Indian Journal of Biotechnology
Vol. 10, January 2011, pp 130-139

Specific and non-specific responses of Hyacinth bean (*Dolichos lablab)* to drought stress

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Drought and salinity stress are the major causes of historic and modern agricultural productivity losses throughout the world. The availability of irrigation water is a challenge for many countries that have scarce water resources, yet are highly dependent on agriculture as a means of revenue. Effect of drought on Hyacinth bean, *Dolichos lablab* (HA-4 cultivar) was evaluated in 10-d-old seedlings for 8 d after withholding water. The stress reduced dry and fresh weight, leaf number, surface area, root and shoot length, total chlorophyll and relative water content. Oxidative stress markers, H_2O_2, glutathione, malondialdehyde, proline, ascorbic acid, total phenols, and total soluble sugars were significantly elevated. Drought enhanced antioxidant enzymes, peroxidase and glutathione reductase, and reduced catalase in a time dependent manner in the leaves. POX and CAT in roots showed inverse relationship with the duration of stress, whereas GR exhibited increased activity. The metabolic activitiy of enzymes, β-amylase and acid phosphatase, increased temporally in leaves and roots. Intensity of isozymes correlated with *in vitro* levels under stress. The plant showed ability to rehydrate and grow upon re-watering, and levels of antioxidant components correlated with drought tolerance of the plant.

Keywords: Antioxidants; antioxidant enzymes; *Dolichos lablab*; drought stress ; hyacinth bean proline; malondialdehyde

Introduction

Since agriculture began, drought has been one of the major crop productivity-limiting factors, causing famine and death. It is more of a constraint in developing countries like India, which depend largely on agriculture for their revenue. It is becoming increasingly important to study plant responses to water stress, as incidences of increasing aridity is being reported in many parts of the globe due to climatic changes. With increasing population and the growing soil aridity, water will become a scarce commodity in the near future. Therefore, a study of plant responses, from the molecular to whole plant level, is needed in order to understand specific and non-specific responses, adaptation reactions and hardening mechanisms. A better understanding of the effect of drought on plants is vital for developing new and improved methods of plant breeding and management. This communication focuses on the specific and non-specific responses of *Dolichos lablab* (Hyacinth bean) to drought stress.

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Note: This paper was presented in the International Conference on 'Challenges in Biotechnology and Food Technology' organized at Annamalai University, Annamalai Nagar during 8-10 October, 2009.

In plants, stress analysis, primary and secondary stresses have been identified, the latter resulting as a consequence of the former. Secondary stress is largely caused due to accumulation of reactive oxygen species (ROS), which are formed as a result of metabolic utilization of reducing power and imbalances in the electron transport rate. A variety of enzymatic and non-enzymatic mechanisms exist in plants to transform ROS into less harmful chemical species. This occurs by a complex interplay of members involved in antioxidant metabolism. Enzymes such as peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and polyphenol oxidase (PPO) form the antioxidant enzymatic component, while the non-enzymatic components include ascorbate (ASC), glutathione (GSH), phenols, etc. The degree of resistance of plants to drought is often related to quantitative and qualitative changes in the antioxidant systems. Other metabolic adjustments to drought stress include accumulation of organic solutes such as sugars, polyols, glycine betaines and proline[1].

Significant changes in water potentials in the environment can impose osmotic stress on plants, disrupting normal cellular activities. Plants subjected to drought stress exhibit a variety of mitigation strategies that include biochemical, physiological, morphological and developmental processes[2].

Adaptation to drought stress is associated with metabolic changes that provoke decrease in growth rate during the vegetative period[3], reduction in leaf growth, increased root length and stomatal closure that helps to resist drought severity, but do not make the plants tolerant to desiccation. Prolonged dehydration may also lead to plant death. Adverse drought stress causes disruption of the balance between production and scavenging of ROS. Increased SOD, APX, POX, CAT and GR have been correlated to salinity and drought tolerance[4,5].

D. lablab is an important pulse crop in South East Asia and Eastern Africa. It is used as an intercrop in India and Australia, as a weed suppressor and soil erosion retardant. It is also an excellent nitrogen fixer, and grown as a cover crop or for livestock fodder. The plant is relatively drought tolerant. In the light of deficient knowledge of biochemical and physiological basis of its response to abiotic stresses, the present study was initiated to evaluate the effect of drought on the plant. The paper attempts to understand the drought stress response of Hyacinth bean in terms of antioxidants, antioxidant enzymes and other markers of abiotic stress.

Materials and Methods

Plant Material and Chemicals

The seeds of *Dolichos lablab* (cv. HA-4) were procured from the National Seed Project, University of Agricultural Sciences, GKVK, Bangalore. Fine chemicals and reagents were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

Growth Conditions

Seeds were surface sterilized with 0.1% (w/v) mercuric chloride for 30 s, rinsed immediately with large volume of distilled water and imbibed overnight in distilled water. The overnight-soaked seeds were sown in plastic trays containing vermiculite and acid-washed sand (1:1 w/w) and irrigated daily 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 Treatment and Experimental Design

Drought stress was induced by withholding water to seedlings 10 d after germination (DAG). Leaf and root samples were collected at 2, 4, 6 and 8 d after stress (DAS) and frozen until further analysis. For the samples used for determination of RWC, fresh and dry weights were measured immediately after collection. The experimental design used was carried out at random factorial scheme, with 4 evaluation points (2, 4, 6 & 8 DAS). Each experiment comprised 16 experimental units (leaf+root samples of control and stressed plants) and done in triplicate. Seedlings watered twice daily were used as control.

Determination of Growth Parameters

Random selection of 10 replicates from each treatment was done. The growth criteria measured were leaf, shoot and root length; fresh and dry weight the whole plant.

