Australian Journal of Crop Science
2(2):40-48 (2008)
ISSN: 1835-2707

High temperature and salt stress response in French bean (*Phaseolus vulgaris*)

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Abstract

Abiotic stresses, such as high temperature, and salt stress are major factors which reduce crop productivity. Effects of high temperature (46-48° C) and salt stress (0.4 M) on French bean (*Phaseolus vulgaris*), a major vegetable crop, were evaluated in terms of antioxidants and antioxidant enzymes in S-9 cultivar. Both stresses caused similar responses in the plant. Oxidative stress indicators such as H_2O_2, TBARS, glutathione, ascorbic acid, and proline were significantly elevated. Similarly, antioxidant enzyme, guaiacol-specific peroxidase (POX) was significantly elevated. Other enzymes, β-amylase and acid phosphatase (AP) activities were marginally enhanced. However, stresses had contrasting effects on glutathione reductase (GR) and catalase (CAT), which were drastically reduced in temperature stress, and elevated in salt stress. No variations were observed in AP, POX, and CAT isozymes. Patterns of GR and β-amylase isozymes differed between temperature and salt stress. SDS-PAGE indicated entirely different sets of proteins in temperature and salt stressed seedlings. Growth rate and fresh mass were affected to same extent, relative to their respective controls. DNA damage was more pronounced under temperature stress than under salt stress. Response mechanism of French bean appears to involve some players which are common to both the stresses, and few specific to individual stress.

Key words: Antioxidants; Antioxidant enzymes; French bean; Isozymes; Temperature stress; Salt stress.

Abbreviations: APX- ascorbate peroxidase; AP- acid phosphatase; Asc-reduced ascorbate; Asct- total ascorbate (reduced plus oxidized); CAT-catalase; DHA-dehydroascorbate; DHAR-dehydroascorbate reductase; GR-glutathione reductase; GSH- reduced glutathione; GSHt- total glutathione (reduced plus oxidized); GSSG-oxidized glutathione; H_2O_2- hydrogen peroxide; ROS- reactive oxygen species; SOD- superoxide dismutase; TBARS-thiobarbituric acid reactive substances

Introduction

Temperature and salinity are major factors which significantly affect plant productivity in arid and semi arid regions (Bray et al., 2000). Exposure of plants to these abiotic stresses results in production of ROSs as byproducts, which damage the cellular components (Noctor and Foyer, 1998). Plants have developed a series of enzymatic and non-enzymatic detoxification systems to counteract ROS, and protect cells from oxidative damage (Sairam and Tyagi, 2004). The Antioxidant enzymes such as SOD, CAT, POX, and GR function in detoxification of super oxide and H_2O_2 (Mittler, 2002). β-amylase expressed during stress has been shown to play a major role in transitory breakdown of starch (Scheidig et al., 2002). Acid

Table 1. Antioxidants and other stress response Factors in French bean*

Antioxidants/ Stress Response Factors	**Amount (µg/g fresh mass)**			
	Temperature		**Salt**	
	Control	**Stressed**	**Control**	**Stressed**
Hydrogen peroxide	28.0 ± 2	87.30 ± 2	21.0 ± 2	67.30 ± 2.0
Ascorbic acid	5.04 ± 4	9.78 ± 1.5	6.01 ± 4	10.18 ± 1.5
Proline	0.27 ± 2	1.04 ± 1.2	1.09 ± 1	2.01 ± 1.1
Glutathione	1.7 ± 0.1	2.2 ± 0.1	1.6 ± 0.1	3.1 ± 0.1
TBARS (µ moles g^{-1})	230 ± 1.3	305 ± 1.3	310 ± 1.3	455 ± 1.2
Soluble protein (mg/g)	232.0 ± 0.2	405.0 ± 0.5	215.2± 1.2	114.7± 0.7

