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Induction of thermotolerance through heat acclimation in lablab bean (*Dolichos lablab*)

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The acclimation of plants to moderately high temperature plays an important role in inducing plant tolerance to subsequent lethal temperatures. This study was performed to investigate the effects of heat acclimation and sudden heat stress on the antioxidant and metabolic profile of lablab bean (*Dolichos lablab*). Following separate pretreatments with heat acclimation (35°C) and NaCl (100 mM), seedlings of lablab bean were exposed to heat stress at 45°C for 5 h and then recovered at 25°C for five days. Pre-treated seedlings performed better under heat stress than the control and it could be associated with the observed increased levels of sugars, proline, glutathione and ascorbate; and increased activities of Peroxidase (POX), glutathione reductase (GR) and ascorbate peroxidase (APX) than just heat shocked seedlings. Seedling growth was dramatically reduced under heat stress but heat acclimation and NaCl pre-treatment were effective in imparting thermoprotection against the lethal heat shock.

Key words: Acclimation, antioxidants, catalase, *Dolichos lablab*, glutathione reductase, heat stress, peroxidase, proline, sugar.

INTRODUCTION

Dolichos lablab, a member of *Fabaceae*, is an ancient crop and has been documented by archaeo-botanical finds in India prior to 1500 BC at Hallur, India's earliest Iron Age site in Karnataka (Fuller, 2003). Despite its label as 'underutilized', substantial cultivation of Lablab bean is seen in certain tropical regions, either as a sole crop or in mixed production systems. Remarkable morphological variations have also been reported throughout India (Sankaran et al., 2007). It also has considerable physiological diversity; a range of adaptation to acidity, low soil phosphorous and drought has been reported for the plant (Mugwira and Haque, 1993; Karachi, 1997). Transitory or constant high temperatures cause an array of morphoanatomical, physiological, and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. Plants have evolved various mechanisms for thriving under higher prevailing temperatures. These include short term avoidance/acclimation mechanism or long term evolutionary adaptations. In case of sudden heat stress, short term response that is, leaf orientation, transpirational cooling and changes in membrane lipid composition are more important for survival (Wahid et al., 2007). Different tissues in plants show variations in responses in terms of developmental complexity, exposure towards the prevailing or applied stress types (Queitsch et al., 2000).

The stress responsive mechanisms established by an initial stress signal are in the form of ionic and osmotic effects or changes in the membrane fluidity. This helps

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Abbreviations: AMY, Amylase; **ASC,** ascorbate; **APX,** ascorbate peroxidase; **CAT,** catalase; **GR,** glutathione reductase; **GSH,** reduced glutathione; **INV,** invertase; **POX,** peroxidase; **ROS,** reactive oxygen species; **RWC,** relative water content; **TBARS,** thiobarbituric acid reactive species; **TSS,** total soluble sugars.

to reestablish homeostasis and to protect and repair damaged proteins and membranes (Vinocur and Altman, 2005). However, irreversible changes in cellular homeostasis may occur due to inadequate response during signaling and gene activation processes that result in the destruction of structural and functional proteins and membranes, ultimately leading to cell death (Vinocur and Altman, 2005; Bohnert et al., 2006). Plants lacking the ability to display rapid heat acclimation responses may be more prone to thermo-damage. Here, acquired thermotolerance may have significant role. Since plants have to face temperature fluctuations during day/night cycle, the acquisition of thermotolerance reflects a more general mechanism that contributes to homeostasis of metabolism on a daily basis (Hong et al., 2003). Some major mechanisms, which make plants thermotolerant include ion transporters, free radical scavengers, late embryogenesis abundant (LEA) proteins, osmoprotec-tants and factors involved in signaling cascades and transpirational control (Wang and Luthe, 2003). Heat stress effects are of greater concern at various levels including plasmalemma, biochemical pathways operative in the cytosol or organelles (Sung et al., 2003).

Studies have revealed that the first target of heat stress is the plasmalemma that shows increased fluidity (Wahid et al., 2007). This leads to the induction of Ca^{2+} influx and reorganization of cytoskeleton and eventually the upregulation of calcium dependent protein kinase (CDPK) and mitogen activated protein kinase (MAPK). Nuclear signaling of such cascades shows the synthesis of cytosolutes and antioxidants. The cytosolutes help to maintain cellular water balance; while the antioxidants scavenge reactive oxygen species (ROS) and are correlated with acquisition of thermotolerance (Maestri et al., 2002). The accumulation of ROS can cause peroxidation of membrane lipids, denaturation of proteins and damage of nucleic acids, ultimately upsetting homeostasis (Mittler, 2002). It is known that plants resist stress-induced production of ROS by increasing the activity of their ROS induced scavenging system (Ali et al., 2008; Goyal and Asthir, 2010). The major ROS-scavenging mechanisms include enzymatic system, which consists of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and non-enzymatic system, which consists of ascorbic acid (ASC) and glutathione (GSH). Previous studies have indicated that the changes in antioxidant enzymes and antioxidants contribute to the plants resistance to high temperature (Almeselmani et al., 2006).

