Acclimatory response to hydrogen peroxide and glutathione under salt-boron stress through their impact on mineral nutrition and antioxidant defense system in pigeonpea (*Cajanus cajan* L.)

S. Chawla · S. C. Goyal · Rajiv Angrish · C. Rani ·
V. Arora · K. S. Datta · S. Madaan · S. Devi

Published online: 30 November 2010

© Prof. H.S. Srivastava Foundation for Science and Society 2010

Abstract Investigations were carried out on a salt tolerant (Manak, H77-216) and a comparatively salt sensitive (ICPL 88039) genotypes of pigeonpea (*Cajanus cajan* L. Millsp.) under NaCl, B and NaCl + B stress to examine the acclimatory response to H₂O₂, glutathione and H₂O₂ + glutathione through their effect on mineral nutrition, morpho-physiological parameters and antioxidant defense system. Both B and NaCl alone and their combinations had deleterious effect on dry biomass of plumule, enhanced relative stress injury (RSI), lipid peroxidation with concomitant increase in Na, Cl and B contents. However it did not bear any correlation with osmotic potential of plumule and K contents. Antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and glutathione reductase (GR) also decreased with salt, B and salt + B treatments. However contents of H₂O₂ enhanced and that of ascorbate declined under aforementioned treatments. These injurious effects are partially alleviated by exogenous application of H₂O₂; glutathione (GSH) and H₂O₂ + GSH treatments. The role of H₂O₂ and GSH in the present study is suggestive of triggering multifunctional signal transduction in plant defense mechanisms to prevent cellular oxidation, membrane injury, lipid peroxidation and protein enzyme inactivation.

Keywords Salt · Boron · Stress · Minerals · Antioxidants · Acclimation · H₂O₂ · Glutathione · Pigeonpea

Introduction

Although boron (B) is one of the essential micronutrient, crops grown worldwide on saline soils under arid and semi-arid environments invariably suffer from boron toxicity (Cartwright et al. 1984; Gupta et al. 1985). This is generally ascribed to build up of soil B to toxic level due to use of underground saline water for irrigation afflicted with B even within safe limits (Keren 1990; Manchanda et al. 1993; Rao et al. 1994; Lauchli 2002). It is as yet not known precisely as to how B affects growth, development and productivity of crop plants particularly under saline conditions (Bingham et al. 1987; Manchanda and Sharma 1991; Alpaslan and Gunes 2001; Lee 2006; Reid 2007; Martínez-Ballesta et al. 2008). Experimental evidences are now available that B is much toxic in saline than in non-saline conditions. Mineral ion studies revealed that B mediated enhanced uptake of toxic ions i.e. Na, Cl, SO₄ and decline in Ca and Mg in different crops (Yadav and Manchanda 1982; Doila et al. 1998a, b).

Further, plant metabolism, cellular homeostasis and major physiological processes are affected by abiotic stresses including salinity (Arora et al. 2002; Srivalli et al. 2003; Foyer and Noctor 2005). A direct result of stress-induced cellular changes in the enhanced accumulation of toxic compounds in cells include reactive oxygen species, ROS (Suzuki and Mittler 2006). These ROS species include superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]) and singlet oxygen (O₂¹). Exposure of plants to stress activates plasma membrane bound NADPH-dependent superoxide synthase (Sagi and Fluhr 2006) producing superoxide (O₂^{•-}), which in turn is converted to hydrogen peroxide (H₂O₂) by superoxide dismutase (SOD). H₂O₂ can be converted further by a

S. Chawla · S. C. Goyal · R. Angrish (✉) · C. Rani · V. Arora ·
K. S. Datta · S. Madaan · S. Devi
Department of Botany and Plant Physiology,
CCS Haryana Agricultural University,
Hisar 125004 Haryana, India
e-mail: angrish@hau.ernet.in

reaction mediated by metals such as Fe^{3+} or Cu^{2+} into one of the most damaging and reactive hydroxyl radical ($\text{OH}^\cdot$) that damages the plant metabolic systems leading to plant senescence and programmed cell death (Halliwell and Gutteridge 1989). These ROS are scavenged from the cell by the action of SOD, catalase, APX and glutathione peroxidase. There are increasing evidences that production of ROS such as ($\text{O}_2^{\cdot-}$) is a common phenomenon in the aerobic organisms under stress conditions (Noctor and Foyer 1998; Vaidyanathan et al. 2003). An antioxidant defense mechanism has evolved in plant cells to prevent the danger posed by these ROS (Vranová et al. 2002). The prevention of damage by ROS in any cellular component depends on the efficacy of the antioxidant system comprising the ascorbate-glutathione cycle, SOD and a number of other antioxidant enzymes (Noctor and Foyer 1998).