Determination of Relative Water Content

The relative water content (RWC) was estimated applying the equation: RWC = (FW-DW) × 100 / (TW-DW) [6]. Leaf discs of 10 mm diameter were weighed to determine the fresh weight (FW), soaked in distilled water at 25°C for 4 h to determine the turgid weight (TW), then oven dried at 80°C for 24 h to determine the dry weight (DW). Similarly, entire shoots and roots were taken for analysis and RWC was computed as before.

Determination of H_2O_2 and Antioxidants

H_2O_2 content in control and stressed seedlings was determined according to Velikova[7]. Estimation of ascorbic acid, GSH was carried out according to Sadasivam and Manickam[8] and Beutler[9], respectively. Total phenols were estimated using catechol as an authentic standard[10].

Determination of Stress Markers

Proline content was estimated using ninhydrin reagent[11]. The amount of total soluble sugars was estimated colorimetrically at 540 nm using anthrone reagent[12]. Chlorophyll content was estimated using acetone (80%) extracts[13]. The concentrations of total chlorophyll, chlorophyll-a, and -b were calculated by the formula of Arnon[14]. The extent of lipid peroxidation was determined[15]. The MDA content was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

Extraction of Enzymes

The frozen samples were homogenized with pre-chilled 50 m*M* sodium phosphate buffer (*p*H 7.0) containing 5 m*M* β-mercaptoethanol and 1 m*M* EDTA using pestle and mortar. L-ascorbate was raised to a final concentration of 2 m*M* for extraction of APX. The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was used as a source of enzymes. Soluble protein content was determined using BSA as the standard[16].

Assay of Enzymes

Standard procedures were followed for the assay of various enzymes[17-25].

Electrophoretic Analysis

Non-denaturing, discontinuous slab gel electrophoresis was carried out essentially according to the method of Davis[26]. SDS-PAGE was carried out according to Laemmli[27], employing 12% resolving gel and 5% stacking gel.

Statistical Analysis

The experiment was performed using a randomized design. All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using GraphPad Prism version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by lowest standard deviations (LSD) test. Comparisons with $P \leq 0.05$ were considered significantly different.

Results and Discussion

Growth Parameters

Plant growth and productivity are primarily dependent on the water availability, and therefore, its decrease has negative effects on plant growth, photosynthesis, solute transport and accumulation. Drought stress in *D. lablab* caused significant reduction in the growth potential in time-dependent manner. The plants showed reduction in number and surface area of leaves as well as curling due to loss of turgidity. The stressed plants failed to grow the second pair of leaves even 8 DAS (Fig. 1). The decrease in growth can be attributed to the reduced cell elongation resulting from decreased turgor, cell volume and cell growth. Drastic reduction in fresh and dry weight of the plant was seen 4 DAS (Fig. 2). Other physical features such as length of stems, roots and leaves exhibited reduction in time dependent manner. The effect was more pronounced in roots. RWC of leaves, stems and roots also showed a similar relationship with the time of exposure (Fig. 3). The RWC of roots showed a steep decline when compared to leaves and stem.

Stress Markers

Photooxidative processes occurring during abiotic stress conditions are responsible for the production of ROS. An increase in activity of SOD and a decrease in activity of CAT lead to an elevation in the levels of H_2O_2. Drought stress resulted in increased production of H_2O_2 in time dependent manner. H_2O_2 concentration in leaves and roots showed greater elevation 6 DAS relative to control tissues. The increase was more pronounced in leaves (Table 1), suggesting greater impact on leaves than roots. Our results are in conformity with acid rain treated French

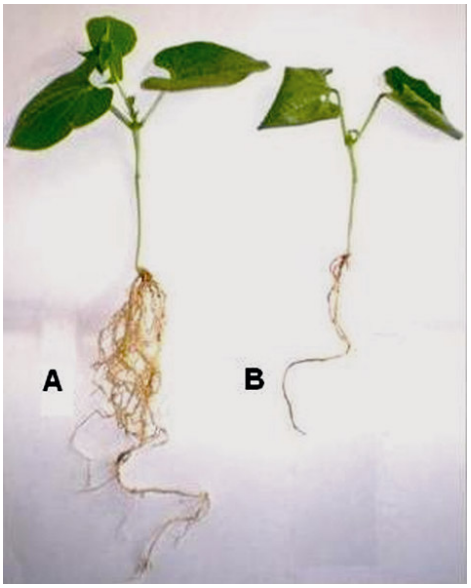

Fig. 1—Effect of drought stress on *D. lablab* seedling: A; Control seedlings, B; Stressed seedlings 6 DAS.

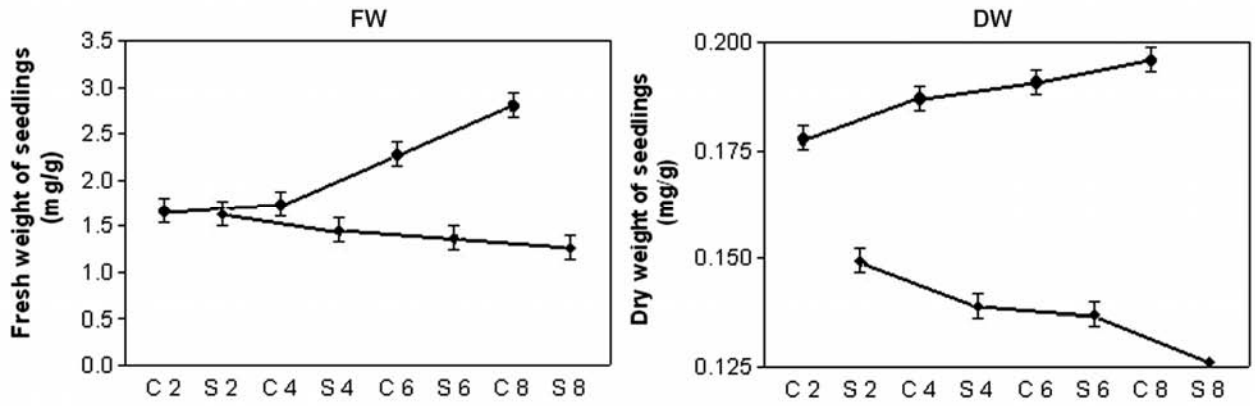

Fig. 2—Fresh and dry weight of *D. lablab* seedlings under 2, 4, 6 and 8 d of water deficit. C2 to C8-Control plants, S2 to S8-Stressed plants.