Heat acclimation, during which the plants develop heat tolerance, is a genetically controlled process that is triggered by exposing plants to mild or sublethal temperatures or by the application of compounds or biomolecules to the growth medium (Charng et al., 2006). The processes involved in temperature acclimation are initiated by the perception of temperature signals and transduction of these signals into biochemical processes that finally lead to the development of heat tolerance (Xu et al., 2006).

The proteins thus expressed facilitate growth and survival of plants not only at transient temperatures, but also under conditions of severe heat stress, whereby lethal temperature can be tolerated for short periods. The present work was initiated to study the effect of high temperature stress on antioxidants and antioxidant enzymes, as well as other parameters, and the role played by these factors in protecting the plant cell from damage occurring due to high temperature stress.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of *D. lablab* (cv. HA-4) were purchased from National Seed Project, University of Agricultural Science, GKVK, Bangalore, India. 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 (3 seeds per pot) 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 25/20°C and ~70%, respectively. The average photoperiod was 12 h light/12 h dark.

Heat acclimation and heat stress treatments

Five day old seedlings were subjected to heat treatments in 1X Hoagland medium (Allen, 1968). For heat acclimation (HA), plants were maintained at 35°C for 2 h and then exposed to heat stress (HS) at 45°C for 5 h. For sudden heat shock (HS), plants were exposed only to 45°C for 5 h. A combination of salt stress and heat stress (SS + HS) was carried out by subjecting salt-stressed plants (100 mM NaCl at 25°C for 24 h) to the heat shock treatment at 45°C for 5 h. All plants, that is, HS, HA + HS, HS + SS were subjected to a recovery period at ~25°C for 3 days in 1X Hoagland media and then sampled. Seedlings kept entirely at 25°C without subjection to any treatment were taken as control (C). Ten seedlings were used in each experiment and each experiment was done in triplicate.

Relative water content (RWC)

The relative water content was estimated according to the method of Turner and Kramer (1980) using the equation:

$$RWC = (FW-DW) \times 100 / (TW-DW)$$

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 shoot and root was taken for analysis and RWC was computed as aforementioned.

Assay of metabolite and antioxidant enzymes

The frozen samples were homogenized with pre-chilled 50 Mm

sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM EDTA using pestle and mortar. L-ascorbate was raised to a final concentration of 2 mM 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 according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

β-Amylase (AMY, E.C. 3.2.1.1)

Activity of β-amylase was measured using the DNS method (Bernfield, 1955). The reaction mixture consisted 0.5 ml of 2% starch solution in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract.

Invertase (INV, E.C. 3.2.1.26)

Invertase activity was determined by the method of Sridhar and Ou (1972). 4.0 ml reaction mixture containing 0.025 M sodium acetate buffer (pH 5.0), 0.625% sucrose and appropriate volume of enzyme extract was incubated at 37°C for 24 h. The reaction was arrested by adding equal volume of DNS reagent. The reducing sugars present were estimated using the method of Miller (1959).

Catalase (CAT, E.C. 1.11.1.6)

Catalase activity was assayed by following the decline in absorbance of H_2O_2 at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) according to the method of Aebi (1984). The reaction mixture consisted of 50 µl of 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 10 mM, and its consumption was measured for 2 min. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of H_2O_2 per min under the assay conditions.

Guaiacol peroxidase (POX, E.C. 1.11.1.7)

Guaiacol peroxidase activity was measured in a reaction mixture of 3.0 ml consisting of 50 mM phosphate buffer (pH 7.0) containing 20 mM guaiacol, 10 mM H_2O_2 and 100 µl enzyme extract (Chance et al., 1955). The formation of tetraguaiacol was followed by an increase in A_{470} nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). One unit of peroxidase is defined as the amount of enzyme needed to convert 1 µmol of H_2O_2 min^{-1} at 25°C.

Glutathione reducatase (GR, E.C. 1.6.4.2)

GR activity was determined by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) according to the method of Carlberg and Mannervik (1985). The reaction mixture contained 50 mM tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH and 250 µl of enzyme extract in a total volume of 1.5 ml. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH per min under the assay conditions.

