocalteu method of Singleton and Rossi (1965). Free proline content was determined using the ninhydrin method of Bates *et al.* (1973). Protein was determined by the method of Bradford (1976).

Enzyme extraction and assays: Fresh tissue (100 mg) was homogenized in an ice cold mortar using 50 mM potassium phosphate buffer pH 7.0 containing 1 mM sodium ascorbate, 1 mM EDTA and 0.5 M NaCl. After centrifugation (15,000 rpm, 20 min) the supernatant was used for

determination of CAT, POD and GST activities. For SOD and GR activity determination, different buffer was used (50 mM potassium phosphate buffer pH 7.8 containing 1mM Dithithreitol (DTT) and 2% Polyvinylpyrrolidone (PVP). SOD activity was measured according to the method of Dhindsa *et al.* (1981). CAT activity was measured according to the method described by Aebi (1984). POD activity was measured by the method of Maehly and Chance (1954). GR activity was measured by following the change in 340 nm as oxidized glutathione (GSSG)-dependent oxidation of NADPH, according to the method of Carlberg and Mannervik (1985). Total GST activity was measured by the method of Habig *et al.* (1974) using CDNB (1-chloro-2, 4-dinitrobenzene) as a substrate.

Statistical analysis: The experiments were conducted in randomized block design. The results presented are the means of three replicates. Sample variability was estimated by standard deviation of the mean. Analysis of variance was conducted on the data at CD 5%. The software used was CPCS 1.

RESULTS

Effect of drought stress on biochemical parameters

Effect of drought stress on RWC: The RWC was found to be higher in leaf tissue of HPK 4 under control and stress conditions (98.08 and 88.68%, respectively) than HPK 2 (95.79 and 80.06%, respectively) (Fig. 1a). However, under stress conditions higher reduction in the RWC was observed for HPK 2 (15.73%) as compared to HPK 4 (9.4%). In case of roots, the RWC decreased from 91.75% (control) to 83.5% (stress) for HPK 2 and from 97.48-92.01%, respectively for HPK 4 (Fig. 1b).

Effect of drought stress on proline content: The increase in the proline content was significantly higher (CD 5%) for the tolerant variety in both the tissues as compared to the sensitive variety (Fig. 2). The percentage increase in leaf proline content from control to stress was higher

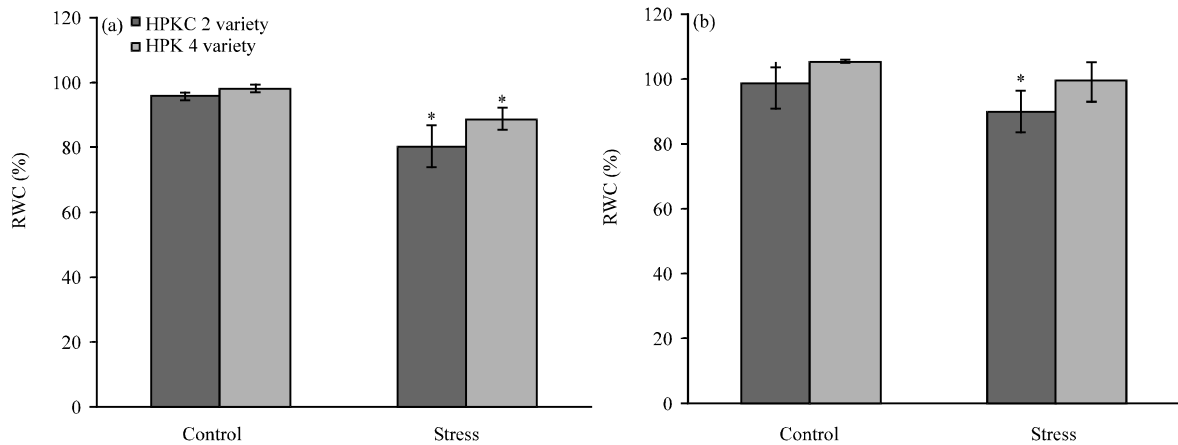


Fig. 1(a-b): Relative water content in the leaves (a) and roots (b) of horsegram. The values are mean of triplicates with SD. The values marked with the symbol '*' are significant at 5% level