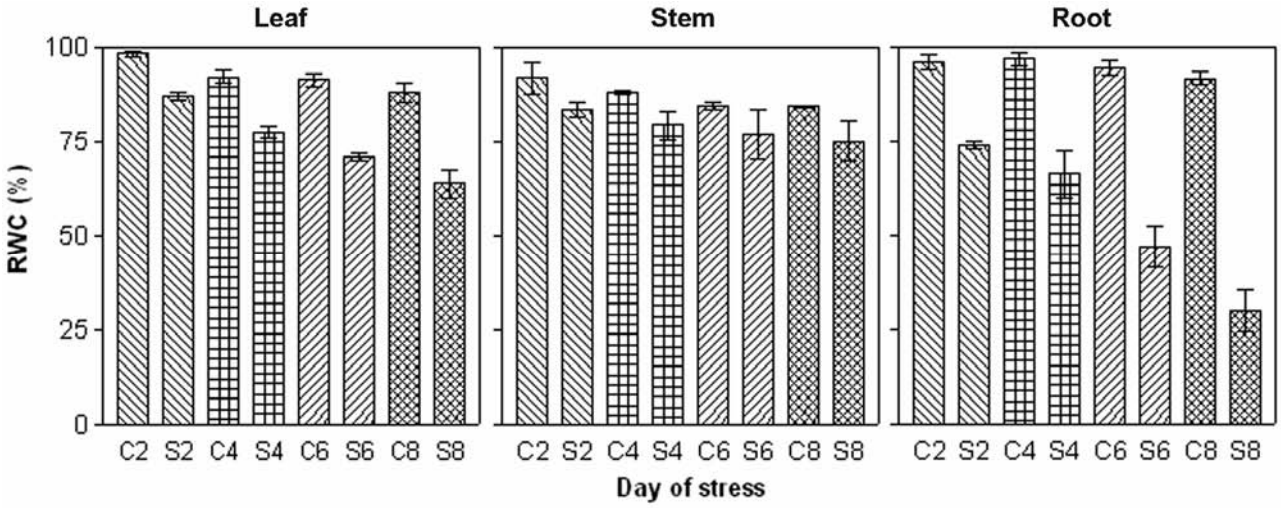

Fig. 3—RWC in leaf, stem and root tissues of *D. lablab* seedlings under 2, 4, 6 and 8 d of water deficit. C2 to C8-Control tissues, S2 to S8-Stressed tissues.

Table 1—Levels of antioxidants and other stress response indicators in drought stressed *D. lablab*

Time	Parameter	Leaves		Roots	
		Control	Stress	Control	Stress
2 DAS	H_2O_2 *	75.33 ± 5.4	71.67 ± 5.5	31.07 ± 2.8	29.30 ± 2.9
	GSH *	324.3 ± 2.4	332.2 ± 2.6	109.7 ± 6.95	157.3 ± 13.3
	Ascorbate *	49.30 ± 4.1	56.63 ± 7.4	37.37 ± 1.4	44.13 ± 3.2
	Proline *	671.60 ± 14.0	771.8 ± 15.5	473.0 ± 18.2	576.3 ± 18.7
	MDA (m moles/g FWt)	5.46 ± 0.49	6.72 ± 0.43	6.56 ± 1.5	11.04 ± 1.2
	Total soluble sugars #	12.60 ± 0.69	12.82 ± 1.4	6.56 ± 1.5	11.04 ± 1.2
	Total phenols #	1.39 ± 0.25	2.26 ± 0.22	2.63 ± 0.24	2.99 ± 0.23
	Total chlorophyll #	6.38 ± 0.42	9.19 ± 0.61	ND♦	ND♦
4 DAS	H_2O_2 *	86.0 ± 6.0	112.7 ± 8.0	30.80 ± 1.6	29.87 ± 0.9
	GSH *	316.5 ± 7.9	376.1 ± 7.9	133.2 ± 8.1	313.3 ± 7.9
	Ascorbate *	42.3 ± 9.0	50.43 ± 6.5	36.67 ± 3.8	53.73 ± 0.0
	Proline *	586.2 ± 9.8	662.0 ± 5.0	528.0 ± 5.2	587.8 ± 8.5
	MDA (m moles/g FWt)	5.11 ± 1.1	10.37 ± 1.1	5.47 ± 0.43	13.85 ± 0.25
	Total soluble sugars #	10.83 ± 0.68	15.48 ± 0.25	8.47 ± 0.43	13.85 ± 0.25
	Total phenols #	1.43 ± 0.23	3.13 ± 0.23	2.53 ± 0.25	2.2 ± 0.26
	Total chlorophyll #	7.61 ± 0.0	9.57 ± 0.25	ND♦	ND♦
6 DAS	H_2O_2 *	105.4 ± 8.8	169.9 ± 4.9	29.07 ± 2.4	32.31 ± 3.1
	GSH *	354.7 ± 9.1	446.2 ± 3.5	177.3 ± 6.1	260.4 ± 9.5
	Ascorbate *	44.43 ± 1.9	54.29 ± 7.0	60.80 ± 4.2	73.90 ± 9.7
	Proline *	616.0 ± 10.2	703.4 ± 5.5	613.0 ± 12.9	734.0 ± 13.5
	MDA (m moles/g FWt)	4.89 ± 0.45	9.83 ± 0.67	6.11 ± 0.81	19.47 ± 0.34
	Total soluble sugars #	10.41 ± 0.68	17.57 ± 0.47	7.11 ± 0.55	19.47 ± 0.34
	Total phenols #	1.73 ± 0.25	4.11 ± 0.23	2.48 ± 0.22	1.96 ± 0.20
	Total chlorophyll #	8.37 ± 0.10	6.17 ± 0.64	ND♦	ND♦
8 DAS	H_2O_2 *	109.73 ± 9.8	194.5 ± 9.1	29.21 ± 0.29	40.63 ± 1.9
	GSH *	373.4 ± 5.1	461.3 ± 3.8	165.7 ± 9.6	191.8 ± 4.1
	Ascorbate *	42.44 ± 4.0	59.73 ± 2.6	57.59 ± 4.8	80.19 ± 1.3
	Proline *	601.0 ± 12.2	983.3 ± 8.3	710.4 ± 9.0	1043.0 ± 8.4
	MDA (m moles/g FWt)	5.29 ± 0.49	9.38 ± 0.61	7.13 ± 0.75	23.28 ± 1.0
	Total soluble sugars #	11.67 ± 0.47	20.13 ± 0.38	7.13 ± 0.75	24.61 ± 1.2
	Total phenols #	2.07 ± 0.23	4.55 ± 0.31	2.58 ± 0.28	1.88 ± 0.08
	Total chlorophyll #	7.71 ± 1.1	4.00 ± 0.44	ND♦	ND♦