**Each value is mean ± SE (n = 5)*

phosphatase activity is known to contribute to resistance under salt and water stress by maintaining a certain level of inorganic phosphate (Olmos and Hellin 1997). Protective roles of the antioxidant enzymes in temperature and salt stress have been reported for a number of plants (Almeselmani et al., 2006; Jaleel et al., 2007; Esfandiari et al., 2007). Antioxidants such as ascorbate and glutathione are involved in scavenging H_2O_2 in conjunction with MDAR and GR, which regenerate ascorbate (Horemans et al., 2000). Proline that accumulates in large quantities in response to environmental stresses acts as a compatible solute, and buffers cellular redox potential (Wahid and Close, 2007). *Phaseolus vulagaris*, French bean, a major vegetable crop with greater nutritional value, accounts for higher consumption and economic importance all over the world. As the bean is mainly grown in poorly irrigated and partly saline conditions (coastal) in India, it is exposed to great deal of temperature and salt stress. Although much supporting evidences on the role of antioxidants and antioxidant enzymes under temperature and salinity are available, there are no specific information pertinent to French bean. The objective of the present study was to evaluate the effect of high temperature and salinity on antioxidants and antioxidant enzymes in French bean, and also to visualize the interrelationship between temperature and salt stress response components.

Materials and Methods

Plant material and Temperature stress

Seeds of French bean (*Phaseolus vulgaris*) genotype (S-9), were surface sterilized with a solution of mercuric chloride (0.1% for 30 S) and were washed immediately with large volume of sterile distilled water, and sown in trays containing vermiculite, and irrigated daily with distilled water. Six days old plants, grown in vermiculite, in a controlled chamber at 25 °C, HR 70%, and a photoperiod of 16 h light: 8 h dark. High-temperature stress was applied by induction treatment at 38-39 °C for 2 h followed by exposure to 45-48 °C for 8 h. the whole plant material was used for studies.

Salt stress treatment

French bean (*Phaseolus vulgaris*) seeds were surface sterilized with a solution of mercuric chloride (0.1% for 30 S) and were washed immediately with large volume of sterile distilled water. Five days old seedlings were then transferred into specially designed dishes containing 1/2 strength sterile Hoagland's nutrient solution with added micronutrients (Allen, 1968) and 0.04 mM ferrous ion as Fe–EDTA, (pH 5.6) as described by (Peters and Mayne, 1974). The seedlings were grown at 26 °C under 16 h light: 8 h dark

photoperiod. Salt stress on *French bean* was induced by incubating plants in half-strength Hoagland's nutrient solution containing NaCl at a final concentrations of 400 mM for 48 h. Plants grown on half-strength Hoagland's medium without NaCl served as control.

Extraction of enzymes and antioxidants

Frozen plant material was homogenized with 100 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, and 5 mM β-mercaptoethanol. The homogenate was filtered through four layers of cheese cloth, centrifuged at 45,000Xg 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 according to the method of Lowry et al. (1951) with BSA as the standard.

Determination of membrane damage

Lipid peroxidation (MDA) in the seedling samples was measured to assess the membrane damage. The amount of MDA–TBA complex was calculated from the extinction coefficient 155 mM^{-1} cm^{-1} (Rubin et al., 1976).

Determination of H_2O_2, ascorbic and proline

The H_2O_2 and ascorbic acid content were measured as described by Mukherjee and Choudhuri (1983). Free proline was extracted from 0.5 g of fresh tissue in 3% (w/v) aqueous sulphosalicylic acid and estimated by ninhydrin reagent (Bates et al., 1973).

Measurements of GSH and GSSG

Plant tissue (1.0 g), frozen in liquid nitrogen was ground to fine powder, and homogenized in 1.0 ml of 6.67% (w/v) sulfosalicylic acid, and centrifuged at 20, 000Xg for 15 min at 4 °C. Reduced and oxidized glutathione were determined according to Griffith (1980). The total glutathione content was calculated from a standard curve of reduced GSH. GSH was determined by subtracting GSSG as GSH equivalents from the total glutathione content.