Recently, the role of H_2O_2 , ascorbate and glutathione as cardinal signaling molecules has gained considerable importance (Mittler 2002). Plants are known to adapt to environmental stresses through specific genetic responses. The molecular mechanisms are associated with signal transduction leading to changes in gene expression at early stage of stress response but are poorly understood (Hernandez et al. 2001). However, there is increasing evidence that gene expressions associated with acclimatory responses in plants is sensitive to the redox state of the cell including its various components which contribute to the redox balance of the cell (Foyer et al. 1997; Vranová et al. 2002; Foyer and Noctor 2003). Two factors have been shown to be crucial in mediating stress responses. Thiol/disulphide exchange reactions, particularly involving the glutathione pool and the generation of the oxidant H_2O_2 (Grill et al. 2001). These molecules are now recognized to be multifunctional triggers, modulating metabolism and gene expression. Both are able to cross biological membranes and diffuse or be transported long distances from their sites of origin (Rennenberg 1982; Bowler et al. 1992). Glutathione and H_2O_2 may act alone or in unison in intracellular and systemic signaling system to achieve acclimation and tolerance to biotic and abiotic stresses (Foyer et al. 1997; May et al. 1998; Neill et al. 1999; Hernandez et al. 2001; De Gara et al. 2003). The rate of H_2O_2 production depends on strength and duration of the imposed stress. Moreover, H_2O_2 levels differ in various cell compartments and these levels are related to type of stress, e.g., excess light stress is responsible for the overproduction of H_2O_2 mainly in the chloroplast (Karpinski et al. 1999; Karpinska et al. 2000; Slesak et al. 2003). Experiments with plant material have demonstrated that plant tissues can tolerate high concentration of H_2O_2 in the range of $10^2 - 2 \times 10^5 \mu\text{M}$. Moreover, plants pretreated with H_2O_2 were more resistant to excess light and chilling stress (Prasad et al. 1994; Karpinski et al. 1999; Karpinska et al.

2000; Yu et al. 2003; Slesak et al. 2007). While the content of glutathione in plant tissue ranges from 0.1 to 3 mM (Grill et al. 2001). Keeping this in view, it was considered worthwhile to investigate the role of H_2O_2 and glutathione on the ROS scavenging system including its enzymes influenced by B and NaCl toxicity stress during early seedling growth of pigeonpea (*Cajanus cajan* L.).

Materials and methods

- (I) Plant material and culture conditions: Seeds of two genotypes of pigeonpea (*Cajanus cajan* L. Millsp.) i.e., salt tolerant (Manak, also numbered H77-216) and salt sensitive (ICPL 88039), were surface sterilized in 0.1% sodium hypochlorite solution for 2 min and subsequently washed twice with distilled water. Ten healthy and uniform seeds were kept in plastic Petri plates of 9 cm diameter (Tarsons, India) lined with filter paper at the bottom containing 10 ml of distilled water in a BOD incubator at $27 \pm 1^\circ\text{C}$ in dark. Seeds produced a dark coloured exudate which diffused in water and inhibited subsequent growth of seedlings particularly that of radicle. To overcome this problem the seeds were gently transferred into other set of Petri plates containing the same amount of water. This operation was repeated every day continuously for 3 days.
- (II) Treatments: Thereafter, the partially germinated seedlings were transferred to Petri plates with 10 ml of different treatment solutions alongwith distilled water control (C); H_2O_2 (100 μM); Glutathione, GSH (500 μM); H_2O_2 (100 μM) + GSH (500 μM) superimposed with S_0B_0 (DW), S_0B_{10} (0 mM NaCl + 10 mM B as $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), $\text{S}_{100} + \text{B}_0$ (100 mM NaCl + 0 mM B) and $\text{S}_{100}\text{B}_{10}$ (100 mM NaCl + 10 mM B) so as to have a total of 16 treatments with three replications in each of the two aforementioned pigeonpea cultivars in a BOD incubator at $27 \pm 1^\circ\text{C}$ in dark for 96 h. Although three concentrations of each of H_2O_2 (50, 100 and 200 μM) and GSH (250, 500 and 750 μM) were used in a preliminary experimentation but the above mentioned concentrations of H_2O_2 and GSH were found more effective. Therefore, samplings for various morpho-physiological and biochemical parameters were carried out after 168 h (i.e., 7 d) of sowing.
- (III) Estimations of various morpho-physiological and biochemical parameters
 - i) Plumule and radicle dry weight: Dry weight of plumule and radicle was expressed as mean dry weight of 20 plumules/radicles separately in each

treatment after 48 h drying of tissues in an oven at 80°C till the constant weight was attained. The dry weight was expressed as mg/20 plumule or radicles.

- ii) Relative Stress Injury (RSI): Quantification of RSI as a measure of membrane stability was done by Dionisio-sese and Tobita (1998) using the formula: RSI (%)

$$RSI (\%) = \frac{EC_a}{EC_b} \times 100$$

Where EC_a denotes electrical conductivity taken at room temperature after 5 h incubation in distilled water and EC_b denotes electrical conductivity after autoclaving the tissues.