Ascorbate peroxidase (APX, E.C. 1.11.1.11)

The activity of APX was determined spectrophotometrically as described by Allen (1968). The assay mixture contained 50 mM HEPES buffer (pH 7.0), 1 mM EDTA, 1 mM H_2O_2, 0.5 mM sodium ascorbate and 50 µl of enzyme extract in a total volume of 2.0 ml. The reaction was initiated by addition of H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290} ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of ascorbate peroxidase is defined as the amount of enzyme necessary to oxidize 1 µmol of ascorbate per min at 25°C.

Determination of H_2O_2 and antioxidants

Hydrogen peroxide content in control and stressed seedlings were determined according to the study of Velikova et al. (2000). Ascorbic acid estimation was carried out according to the procedure of Sadasivam and Manickam (1997). Glutathione (GSH) was estimated according to the study of Beutler (1963). Total phenols were estimated by the method of Slinkard and Singleton (1977) using catechol as an authentic standard.

Determination of stress response factors

Proline content was estimated using ninhydrin reagent according to the study of Bates et al. (1973). The amount of total soluble sugars was estimated colorimetrically at 540 nm using anthrone reagent, according to Roe (1955). The extent of lipid peroxidation was determined according to Heath and Packer (1968). The TBARS content was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

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 PrismGraph version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by least significant difference (LSD) test. Comparisons with $P < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Effect of stress treatments on growth and RWC

The efficacy of various pre-treatments like heat acclimation and use of salt was studied by inducing thermotolerance in Lablab bean. Growth is an irreversible increase in volume and structural biomass involving cell division, cell enlargement, maturation and specialization to form tissues and organs. A quantitative understanding of the plant growth dependence on temperature is essential for the selection of cultivars to optimize growth in different climates, to understand the physiological responses to climate change and to identify and quantify thermotolerant species. Direct analysis of plant growth rates involves the measurement of seedling length, fresh/ dry weights and RWC. Exposure to heat shock (HS) alone caused inhibition of shoot growth in terms of seedling length, fresh/ dry weights and RWC (Figure 1). HS severely limits water uptake causing a reduction in growth. Both pre-treatments that is, HA + HS and SS + HS helped seedlings to recover from heat stress wherein the best heat tolerance based on morphological analysis was conferred by the former. The fresh/dry weights of pre-treated seedlings increased when compared to control

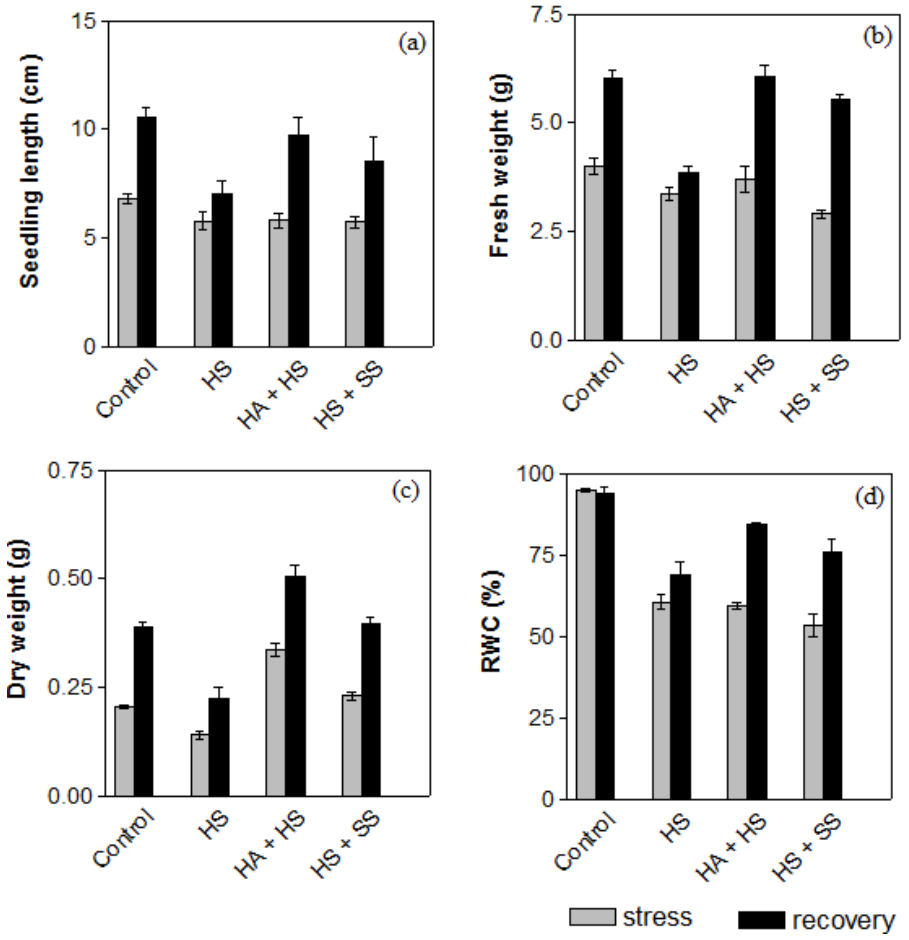

Figure 1. Seedling length (a), fresh weight (b), dry weight (c) and RWC (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