Isolation of total DNA

Ten grams of control and stressed seedlings of French bean were frozen in liquid nitrogen and total DNA was isolated by CTAB method (Sambrook, et al., 1989).

Electrophoretic analysis

Non-denaturing, discontinues slab gel electrophoresis was carried out essentially according to the method of Davis (1964). SDS-PAGE was carried out according to Laemmli (1970), employing 12% resolving gel and 5% stacking gel

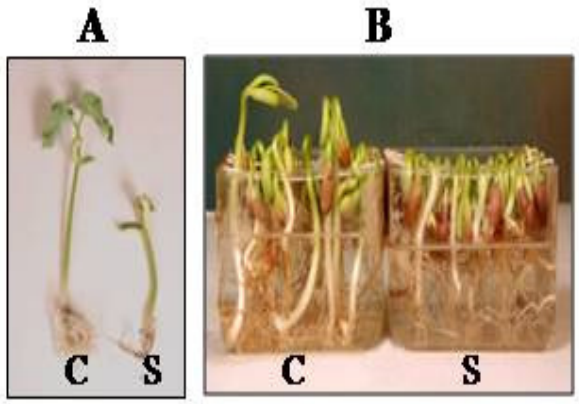

Fig 1. Effect of high temperature and salt stress on French bean; A: Six days old seedlings subjected to temperature stress for 10 h. Control [C], stressed [S].
B: 5 day old seedlings subjected to salt stress for 48 h. control [C], stressed [S].

Assay of enzymes

β-Amylase *(E.C. No. 3.2.1.1)* activity was assayed according to the method of Shuster and Gifford (1962). The activity unit was expressed in terms of µmoles of maltose formed per minute. β-amylase isozymes were separated on non denaturizing polyacrylamide gels (9%) at 100 V for 2 h at 4 °C. Gels were then soaked in substrate (2% soluble starch) for 30 min at 27 °C and incubated in 0.025% acidified iodine solution for 5 min.

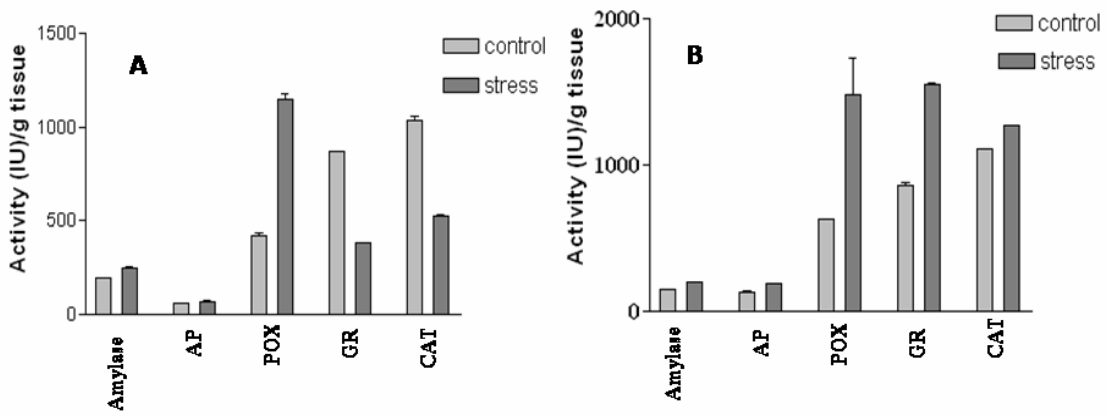

Fig 2. Antioxidant and other stress-specific enzyme activities in French bean during abiotic stress. **A;** Enzyme activities during temperature stress. **B;** Enzyme activities during salt-stress. Data are mean values ± SD (n= 5).