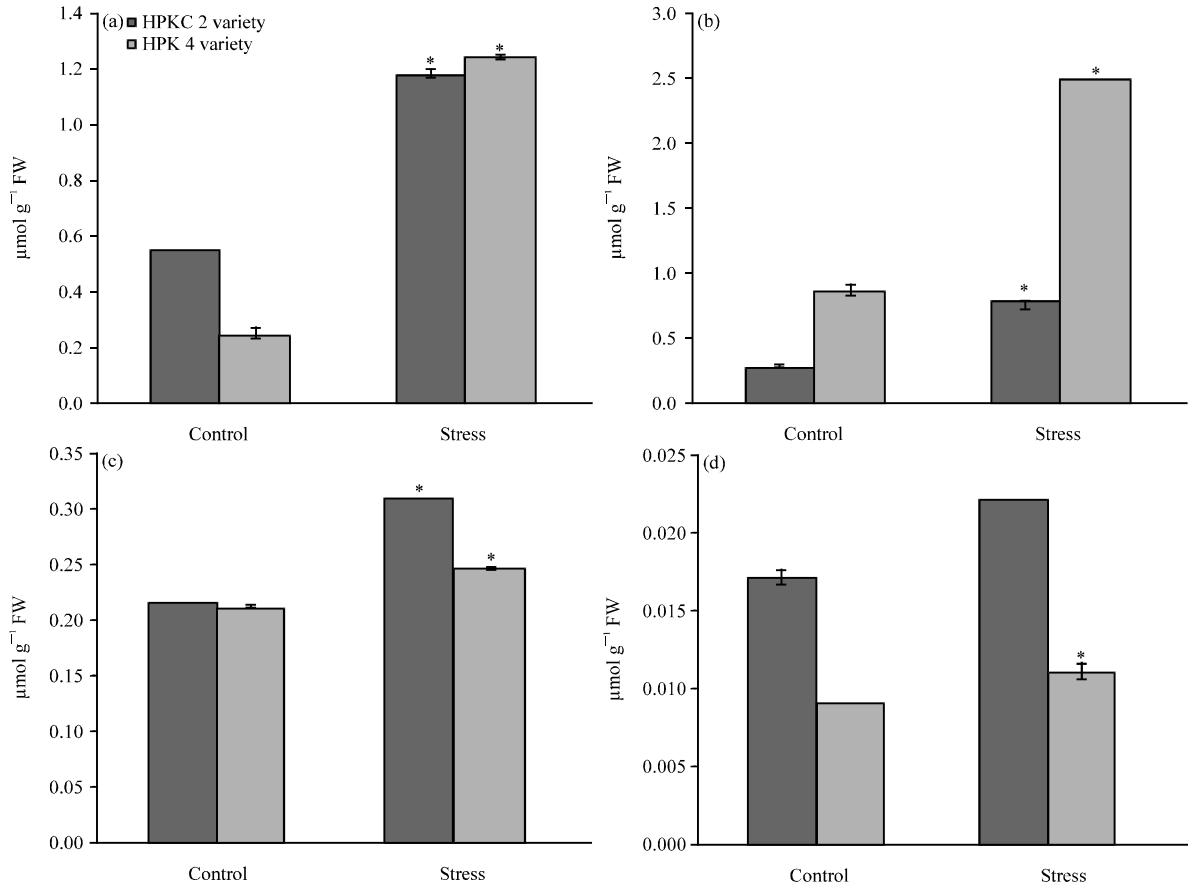


Fig. 2(a-d): Proline content in leaves (a) and roots (b) of horsegram. Malondialdehyde content in the leaves (c) and roots (d) of horsegram. The values are mean of triplicates with SD. The values marked with the symbol ‘*’ are significant at 5% level

for HPK 4 (406% increase) than HPKC 2 (113% increase) (Fig. 2a). However, the equal increment was observed in the roots from control to stress for the two varieties (Fig. 2b).