(Figure 1b and c). The increase in dry weight in these seedlings may have been due to accumulation of osmolytes, TSS and proline. Accumulation of osmolytes either active or passive is an important adaptation mechanism for stressed plants to protect cellular components form the injury caused by dehydration (Wahid and Close, 2007; Ashraf and Foolad, 2007). Several studies have reported biomass accumulation in heat acclimated *Brassica* (Kaur et al., 2009) and wheat (Asthir and Deep, 2011) under heat stress conditions.

Plant RWC status is the most important variable under changing ambient temperatures (Mazorra et al., 2002). In general, plants tend to maintain stable RWC regardless of temperature when moisture is abundant; however, high temperatures results in limited availability of water (Simoes-Araujo et al., 2003). In Lablab bean, elevated temperatures caused reduction in RWC in all treated samples when compared to control, the decrease being greater in HS treated seedlings (Figure 1d). A decrease in RWC in relation to raised temperature was also reported in *Lotus creticus* (Anon et al., 2004) and tomato (Morales et al., 2003). Reduction in tissue water causes a decrease in water potential thereby leading to perturbation of many physiological processes (Tsukaguchi et al., 2003) such as reduction in rate of transpiration, protein synthesis, enzymes and ion uptake and transport (Khalil et al., 2009). This explains the growth inhibition observed in HS treated seedlings even after the removal of the stressing conditions.

Response of hydrolytic enzymes and soluble sugars

Metabolites have a number of functions in addition to those of intermediary metabolism. They act as signaling/regulatory agents, compatible solutes, antioxidants or defense molecules against pathogens. The results obtained with Lablab bean provide an insight into the roles of two known signaling molecules and protectants, namely total soluble sugars (TSS) and proline.