Acid phosphatase *(E.C. No. 3.1.3.2)* activity was assayed according to the method of Hoerling and Svensmark (1976) employing α-naphthyl phosphate or *p*-nitrophenyl phosphate as substrates. Each unit of activity is defined as the number of µmoles of α-naphthol or *p*-nitro phenol released per minute. In-gel assay was carried out after electrophoretic separation of phosphatase isozymes on native (9%) polyacrylamide gels at 4 °C. The enzyme bands were detected using α-naphthyl phosphate as substrate and fast blue -RR as coupling dye.

Catalase *(E.C. No.1.11.1.6)* activity was measured by following the decline in $A_{240\ nm}$ as H_2O_2 ($\varepsilon = 36 M^{-1}cm^{-1}$) was catabolized, according to the method of Aebi (1984) in a reaction mixture containing 20 µl enzyme extract in 50 mM sodium phosphate buffer (pH 7.0). The reaction was started by addition of H_2O_2 to a final concentration of 15 mM, and its consumption was measured for about 30 S at 240 nm. In-gel assay for CAT isozymes were performed on non-denaturizing gels (9%) electrophoresed at 100 V for 2 h at 4 °C. Gels were soaked in 3.27 mM H_2O_2, for 15 min, rinsed with water, and stained in 2% potassium ferricyanide followed by 2% ferric chloride to visualize the bands (Prasad et al., 1995).

Glutathione reductase *(E.C.No.1.6.4.2)* was assayed by monitoring the GSSG dependent NADPH oxidation according to the method of Edwards et al. (1994). GR isozymes were separated on non denaturizing gels (9%) at 100 V for 2 h at 4 °C., soaked in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mg MTT, 10 mg 2, 6-dichlorophenol indophenol, 3.4 nM GSSG, and 0.4 mM NADPH.

Guaiacol peroxidase activity *(EC No. 1.11.1.1)* was measured by monitoring the formation of tetra guaiacol at 470 nm ($\varepsilon = 26.6\ mM^{-1}cm^{-1}$) using H_2O_2 as substrate according to Chance and Machly (1955). One unit of peroxidase is defined as the amount of enzyme that caused the formation of 1 mM of tetra-guaiacol per minute. POX isozymes separated on 9% native acrylamide gels were incubated in a mixture of *o*-dianisidine-HCl in acetate buffer (pH 5.5) for 30 min at room temperature. Gels were then transferred to 100 mM H_2O_2 until visible bands developed.

Statistical analysis

All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using SPSS 9.0 software. 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 $P< 0.05$ were considered significantly different.

Results

Morphological features and overall growth pattern of the seedlings indicated that both, high temperature and salt stress in French bean caused inhibition of shoot, and root growth and reduction in the fresh mass of seedlings (Fig. 1). Development of leaf and root was severely affected in temperature stress, wherein the stunted seedling had ~one half height, ~one eighth leaf area, and one fifth of root length of controls. Seedlings stressed with high salt exhibited much better performance than those stressed with temperature. The seedlings exhibited variable recovery between the two stresses, wherein the recovery from temperature and salt stress were 40% and 60%, respectively.

Antioxidants such as ascorbic acid and glutathione, which are found in mM concentrations in cellular compartments, are crucial for plant defense against oxidative stress. Stressed French bean seedlings exhibited elevated levels of glutathione, and ascorbic acid. Whereas ascorbic acid contents doubled in both stresses, glutathione content showed a two fold increase in salt stress and a marginal but significant increase in temperature stress (Table 1).

H_2O_2, a product of oxidative stress, and major signal molecule for oxidative stress response showed a three-fold increase relative to control under both stresses. Plants grown under abiotic stresses such as salinity, high temperature and drought accumulate compatible osmolytes like glycine betaine and proline. Elevation in the proline content was observed in salt as well as temperature stressed French bean. However, the increase was a remarkable five-fold during temperature stress, against a moderate two-fold under salt stress. Stress induced ROS cause membrane damage in plants. A raise in TBARS, an indicator of membrane damage was observed under both stresses. Although the raise in TBARS was moderate, its level under salt stress was slightly higher than levels under temperature stress. Cell death has been attributed to membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage induced by ROS. Electrophoretic analysis of genomic DNA from stressed French bean indicated moderate damage only under temperature stress.