Effect of drought stress on MDA content: The MDA content was found to be similar at the basal level in the leaves of both varieties (Fig. 2c). However, under drought stress conditions the increase was more significant (CD 5%) for HPKC 2 (43.92% increase) than for HPK 4 (17.14% increase). In roots, the extent of increase from control to stress was significant for both the varieties being higher for HPKC 2 (29.41% increase) compared to HPK 4 (22.22% increase) (Fig. 2d).

Effect of drought stress on protein content: The increment in the leaf protein content from control to stress level was found to be significantly higher (CD 5%) for HPKC 2 (22.15% increase) than HPK 4 (4.5% increase) (Fig. 3a). In case of roots also, the increase was significantly higher for HPKC 2 (69.27% increase) than HPK 4 (4.93% increase) (Fig. 3b).

Effect of drought stress on phenols content: The basal level of phenol content in leaves and roots was found to be higher for HPKC 2 than HPK 4 (Fig. 3). However, the increment in the leaf phenol content from control to

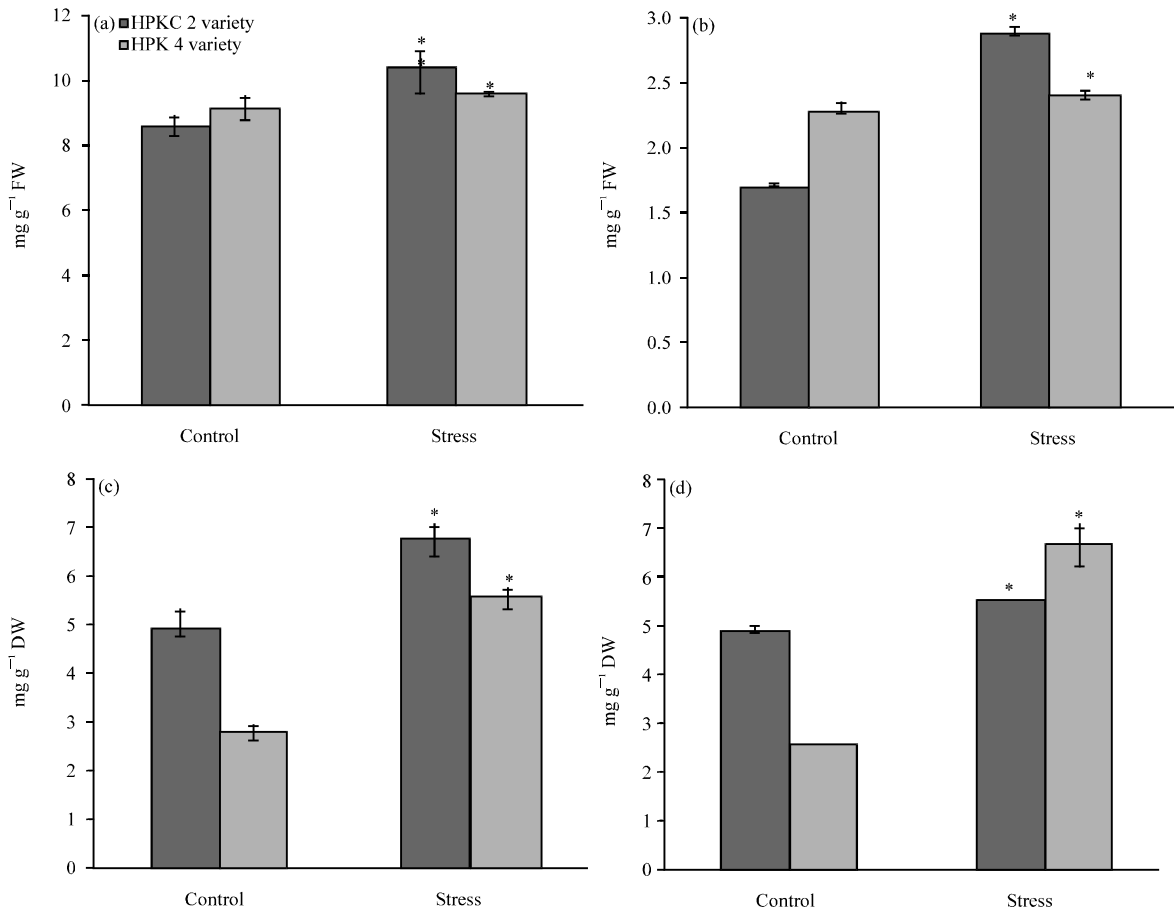


Fig. 3(a-d): Protein content in leaves (a) and roots (b) of horsegram. Phenol content in the leaves (c) and roots (d) of horsegram. The values are mean of triplicates with SD. The values marked with the symbol ‘*’ are significant at 5% level

stress was found to be significantly higher (CD 5%) for HPK 4 (96.01% increase) than HPKC 2 (36.84% increase) (Fig. 3c). Similarly, for roots the extent of increase in phenol content was significantly higher for HPK 4 (159% increase) than HPKC 2 (12% increase) (Fig. 3d).

Effect of drought stress on antioxidant enzymes

Effect of drought stress on CAT activity: The CAT activity in the leaves was found to remain same at the basal and stress level for both the varieties (Fig. 4a). In case of roots, the activity was found to be same for HPKC 2 at both levels. However, for HPK 4 a significant decrease (CD 5%) of 120% was recorded from control to stress level (Fig. 4b).

Effect of drought stress on POD activity: The levels of POD activity in the tolerant variety were found to be significantly higher (CD 5%) for both the tissues under control and stress conditions (Fig. 4c, d). In case of sensitive variety, only a marginal increase was observed in the roots (1.7%) (Fig. 4c, d).