* µg/g fresh weight tissue; # mg/g fresh weight tissue; ♦ Not determined

bean[28] and chill stressed cucumber[29]. GSH provides a source of reducing equivalents that buffer the plant from a number of oxidative stresses. It acts directly by scavenging free radicals and indirectly by participating in the ascorbate/GSH cycle. Elevation in the levels of reduced GSH proportionate to time of drought stress in root and leaf (Table 1) is in consonance with observed induction of GSH synthesis by H_2O_2[30]. Another antioxidant, ASC behaves as a reductant in many enzymatic reactions as well as exhibits a non enzymatic role as a free radical scavenger of H_2O_2. Its levels in leaves and roots showed an exponential rise from the second day of drought stress (Table 1). This supported a significant role of ascorbate in scavenging of ROS in *Dolichos*. Parallel increase in GSH and ascorbate under drought stress, further suggested the efficient working of the ascorbate-gluathione cycle.

One of the strategies in drought stress tolerance is overproduction and accumulation of osmolytes. Proline has been shown to serve as a signal of senescence and a soluble nitrogen sink. Proline and TSS levels in leaves raised quickly and persistently in drought stressed *lablab* 8 DAS. Both leaves and roots exhibited ~ 1.5 fold increase in proline. The TSS rose by 1.7 and 3.4 fold respectively in leaf and root (Table 1). Such responses have been observed in a variety of leguminous plants including chickpea[31] under salt stress. The synthesis of proline bears a direct correlation with carbohydrate levels. The observed elevation of TSS in *lablab* indicated the prevalence of such a correlation. The increase in concentration of soluble sugars may be due to starch degradation, inhibition of starch synthesis and inhibition of INV. Elevated levels of TSS is important for energy production, stabilization of cellular membranes, maintenance of turgor, and signaling, this accounts for tolerance to dehydration stress.

The accumulation of free radicals in stressed plants causes oxidation of polyunsaturated fatty acids in the plasma membrane, resulting in the formation of MDA, an indication of membrane lipid peroxidation. MDA content of drought stressed leaves of *D. lablab* exhibited 50% increase at 2 DAS, but remained unaffected beyond 2 DAS. MDA content in roots, on the other hand, demonstrated a linear increase with time of exposure (Table 1). This suggested greater lipid peroxidation in roots, and a possible protective system in leaves of *lablab*. Total chlorophyll content in leaves declined steadily 6 DAS onwards (Table 1) and can be attributed to oxidative damage and increased chlorophyllase activity.

Phenols protect the cells from potential oxidative damage and increase the stability of cell membrane. The total phenol content of the drought stressed leaves exhibited a time dependent linear increase (Table 1) suggesting that signals for this secondary metabolite-mediated response is elicited under extreme dehydration stress in Hyacinth bean. Contrary to leaves, the total phenols in roots showed a less steep linear decline (Table 1). Reduced membrane damage in leaves as evidenced by the MDA content correlated with the elevated levels of phenols, thereby further substantiating the role of phenols in membrane protection.

Enzyme Activity

Along with antioxidants AOX enzymes contribute to alleviation of oxidative damage. The increase in activity of APX (Fig. 4) and POX (Fig. 5) with increased levels of H_2O_2, indicated their role in detoxification of H_2O_2. Higher levels of GR (Fig. 6), GSH and ASC (Table 1), which coincided with enhanced levels of POX, further suggested that GR activity provided the reductant (reduced ASC), necessary for POX to reduce H_2O_2 under drought stress. A marginal decrease of POX in roots indicated that it does not contribute to the removal of H_2O_2 in roots. However, the activity of GR in roots was found to increase suggesting the role of ASC/GSH cycle playing a major role in the root. The isozyme patterns of the POX and GR in roots and leaves differed, indicating a different set of genes being involved in roots and leaves of *lablab*. The isozyme patterns in leaves indicated an enhanced expression of these genes rather than expression of newer ones. A new isozyme was observed in roots of stressed *lablab*.

The break down H_2O_2 by CAT has been attributed in stress tolerance. Increase in CAT activity has been reported in many plants under water stress. A steep decline in its activity in leaf and a moderate drop in roots of drought stressed *lablab* indicated no significant role of the enzyme in stress response (Fig. 7). This drop in CAT and elevation of GR and APX further reinforced the fact that H_2O_2 detoxification is mainly through GR, APX and Asc/GSH cycle in *lablab*.