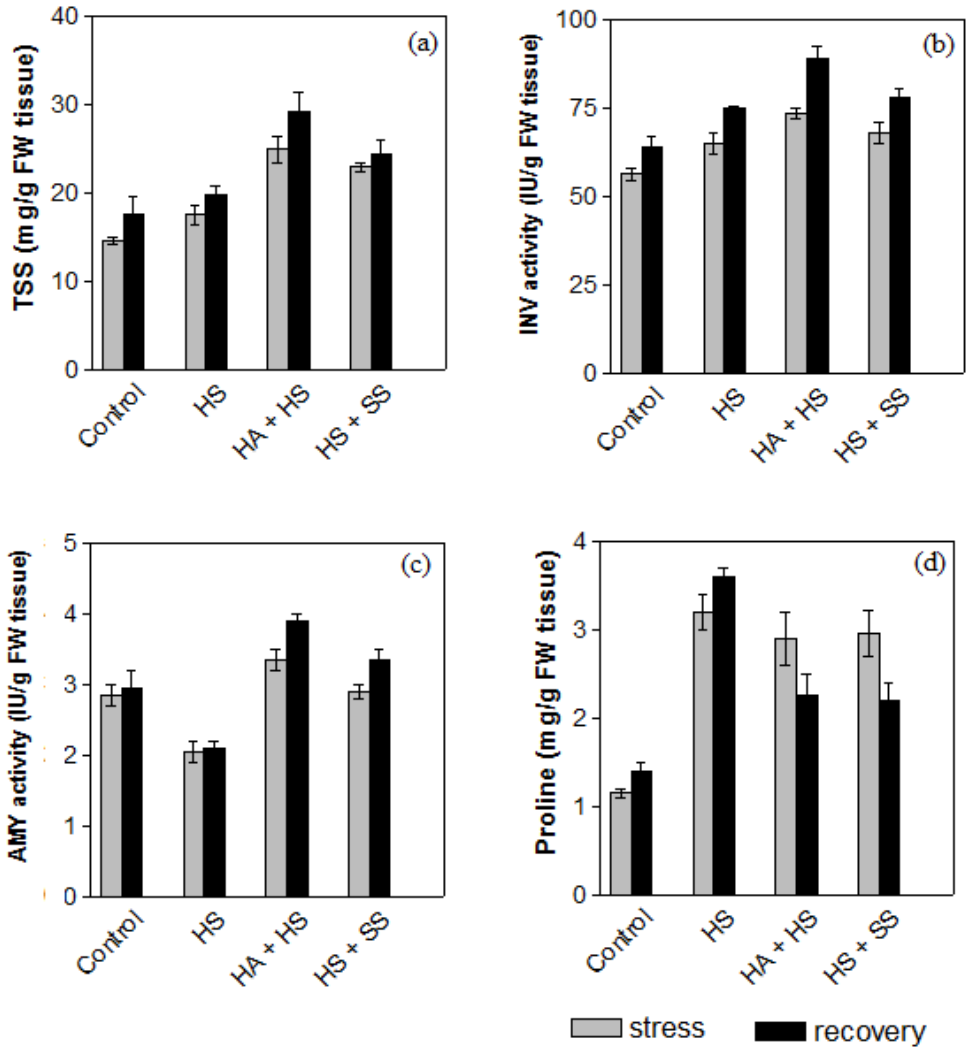

Figure 2. Total soluble sugars (a), invertase activity (b), amylase activity (c) and proline (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

Accumulation of total soluble sugars (TSS) under heat stress has been implicated in the establishment and maintenance of thermotolerance (Wahid and Close, 2007; Rizhsky et al., 2004). Sugars serve as signalling molecules during abiotic stress in stress-tolerant phenotypes (Rosa et al., 2009). Sugar signaling pathways interact with stress pathways in a complex network to modulate the metabolic responses of plants (Gill et al., 2003; Tran et al., 2007). The effect of HS on carbohydrate metabolism in Lablab bean showed a small increase in the TSS while HA and SS + HS pre-treatments resulted in a significant increase (Figure 2a) implying better signalling in place in pre-treated seedlings. Accumulation of TSS has also been reported in heat acclimated grape (Greer and Weston, 2010) and sugarcane (Wahid and Close, 2007) as a means of establishing thermotolerance. Invertase (INV) plays an important function in cell elongation and plant growth through carbon allocation (Gibeaut et al., 1990). Secondly, it also helps in sucrose metabolism, which in turn, has a crucial role in germination, seedling growth and in increasing the osmotic potential of the stressed cell (Ruan et al., 2010).

Increased INV in HS and pre-treated seedlings (Figure 2b) corroborates the higher level of TSS in these seedlings. An increase in INV was also reported in Brassica (Kaur et al., 2009) and potatoes (Lorenzen et al., 2002) where it was shown to contribute to theromtolerance of these plants. β-Amylase (β-AMY) is important in the transitory starch breakdown (Scheidig et al., 2002) needed to combat heat stress (Mansoor and Naqvi, 2012). The activity of β-AMY was reduced under HS;

however, HA treatment prior to HS (HA + HS) induced AMY (Figure 2c). Lethal temperatures retard seedling growth as well as β -AMY in winter wheat (Sultana et al., 2000). Kaplan and Guy (2004) demonstrated the appearance of maltose after β-AMY induction and also highlighted the contribution of stress-induced maltose accumulation towards the protection of the photosynthetic electron transport chain, proteins and membranes inside the chloroplast during acute temperature shock.

Response of the osmolyte, proline

Proline accumulation is a widespread phenomenon in higher plants in response to various environmental stresses and is demonstrated to be protective for plants under adverse conditions. Proline so accumulated is proposed to act as a compatible osmolyte, free radical scavenger, cell redox balancer, potential inhibitor of programmed cell death (PCD), cytosolic pH buffer and stabilizer for subcellular structures during various stresses (Kavi Kishore et al., 2005; Trovato et al., 2008; Gill and Tuteja, 2010). Under supra optimal temperature, free proline is known to accumulate in different crops (Rasheed et al., 2011). It is therefore, considered to be a useful component for evaluating the degree of heat stress (Kou et al., 1986). Proline content in leaves was significantly higher in HS treated Lablab bean while its levels declined considerably in HA + HS (Figure 2d) after recovery. This suggests that the HS seedlings were still unable to recuperate from stress even after 3 days of recovery.

The exact physiological function of proline is still controversial, and several researchers have attributed its beneficial function to the process of proline metabolism rather than to the proline molecule itself. The inter conversion of pro and pyrroline-5-carboxylate (P5C) in different cellular compartments might be involved in metabolic signaling, regulation of intracellular redox potential in higher plants and generation of ATP required for recovery from stress (Miller et al., 2009). Compared with other stresses; however, only a few reports demonstrated proline accumulation during heat stresses (Chakraborty and Tongden, 2005; Rasheed et al., 2011).