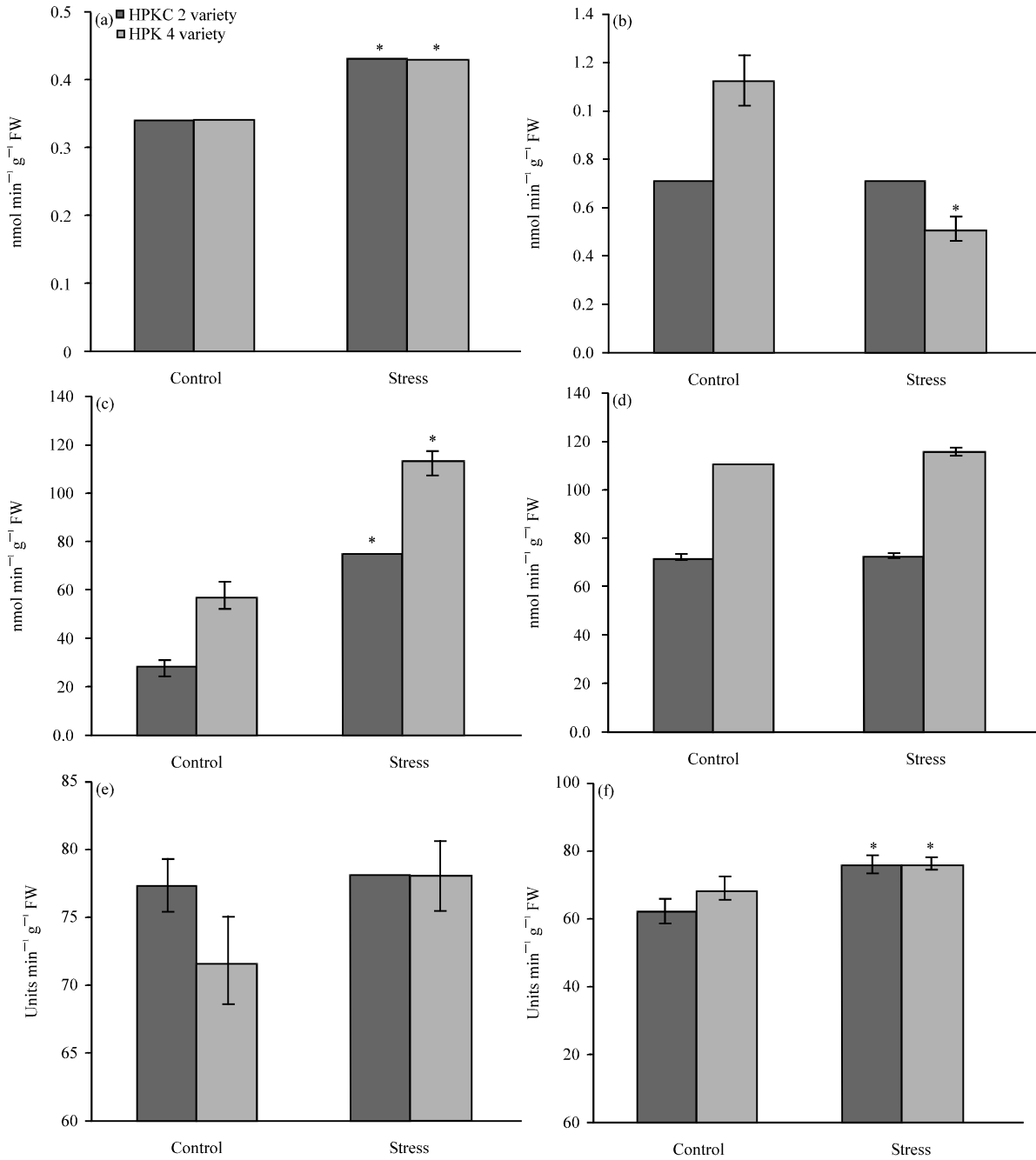


Fig. 4(a-f): CAT activity in leaves (a) and roots (b) of horsegram. POD activity in leaves (c) and roots (d) of horsegram. SOD activity in the leaves (e) and roots (f) of horsegram. The values are mean of triplicates with SD. The values marked with the symbol '*' are significant at 5% level

Effect of drought stress on SOD activity: An insignificant increase of 1% was observed in the SOD activity of leaves for HPKC 2 from control to stress as compared to a significant increase