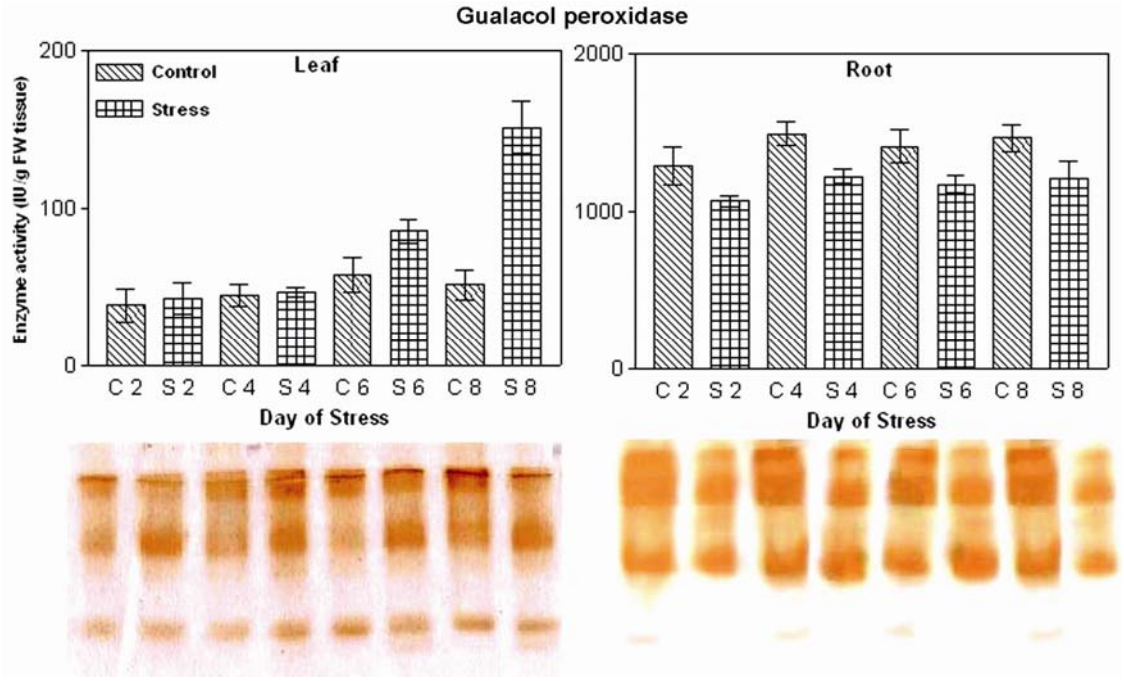

Fig. 4—Ascorbate peroxidase activity, in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissues, S2 to S8-Stressed tissues.

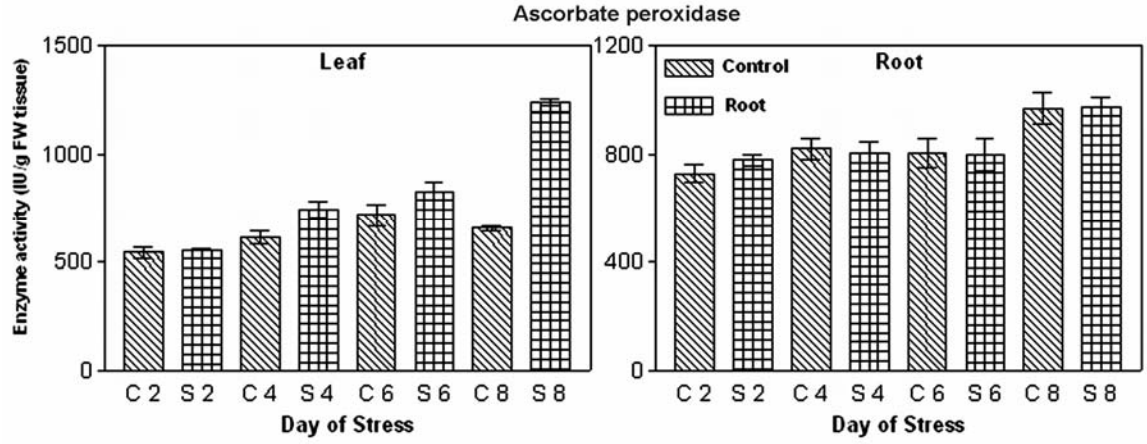

Fig. 5—Guaiacol peroxidase activity in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissuess, S2 to S8-Stressed tissues. Lower panel: Zymogram of POX during drought stress; 100 μg protein of each sample was separated on non-denaturing PAGE (10%) and stained for enzyme activity as described under materials and methods.

This kind of increased POX and GR associated with decreasing CAT in leaves has been reported in chickpea subjected to salt stress[31].

PPO has been shown to be associated with drought and salt tolerance[32]. Levels of PPO in *lablab* indicated a substantial role of the enzyme in drought response (Fig. 8). A similar correlation between PPO level and freezing tolerance has been reported for peach seedlings[33].

The role of antioxidant enzymes in stress response is indisputable. In addition, there are reports of the induction of metabolite enzymes such as; β-AMY[34], AP and INV under abiotic stress. AMY (Fig. 9) and AP (Fig. 10) activities increased in both leaves and roots of stressed seedlings and the enhancement of isozyme intensity was also correlated with *in vitro* levels. APs are known to act under water stress by maintaining a certain level of