Quantitative and qualitative alterations in antioxidant enzyme system are often related to level of resistance to stress. Temperature and salt stress in French bean altered POX, AP, and amylase activities in same manner, wherein POX activity doubled, and the latter two showed slight increase (Fig 2). Contrary to these effects, the applied stresses differed in their effects on GR and CAT activity. Temperature stress resulted in reduction (~ 50%) of the activities, and the salt stress caused increase in these enzyme activities. The increase during salt stress was more pronounced for GR than for CAT activity.

With quantitative changes in the enzyme levels, alterations were also observed in intensities and number of isozyme bands during applied stresses. In-gel assays indicated variation in intensities of AP, POX and CAT during stresses. The POX and AP bands were intensified under both stresses, and the CAT isozymes showed reduced intensity during temperature stress and enhanced intensities under salt stress. Isozyme patterns of POX, CAT, and AP did not show any alterations relative their respective controls during both stresses. GR and amylase differed between temperature and salt stress. A new isozyme and all the five isoforms of GR were enhanced during the salt stress. Excluding an isozyme that remained enhanced, all the other isoforms were significantly reduced during temperature stress (Fig 3). Although a new isozyme of amylase was observed in both stresses, temperature stress differed in disappearance of a minor isozyme.

In addition, different SDS-PAGE patterns were observed between salt and temperature stressed seedlings (Fig 4). The band patterns also reflect the differences observed in soluble protein contents.

Discussion

High temperature, salt stress, and drought, are major ecological factors, which prevent crop plants from realizing their full genetic potential. Of the three, temperature is more pervasive and economically damaging. High temperature causes reduction in shoot dry mass, growth and net assimilation rates in a number of plants (Wahid et al., 2007). Similarly, salinity stress affects development processes such as seed germination, seedling growth and vigor, vegetative growth, flowering and fruit set (Sairam and Tyagi,