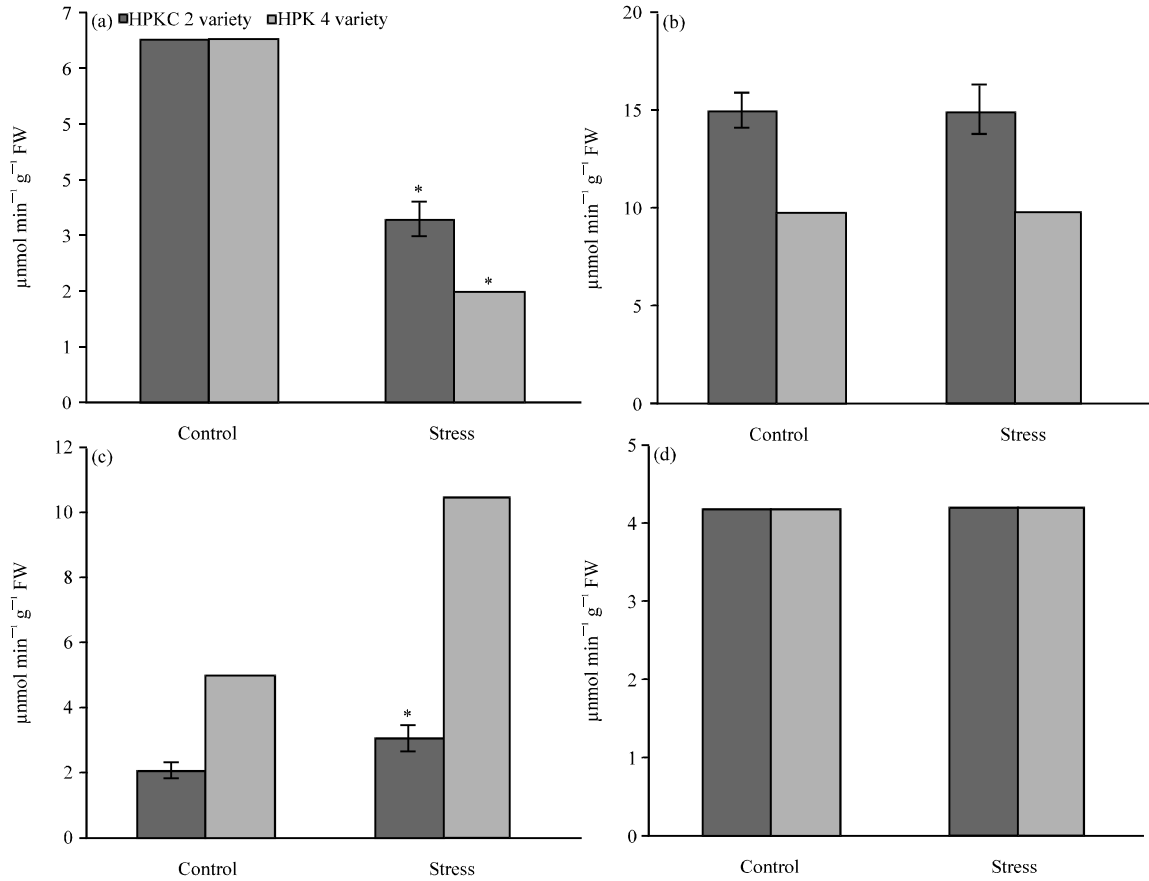


Fig 5(a-d): GR activity in leaves (a) and roots (b) of horsegram. GST activity in the leaves (c) and roots (d) of horsegram. The values are mean of triplicates with SD. The values marked with the symbol '*' are significant at 5% level

(CD 5%) of 8.85% in HPK 4 (Fig. 4e). In case of roots, the increment in the activity was significant for HPKC 2 (21.22% increase) than HPK 4 (9.93% increase) under stress conditions (Fig. 4f).

Effect of drought stress on GR activity: The GR activity in leaves was found to be same for both the varieties at basal level. However, under stress conditions the activity was found to be decreased more significantly (CD 5%) for HPK 4 (234% decrease) than HPKC 2 (100% decrease) (Fig. 5a). The GR activity in roots was found to be same at both the levels for the two varieties although the values were higher for HPKC 2 as compared to HPK 4 (Fig. 5b).

Effect of drought stress on GST activity: The GST activity in leaves was found to be significantly higher (CD 5%) at both the levels for the tolerant variety (Fig. 5c). While only 50% increment was recorded for HPKC 2 from control to stress conditions, HPK 4 showed 108% increase under same conditions. The GST activity in the roots was found to be unchanged for both the varieties under the control and stress conditions (Fig. 5d).