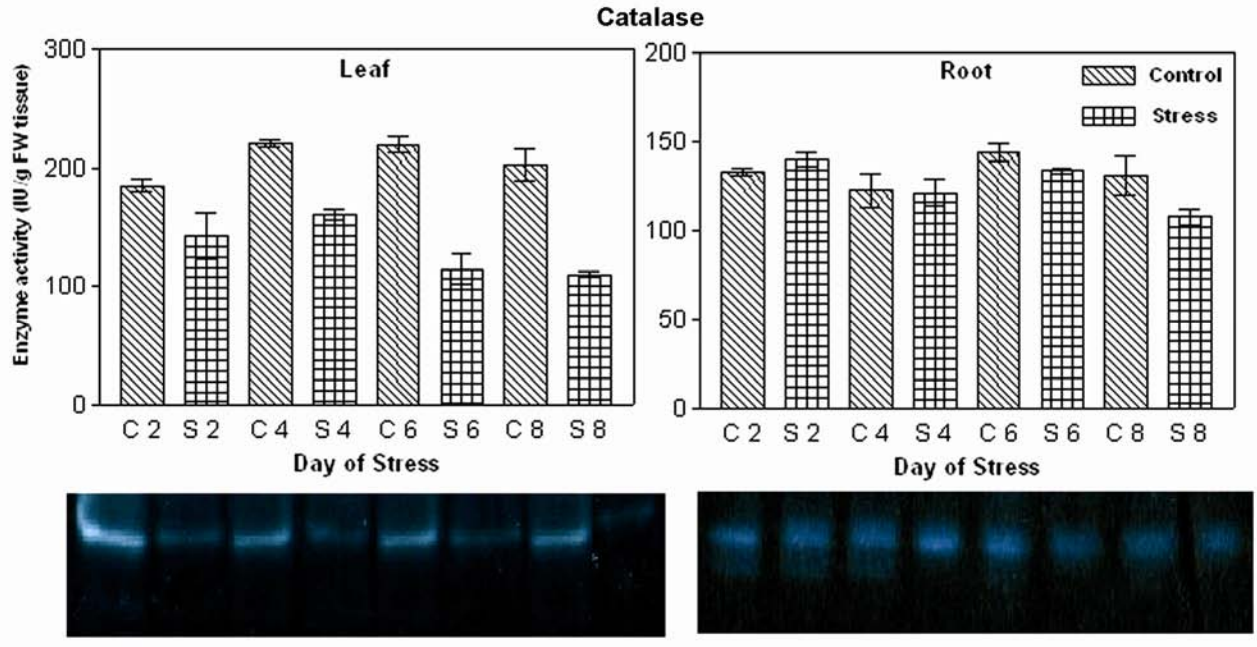

Fig. 6—Glutathione reductase activity in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissuess, S2 to S8-Stressed tissues. Lower panel: Zymogram of GR during drought; 100 µg protein of each sample was separated on non-denaturing PAGE (10 %) and stained for enzyme activity as described under materials and methods.

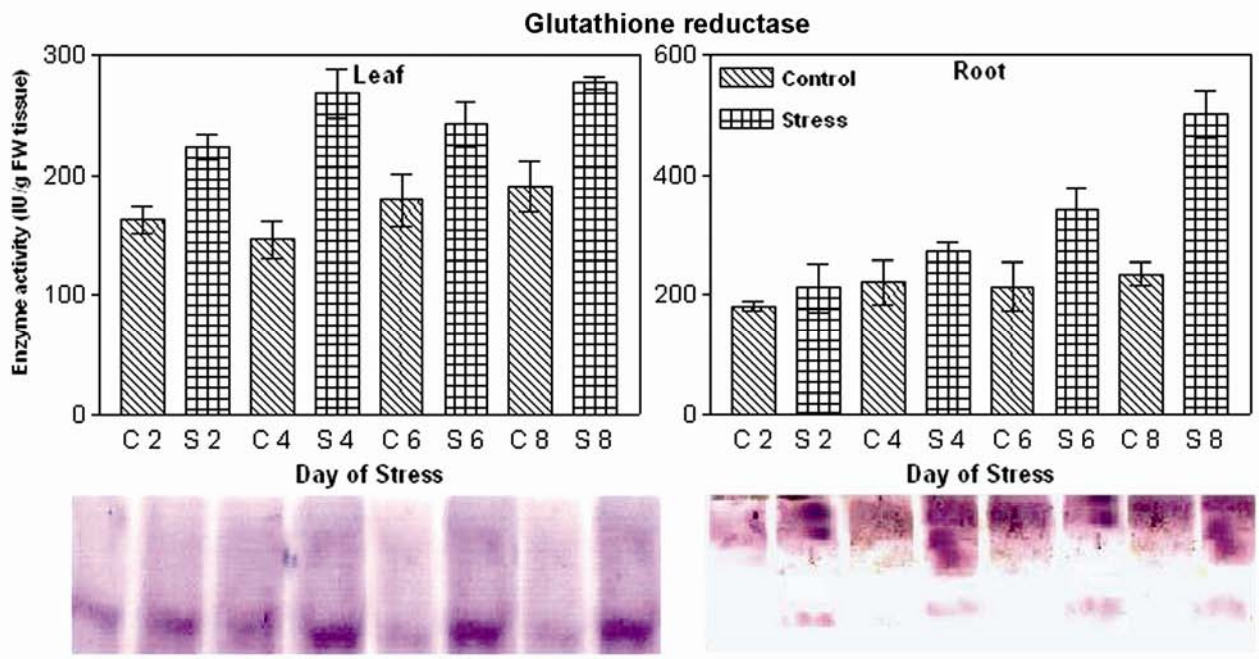

Fig. 7—Catalase activity in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissuess, S2 to S8-Stressed tissues. Lower panel: Zymogram of CAT during drought; 100 µg protein of each sample was separated on non-denaturing PAGE (10%) and stained for enzyme activity as described in materials and methods.