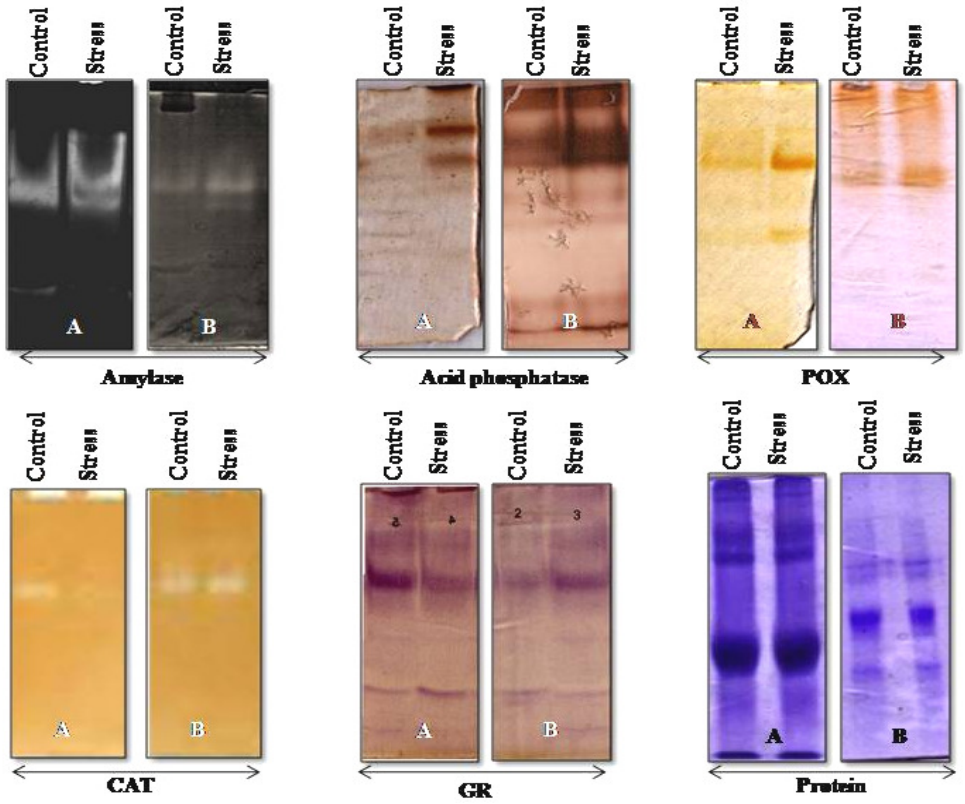

Fig 3. In-gel assay of enzymes during temperature and salt stress in French bean. Proteins, 20 µg each (except GR, 50 µg) were separated by non-denaturing PAGE (9%) and stained for enzymes as described under materials and methods. Panel-A; zymogram of control and temperature stressed seedlings. Panel-B; zymogram of control and salt stressed seedlings.

2004). Reduction in shoot and root growth rates, as well as fresh mass observed in both temperature and salt stressed French bean seedlings (Fig.1) are in conformity with proven effects of these stresses. The variations in the parameters between salt and temperature stressed seedlings suggested involvement of distinct biochemical components. ROS scavengers GSH and ascorbic, which accumulate in response to oxidative stress, are part of a well established ascorbate cycle. Remarkable increase in GSH and ascorbic acid levels (Table 1) indicated the induction of antioxidant mechanism such as GSH-ascorbate cycle in French bean, as reported for a number of plants (Halliwell and Gutteringe, 1989; Koca et al., 2007). Proline accumulation occurs normally in cytosol in response to drought and salinity stress. Apart from its role as osmolyte, proline contributes to stabilization of sub-cellular structures, scavenging ROS, and buffering cellular redox potential under stress (Ashraf and Foolad, 2007). Five-fold and two-fold increase in proline content during temperature, and salinity stress, respectively, implied a more crucial role of proline in temperature stress or drought induced oxidative damage than in salinity stress. The parallel increase in ascorbic acid and proline in French bean resembles the response of maize to water deficit (Ashraf and Foolad, 2007). Alteration of H_2O_2 levels to similar extents in salt and temperature stress suggested that both stresses equally influenced the antioxidant response mechanisms in French bean.

Free radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level (Jain et al., 2001). Increase in the level of MDA, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. Higher levels of TBARS recorded in French bean, also indicated membrane damage during temperature and salt stress. The chemistry of ROS-induced DNA damage has been extensively studied *in vitro* and *in*

vivo (Beckman and Ames, 1997). Stress induced production of ROSs, and concomitant oxidative damages include damage to proteins and nucleic acids. The preferential DNA damage during temperature stress than salt stress may be due to greater levels of ROSs generated during the temperature stress and/or more protective factors which prevailed during salt stress. From these facts, it is evident that majority of non enzymatic antioxidant components responded differently to temperature and salt stress except ascorbic acid, hydrogen peroxide.

Antioxidant enzymes play a crucial role in detoxification of ROS and generation of antioxidants in response to prevailing stress. Accumulation of the ROS, H_2O_2, induced by various environmental stresses result in the combined activity of CAT and POX. Severe deactivation of CAT (one half) and enhancement of guaiacol-POX (two-fold) accompanied by increased H_2O_2 during temperature suggested the operation of a similar antioxidant mechanism in French bean as has been observed in many species (Blokhina et al., 2003). Our results are also in agreement with heat stressed mustard (Dat et al., 1998) and drought stressed pea (Moran et al., 1994), which exhibited a significant increase in endogenous H_2O_2 and POX, and marked decline in CAT. Contrary to decrease under temperature stress, the CAT activity was elevated under salt stress. Increased activity of CAT and POX has been suggested as an adaptive mechanism to reduce the H_2O_2 and offer protection against oxidative damage (Agarwal and Pandey, 2004). The enhanced activities of CAT and POX under salt stress in French bean suggest an effective scavenging of H_2O_2 and tolerance against salt. Isozyme patterns of POX, indicated over expression of all the five constitutive isozymes under stress, and their role in detoxification of ROS. A total decline of CAT under temperature, and over expression of all the isozymes under salt stress, further strengthened the view of divergent response to salt and temperature stresses. Another antioxidant enzyme, GR was drastically reduced, implying an elevated GSH (Fig 2 and Table 1). Our observations are in contrast to those patterns reported during low temperature stress in pea and maize, wherein a rise in the GR activity and reduction in GSH (Edwards et al., 1994 and Prasad et al., 1995). The isozyme pattern of the GR were