DISCUSSION

RWC is the measure of the health and sturdiness of a plant and is lowered in the state of stress. The higher reduction of RWC in sensitive variety of horsegram suggested more profound effect of drought stress as compared to tolerant one. It has been reported earlier that RWC is lowered in the roots of *Zea mays* under salt stress, in both shoots and roots of wheat and in leaves of mulberry (Rodriguez *et al.*, 1997; Gajewska and Sklodowska, 2008; Harinasut *et al.*, 2000). MDA is a product of peroxidation of unsaturated fatty acids in phospholipids and the level of lipid peroxidation has been used as an indicator of free radical damage to cell membranes under stress conditions. Under drought stress conditions the MDA content was found to be increased in both the varieties but extent was higher in the sensitive variety. This suggested that the extent of response to drought stress was more in the sensitive variety and the tolerant variety seems to be capable of withstanding such stress. MDA has been widely used to assess abiotic stress injury as criterion in various plants (Jain *et al.*, 2001; Katsuhara *et al.*, 2005; Abdul Jaleel *et al.*, 2007). It has been reported earlier that lower MDA level in the salt tolerant cultivar of maize suggests its tolerance towards stress (Azooz *et al.*, 2009). The possible reason for this could be that free radical generation and membrane damage would be low in tolerant plants leading to lower levels of MDA content. Therefore, in the present study higher RWC and relatively lower degree of increase in MDA content in the tolerant variety of horsegram due to drought stress supported its tolerant nature.

Proline is one of the most important organic solute that has been reported in plants to maintain the water content under stressful conditions by acting as osmoprotectant for membrane stabilization (Kavi Kishor *et al.*, 2005). In this study, higher accumulation of proline content in the tolerant variety might be the possible reason for its enhanced capacity to resist drought stress. Higher proline accumulation was reported under dry habitat in the root and shoot tissues of *Tephrosia purpurea* Pers and Ragi (*Eleusine coracana*). It was positively related with survival capability and drought tolerant nature of these plants (Erakar and Murumkar, 1995; Kandpal *et al.*, 1981). Proline accumulation has also been reported to increase under salinity stress in green gram plants (Misra and Gupta, 2006b). Present results depicting accumulation of proline may provide a biochemical adaptation for plants during drought stress.

The increase in protein content of horsegram during drought stress was in contradiction to that of reported earlier (Ghasempour and Kianian, 2007; Saleh, 2007). However, Ting *et al.* (2009) have reported an increase in the protein content of *Orthosiphon stamineus* under PEG induced water stress and NaCl stress as an adaptive mechanism of stress tolerance in plants. Kandpal *et al.* (1981) also reported higher protein content in the Ragi leaves under drought stress. The possible reason suggested for the increase in the protein content was either increased *de novo* synthesis or decreased protein degradation. Proteins are assumed to protect the plants against stress by supporting the leaf structure during wilting process (Kandpal *et al.*, 1981). Earlier study has documented the increase in proline and protein content of wheat upon the influence of abiotic stresses (Azooz and Youssef, 2010).

The functioning of protective mechanisms involving the elevated levels of phenolics e.g., ferulic acid in leaf tissues has been regarded as one of the most important criterion for categorizing plants to be more drought tolerant (Hura *et al.*, 2007, 2009). The higher level of phenols in both leaf and root tissues of the tolerant variety suggested their contribution in drought tolerance. In an earlier study also, higher levels of phenols have been reported in more resistant varieties of grapes (Satisha *et al.*, 2007).

ROS are natural products of cell metabolism, however, under conditions of environmental stress their generation may be greatly increased (Gajewska and Sklodowska, 2008). Superoxide dismutase (SOD, EC 1.15.1.1) which catalyzes disproportionation of superoxide anion ($O_2^{\cdot -}$) to H_2O_2 and O_2 constitutes the first line of antioxidative defence (Hamilton and Heckathorn, 2001). Hydrogen peroxide so produced may be scavenged by catalases and peroxidases. Catalase (CAT, EC 1.11.1.6) removes H_2O_2 by converting it to H_2O and O_2 . Other peroxidases, including guaiacol peroxidase (POD, EC 1.11.1.7.) eliminate H_2O_2 using various reductants, e.g. phenolic compounds. Plants with high constitutive and induced antioxidant levels have better resistance to damage (Parida and Das, 2005).