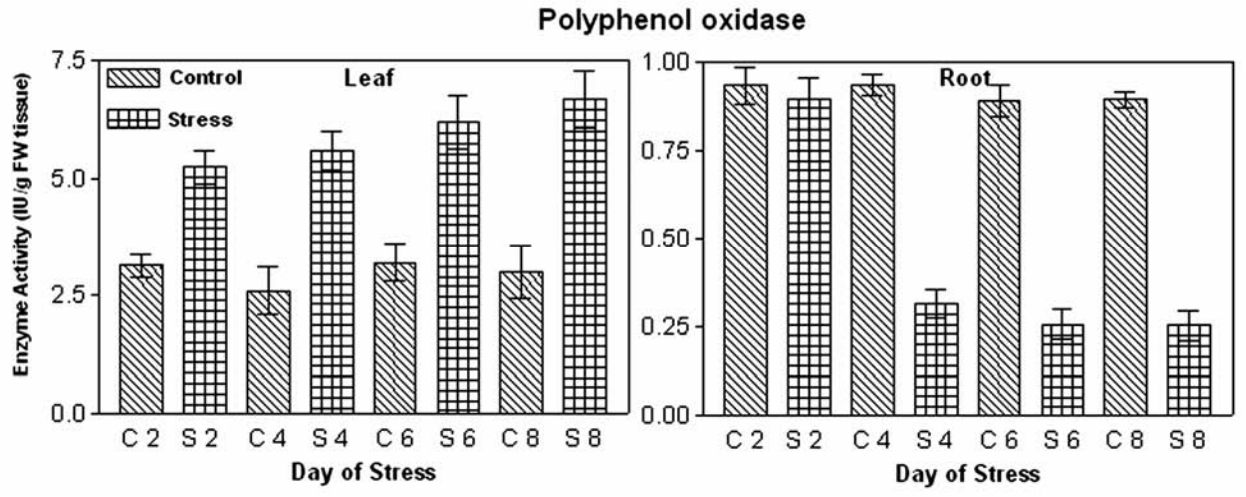

Fig. 8—Polyphenol oxidase activity in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissuess, S2 to S8-Stressed tissues.

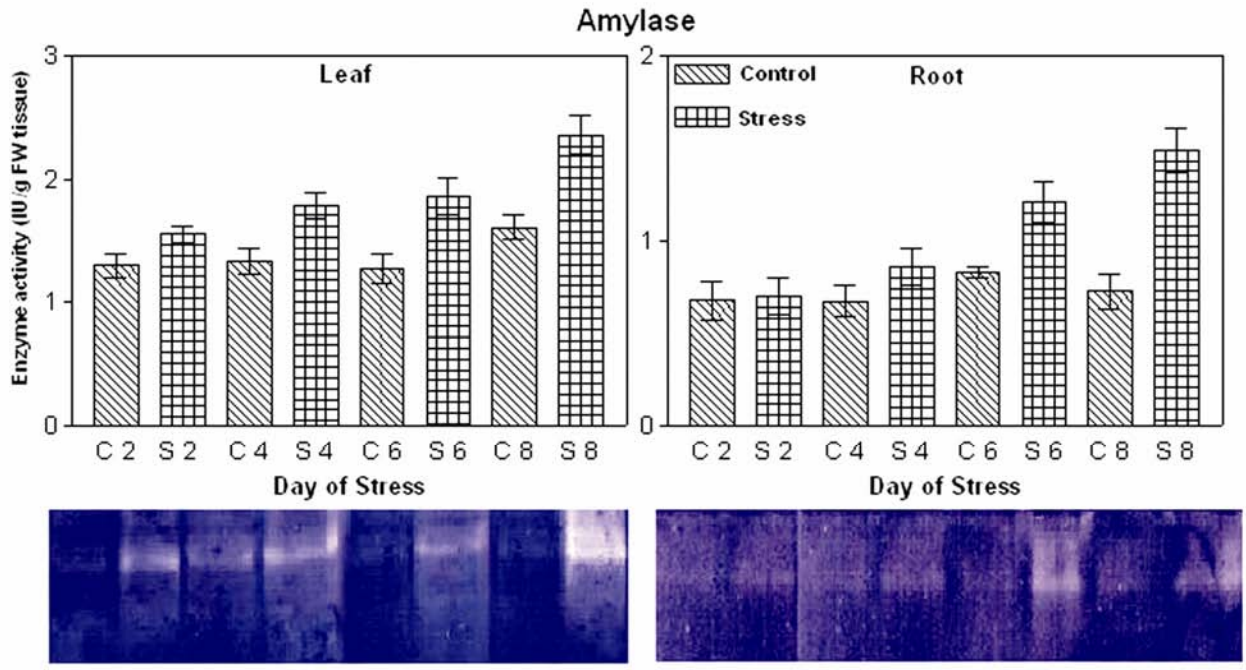

Fig. 9—Amylase activity in leaf and root tissues of *D. lablab* during drought stress. C2 to C8- Control tissuess, S2 to S8- Stressed tissues. Lower panel: Zymogram of AMY during drought; 100 µg protein of each sample was separated on non-denaturing PAGE (10 %) and stained for enzyme activity as described under materials and methods.

inorganic phosphate, which can be co-transported with H^+ along a gradient of proton motive force[35]. INV activity decreased steadily in leaf and only marginally in root tissue (Fig. 11). Inhibition of INV could explain the tendency to accumulate sugars in stressed leaves.

SDS-PAGE patterns of the leaves exposed to varying lengths of stress showed no remarkable alterations in protein band pattern with time of exposure. Nevertheless, two bands of very high molecular weight exhibited intensification under stress and another of lower size decreased in intensity 6 DAS (Fig. 12).