Response of antioxidants and antioxidant enzymes

In plants, ROS has been demonstrated to cause oxidative damage leading to cellular injury during various environmental stresses, including extreme temperature (Larkindale and Knight, 2002; Apel and Hirt, 2004). Even very short heat stress is able to bring about the increase in ROS, among which hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are believed to be the most important components (Apel and Hirt, 2004). The scavenging of O_2^- by superoxide dismutase (SOD) results in the production of H_2O_2, which is then removed by POX or CAT. H_2O_2 is primarily associated with the stress-induced stomatal closure that, in turn, causes a decrease in the CO_2/O_2 ratio in the chloroplasts (Cavalcanti et al., 2004). It has been demonstrated that this CO_2/O_2 ratio reduction in leaves inhibits CO_2 fixation, increasing the rate of ROS formation by enhancing electron leakage to oxygen molecules, and also, therefore, increasing the photorespiration process (Foyer and Noctor, 2003). To explore whether increased heat tolerance in HA seedlings is related to ROS generation during the acclimation phase, seedlings were analyzed for H_2O_2 levels upon recovery from heat stress. In Lablab bean, even after recovery, H_2O_2 levels were highest in HS treated seedlings than others (Figure 3a). Pre-treatment of seedling that is, HA + HS and SS + HS resulted in lower H_2O_2 content indicating that pre-treatments induced antioxidative components more efficiently than direct HS.

The accumulation of free radicals in stressed plants cause oxidation of polyunsaturated fatty acids in the plasma membrane resulting in the formation of thiobarbituric reactive species (TBARS) (Garg and Manchanda, 2009). TBARS level is used as an index of lipid peroxidation of cell membranes (Gechev et al., 2002). Temperature regulates membrane fluidity based on its composition and the degree of unsaturation (Los and Murata, 2004). Saturation of membrane lipids as a means of acclimation to high temperature is known to enhance thermal stability of PSII in thylakoid membranes (Sato et al., 1996). During the recovery stage after heat treatment, TBARS levels mimicked those of H_2O_2 (Figure 3b). These results demonstrate that oxidative stress is an important component of heat stress injury in Lablab bean and that HS induced more severe oxidative damage than pre-treated seedlings which were better equipped to scavenge ROS upon removal of heat stress. The increase in the content of lipid peroxides commonly associated with high temperature stress could serve as an activation signal for the expression of heat-shock genes which code for proteins and enzymes needed for the cell to tolerate high temperature (Vigh et al., 1998). Plants have multiple strategies to prevent oxidative damage to cells, employing enzymatic and nonenzymatic antioxidants. Superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) are among the enzymatic antioxidants. It is a well known fact that dismutation of O_2^- catalyzed by SOD produces H_2O_2 and O_2 (Asada and Takahashi, 1987). CAT exterminates H_2O_2 and is thought to be one of the most important antioxidant enzymes.

GR and APX act in the stress-regulated glutathione-ascorbate cycle. The activities of these enzymes have been proved to be inducible by the rise in intracellular ROS levels (Apel and Hirt, 2004). In Lablab bean, POX was found to be higher in HA + HS followed by SS + HS (Figure 3d). The maintenance of higher POX activity may provide further oxidative protection by detoxifying H_2O_2.