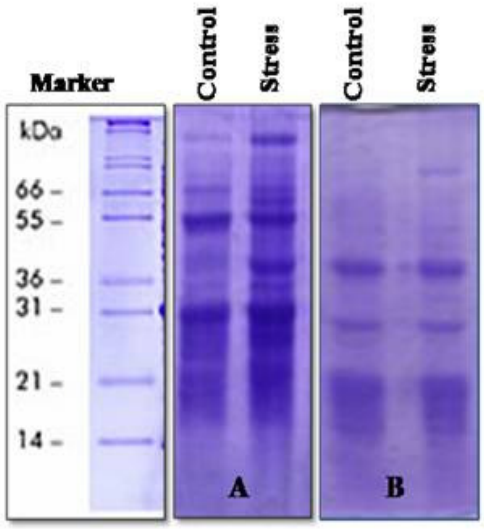

Fig 4. SDS-PAGE pattern of temperature (A) and salt (B) stressed seedlings of French bean. Soluble protein extracted from control and stressed seedlings were resolved on 12% gel and stained with coomassie brilliant blue.

also altered significantly, in that, only one of the five isoforms became prominent, and persisted under temperature stress (Fig 3). This suggested an exclusive role for the induced isozyme in temperature stress. Under salt stress, all the five isozymes were intensified suggesting a response mechanism different from temperature stress response.

Variation in the transcript and enzyme levels of major antioxidant enzymes during stress is well documented. There are also scanty reports of induction of either transcripts and/or activity of amylase (Datta et al. 1999; Kreps et al., 2002, Seki et al., 2001, and Jung et al., 2003,). Increased amylase activity has been linked to increased maltose content (Kaplan and Guy 2004, Nielsen et al., 1997), and its levels are modulated in response to osmotic (Datta et al., 1999, Dreier et al., 1995), drought (Yang et al., 2007), salt (Datta et al. 1999, Dreier et al., 1995), and heat stress (Dreier et al., 1995 and Sung, 2001). Moderate elevation of activity and isozyme pattern of □-amylase in French bean also indicated difference between salt and temperature stress responses. Higher level of acid phosphatase activities in plant increases its tolerance to stress. Our results also indicated increased acid phosphatase activities under

both stresses. Olmos and Hellin (1997) observed that acid phosphatase are known to act under salt and water stress by maintaining a certain level of inorganic phosphate which can be co-transported with H^+ along a gradient of proton motive force. In few cases, phosphatase activities are independent of phosphate levels (Szabo-Negy et al., 1992). Parallel to the alterations in isozyme intensities and patterns, SDS-PAGE patterns of stressed French bean also showed intensification of few protein bands and appearance of, at least, five new bands. Interestingly, the SDS-PAGE patterns of salt and temperature stress differed significantly, indicating the involvement of different players.

In conclusion, both non enzymatic and enzymatic antioxidant mechanisms responded distinctly to temperature and salt stress, suggesting divergent of response mechanisms. Response of French bean to abiotic stresses involves few common enzymatic and non enzymatic components, and few exclusively specific to the applied stress.

Acknowledgements:

The authors sincerely acknowledge the University Grant Commission, New Delhi, and Bangalore University, Bangalore, for JRF to Nagesh Babu.

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