It can be concluded that prolonged drought leads to oxidative stress and in turn causes a significant increase in antioxidative responses. Several defense strategies function in the shoots and roots against

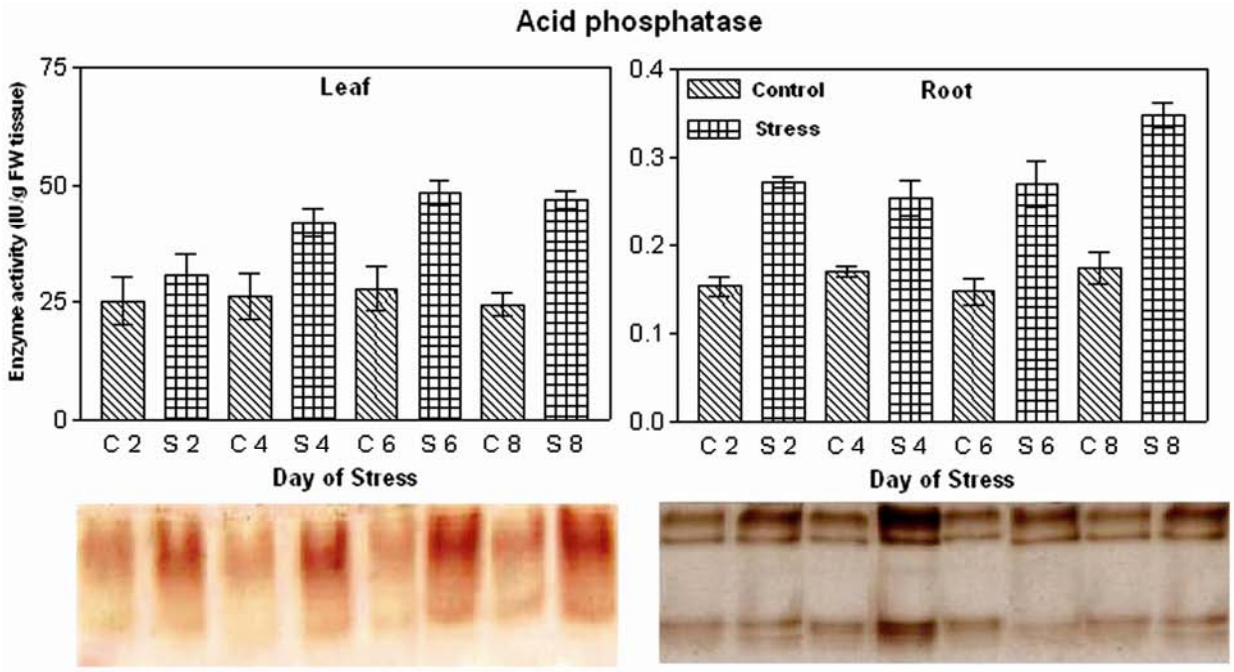

Fig. 10—Acid phosphatase activity levels in leaf and root tissues of *D. lablab* during drought stress. C2 to C8-Control tissuess, S2 to S8-Sressed tissues. Lower panel: Zymogram of AP during drought; 100 µg protein of each sample was separated on non-denaturing PAGE (10%) and stained for enzymes as described under materials and methods.

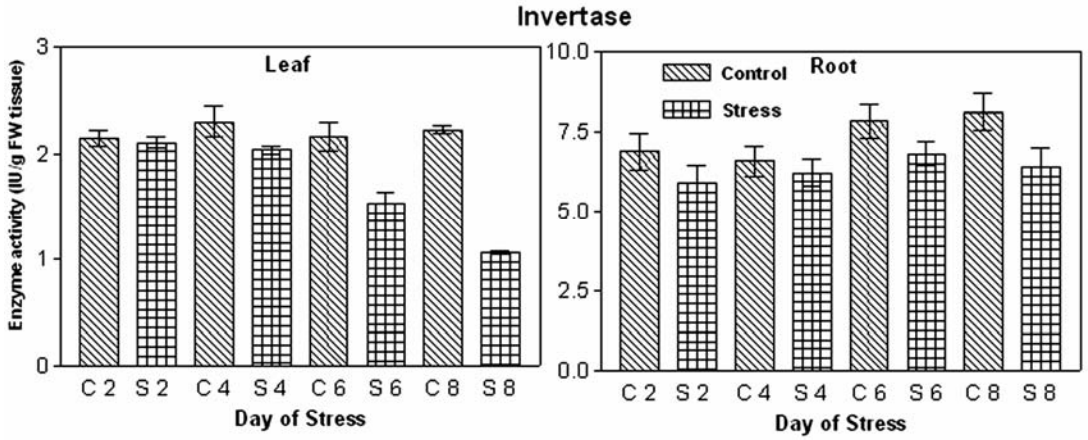

Fig 11—Invertase activity levels in leaf and root tiassuess of *D. lablab* during drought stress. C2 to C8- Control tissues, S2 to S8- Sressed tissues.

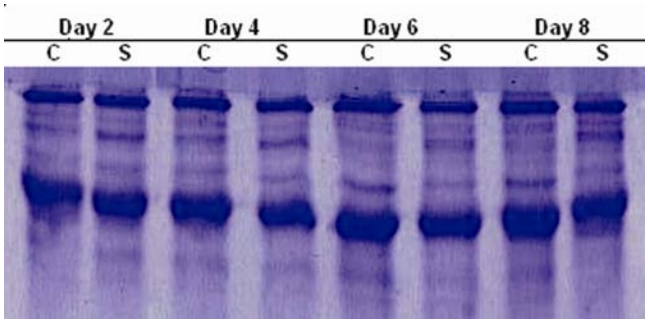

Fig 12—SDS-PAGE pattern of drought stressed seedlings of *D. lablab*. 100 µg soluble protein extracted from control (C) and stressed seedlings (S) were resolved on a 12% gel and stained with Coomassie brilliant blue R-250.

oxidative stress that lead to the positive adaptation of the plant to drought. The antioxidant system in leaves involves the non-enzymatic components GSH, ASC and proline and enzymatic components such as POX, APX, GR and to a lesser extent, PPO. These parameters and the recovery of the plant after drought stress correlates well with tolerance exhibited by plants.

Acknowledgement

M R D'souza acknowledges the Council for Scientific and Industrial Research, New Delhi, India for JRF (Award No. 09/039(0080)/2007-EMR-I).

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