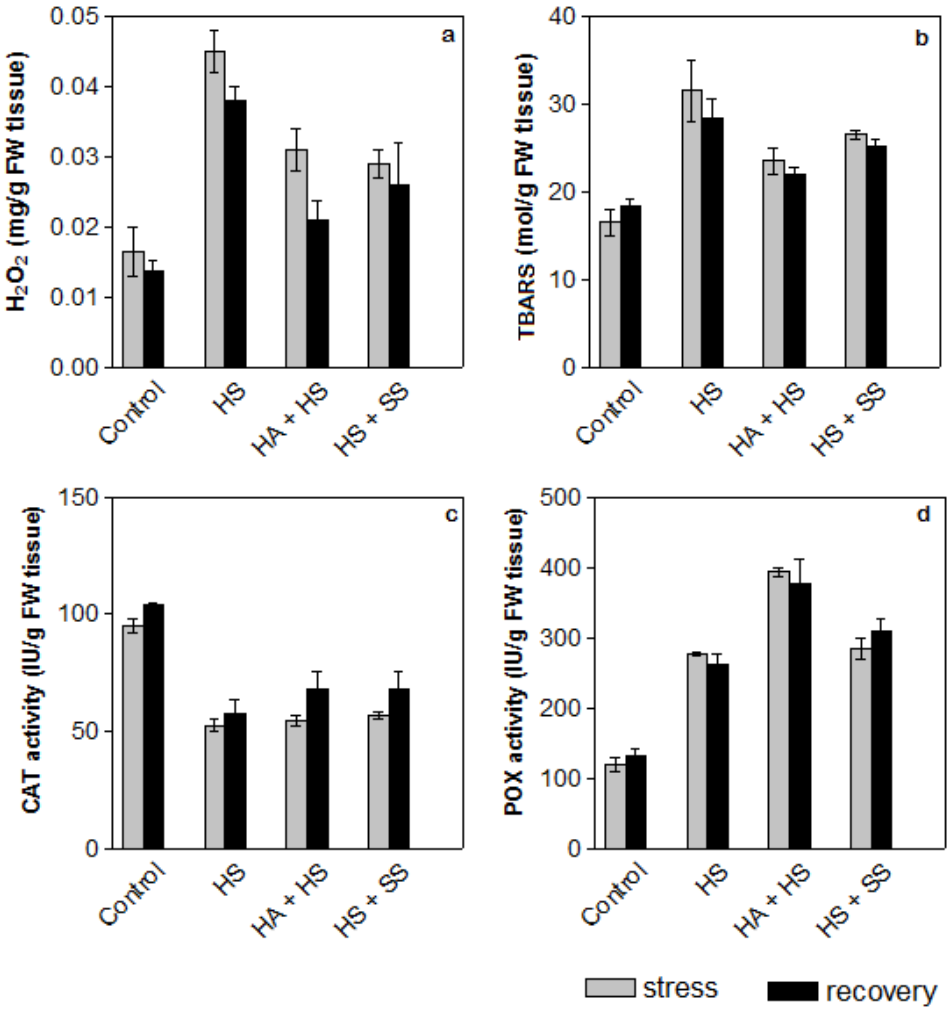

Figure 3. H_2O_2 (a), TBARS (b), CAT activity (c) and POX (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

These results are in consonance with POX activity reported in heat acclimated wheat (Asthir and Deep, 2011) and turfgrass seedlings (Du and Wang, 2009).

The reduction in CAT activity indicated that CAT may not be involved in antioxidant defence against heat stress in the Lablab bean (Figure 3c). A decrease in CAT activity was also reported in turfgrass (Du and Wang, 2009). The protective action of CAT is limited because it has relatively poor affinity for its substrates and is sensitive to light-induced inactivation compared with other antioxidant enzymes (Engel et al., 2006). Peroxisomal CAT is known to be sensitive to high temperature stress (Foyer and Noctor, 2000) probably because of an imbalance that occurs between its synthesis and degradation.

Also, as CAT has a rapid turnover, conditions inhibiting its synthesis will lower the steady-state level of this enzyme (Scandalios et al., 1997). Thus, heat shock and oxidative stress will enhance inactivation of CAT by preventing synthesis of new enzyme (Feierabend and Dehne, 1996), resulting in a decline in CAT activity. On the other hand, the absolute absence of recovery in leaf CAT activity in the recovered plants, even after 3 days, suggests that the enzyme suffered irreversible damage to its structure and/or that very low rates of *de novo* synthesis occurred. Available data suggests that signaling molecules like H_2O_2 may cause an increase in the antioxidant capacity of cells (Gong et al., 2001) by raising levels of GSH and ASC (Xu et al., 2006). GSH plays an important role in physiological functions such as redox regulation, conjugation of metabolites, detoxification of xenobiotics, homeostasis and cellular signaling that trigger adaptive responses (Foyer and Noctor, 2005; Rouhier et al., 2008). Nieto-Sotelo and Ho (1986) were the first to show that elevated synthesis of GSH occurs during temperature stress in plant cells.