SOD is the key enzyme in the active oxygen scavenger system (Azooz *et al.*, 2009). The SOD enzyme has been found in seeds, shoots and roots of the pea seedlings (Giannopolitis and Ries, 1977). Abiotic stresses are known to induce and enhance the expression of antioxidant genes encoding SOD, CAT, POD and GR. (Guan *et al.*, 2000; Jiang and Zhang, 2002; Unyayar and Cekic, 2005). The increment in the activity of SOD in both the tissues suggested its role in the defence system against drought stress of horsegram. SOD activity has earlier been shown to be increased in two *Datura* species under the effect of triadimefon, a fungicide which creates stress like symptoms (Sivakumar and Panneerselvam, 2011). Other researchers have also reported that ABA treatment or drought increased the activity of SOD in mature leaves of *Arabidopsis* (Jung, 2004) and wheat (Keles and Oncel, 2002). The higher increase of POD activity in both the tissues of the tolerant variety suggested its potential role against drought stress. However, the CAT activity was observed to be either decreased or remain same under stress conditions. Earlier studies have reported the decrease in CAT activity under salinity stress in soybean (Amirjani, 2010). Hence, decline in the CAT activity and increase in the POD activity indicated that POD rather than CAT might be more active in the protection of leaf tissues of horsegram against H_2O_2 toxicity. In addition to its role in the removal of H_2O_2 , POD is involved in lignification and other processes leading to cell wall strengthening. Enhancements in the POD activity after Ni exposure have previously been found in the shoots of maize (Baccouch *et al.*, 1998) and wheat (Gajewska and Sklodowska, 2008). Increased peroxidase activity under chilling stress has been reported to be associated with tolerance capacity of genotypes of sweet potatoes and cowpea (Islam *et al.*, 2011, 2009). Tolerant variety of canola showed higher increase in proline content, POD and CAT activity than sensitive variety towards drought stress (Omidi, 2010). This study further suggested the importance of antioxidant enzyme activity and proline content in drought stress tolerance.

Glutathione reductase (GR; EC.1.8.1.7) catalyzes the reduction of the disulfide form of glutathione (GSSG) to reduced Glutathione (GSH) by utilizing NADPH in the ascorbate glutathione cycle (Marrs, 1996). No change in GR activity in roots from control to stress conditions suggested that other enzymes of ascorbate-glutathione cycle like MDHAR, DHAR and APX instead of GR might be involved against cytotoxicity due to stress. Glutathione-S-transferase (GST, EC 2.5.1.18) catalyzes the conjugation of electrophilic substrates to reduced glutathione (GSH) and the resulting complexes are transported to a vacuole for further processing or degradation (Marrs, 1996). Apart from participation in the metabolism of natural plant secondary compounds and detoxification of xenobiotics, GST plays an important role in the removal of toxic products of lipid and protein peroxidation (Edwards and Dixon, 2004). The increase in the GST activity of the tolerant horsegram variety suggested its important role in protection against drought stress. Similar, results have been reported in a salt tolerant and a salt sensitive variety of green gram

under salt stress (Misra *et al.*, 2006). The unaltered activity of GST in roots may be explained such that its constitutive activity is probably high enough to protect the root cells against ROS arising due to the drought stress.

CONCLUSION

The biochemical factors can be used for categorizing drought tolerant and sensitive varieties in plants. Higher accumulation of proline in leaves of tolerant variety and phenolics in both the tissues of the tolerant variety of horsegram suggested their important role in adapting the plant towards stressful conditions. Higher extent of increase in MDA content of the sensitive variety suggested its sensitivity towards drought stress. The increase in the protein content might be an adaptive mechanism to overcome the harmful effects of drought besides the accumulation of proline and phenolics. Drought sensitive and tolerant varieties of horsegram had different dismutating capacities. Peroxidase enzyme was observed to be more active in scavenging H₂O₂ than catalase in horsegram. In roots of horsegram oxidative mechanisms other than GST might be involved in the degradation of the toxic radicals. Differential biochemical and antioxidative responses of the leaves and roots of horsegram towards drought stress might be variety dependent.

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