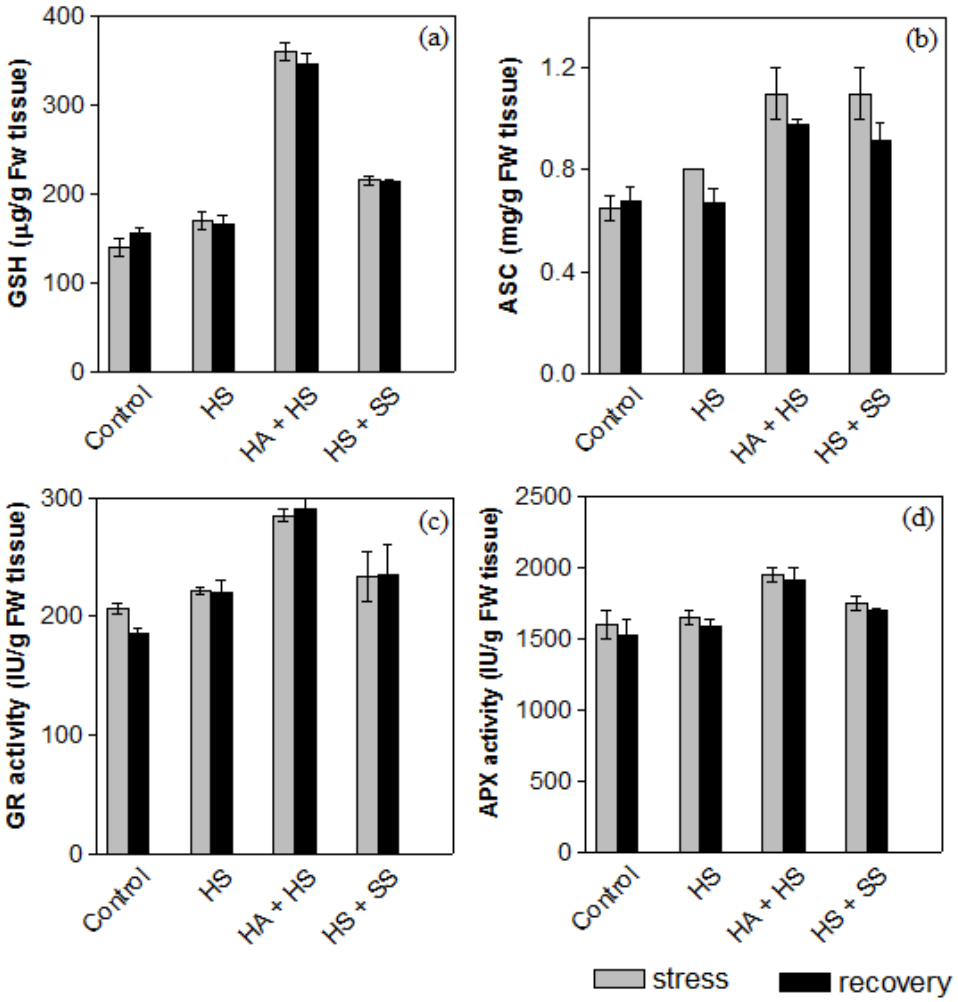

Figure 4. GSH (a), ASC (b), GR activity (c) and APX activity (d) of Lablab bean after 3 days recovery following heat treatments. Data plotted are mean ± SE of duplicates of three separate replicates; mean values were compared by one way ANOVA (P≤ 0.05).

Studies with heat-stressed Lablab bean suggested that seedlings that were pre-treated with heat stress had lower H_2O_2 levels as a result of enhanced synthesis of GSH (Figure 4a) and ASC (Figure 4b). Several authors have shown that elevated GSH content is correlated with the ability of plants to recover from heat stress when acclimated using sub-lethal temperatures (Nieto-Sotelo and Ho, 1986; Chao et al., 2009). Chao et al. (2009) have also demonstrated that HS signals lead to an early accumulation of H_2O_2 which in turn prevented rice seedlings from oxidative damage by Cd. The ASC pool in the chloroplast is regulated by the ascorbate-glutathione cycle involving four enzymes; APX, GR, DHAR and MDHAR (Noctor and Foyer, 1998). Our results show that GR and APX activities were significantly elevated in HA + HS (Figure 4c and d) resulting in a higher ASC content in these seedlings. It has been reported that the over-expression of GR leads to an increase in the ASC pool (Foyer et al., 1995) while overexpression of thylakoid membrane-bound APX functions to maintain the ASC content and the reduced status of ASC under stress conditions (Yabuta et al., 2002). In addition, enhanced choroplastic GR activity in transgenic plants have shown increased protection against oxidative stress (Melchiorre et al., 2009).

The increase in activity of POX, GR and APX in the recovery period of Lablab bean was in keeping with the work of Almeselamni et al. (2006) who have proposed that this type of response is characteristic of heat tolerant wheat genotypes upon recovery from high temperatures. The enhanced activities of GR and APX, concomitant with the enhanced content of ASC and GSH observed in this study could help to quench ROS and prevent cellular damage. According to the results obtained, it can be opined that Lablab bean plants may develop tolerance against superoptimal temperature stress caused at 45°C,

a temperature well above the optimal growth temperature of ~30°C through exposure to sub-lethal temperature of 35°C for 2 h. Thermotolerance acquired by plants through autonomous synthesis of pertinent compounds or induced through gradual exposure to sub-lethal temperatures (HA + HS), though cost intensive, is an important and potentially vital strategy. This phenomenon is principally related to display of heat shock response by antioxidants, antioxidant enzymes and compatible solutes; and accomplished by reprogramming of gene expression, allowing plants to cope with the heat stress.

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