- iii) Lipid peroxidation: The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) present in the tissues (fresh plumule and radicle). MDA is a product of lipid peroxidation and was measured by thiobarbituric acid (TBA) reaction with minor modification of the method of Heath and Packer (1968). and expressed in mg MDA/g fresh weight.
- iv) Osmotic potential: Osmotic potential was determined by a modified method of Gorham et al. (1984) using vapour pressure osmometer 5100-B, Wescor Inc., Logan Utah, USA and was expressed in mega pascals (MPa)
- v) Mineral estimation: Minerals i.e., Na, K, Cl and B content was determined from the oven dried material of plumule and radicle. While contents of Na and K were determined in an acid digest with a flame photometer (Elico, India, Model 128), that Cl estimation was done by ion analyzer (Elico, India made, Model-LI126) using chloride specific electrode supplied by pH products Co., Hyderabad. Whereas, B content was estimated after Bingham (1982) colorimetrically with azomethine -H as outlined in USDA Handbook no 60 (1954).

(IV) Enzyme analysis

Extraction: 500 mg plumule and radicle each were obtained 48 h after treatment and crushed in 5 ml of 0.1M phosphate buffer (pH 7.0). The extract was then centrifuged at 10,000×g at 40°C for 20 min. The supernatant was then used for the estimation of the following enzymes.

- (i) Superoxide dismutase (SOD) activity was measured according to the method of Jiang and Huang (2001) and expressed as units mg^{-1} protein min^{-1} .
- (ii) Catalase (CAT) activity was estimated by the method of Aebi (1983) and expressed as units mg^{-1} protein min^{-1} .

- (iii) Ascorbate peroxidase (APX) activity was assayed by the method of Nakano and Asada (1981) and expressed as units mg^{-1} protein min^{-1} .
- (iv) Peroxidase (POX) activity was measured by measuring the oxidation of guaiacol at 470 nm (Rao et al. 1996) and was expressed as units mg^{-1} protein min^{-1} .
- (v) Glutathione peroxidase (GPX): This enzyme was estimated as in case of ascorbate peroxidase replacing ascorbate with glutathione (GSH) by the method of Nakano and Asada (1981) and expressed as units mg^{-1} protein min^{-1} .
- (vi) Glutathione reductase (GR) activity was estimated by the method of Goldberg and Spooner (1983) and was expressed as units mg^{-1} protein min^{-1} .
- (V) Estimation of H_2O_2 and Ascorbic acid: While H_2O_2 content was determined by the method given by Evans et al. (1999) that ascorbic acid (AA) content was measured by the method of Schopfer (1966) and was expressed in n moles g^{-1} fw.

Results and discussion

At the outset it is worthwhile to mention here that the trends of the results both with respect to plumule and radicle were almost similar, so for the sake of brevity only the results with respect to plumule have been described here.

- (i) Dry mass of plumule: The magnitude of plumule growth when compared to untreated seedlings (S_0B_0), the treatment effect on growth as indicated by seedling dry mass (Fig. 1a) decreased considerably and that the dry mass of plumule in S_0B_0 did not differ much in both the cultivars. Manak (H77-216) registered higher dry mass of plumule than ICPL 88039 in all the comparable treatments. It was interesting to note that the maximum reduction in dry mass was 26.2% and 34% in $S_{100}B_{10}$ treatments in Manak and ICPL 88039, respectively in comparison to respective controls (S_0B_0).

It was interesting to note that application of H_2O_2 , GSH and $H_2O_2 + GSH$ caused enhancement in dry weight of plumule. The order of increase being $H_2O_2 + GSH > H_2O_2 > GSH > C$. Further, the increase in the highest stress level ($S_{100}B_{10}C$ treatment) over different treatments was 22.9% and 16.7% in $S_{100}B_{10} H + G$, 12.0% and 9.0% in $S_{100}B_{10} H$ and only 7.3% and 5.4% in $S_{100}B_{10} G$ in the genotypes Manak and ICPL 88039, respectively.

Fig. 1 Effect of H₂O₂ and GSH on (a) dry mass (b) relative stress injury (c) MDA content and (d) Osmotic potential of relatively tolerant (Manak) and sensitive (ICPL 88039) genotypes of pigeonpea. Bars on the histograms show the standard error mean

The decline in dry mass of plumule of seedlings under salt stress could be attributed to the reduced mobilization of the reserve food materials from endosperm (cotyledons) to growing axis due to inhibitory effects of both B and salt alone and in their combinations (Sheoran 1975; Mehta and Bharti 1983). Another pivotal observation is that GSH and H₂O₂ and their combination partially alleviated the B-salt toxicity which might be ascribed to intracellular and systemic signaling of GSH and H₂O₂ for inducing acclimation and tolerance to biotic and abiotic stresses (Karpinski et al. 1999; Neill et al. 1999; Ogawa and Iwabuchi

2001; Sairam and Srivastava 2001; De Gara et al. 2003).

- (ii) Relative Stress Injury (RSI): The relative stress injury (RSI) increased upon imposition of both salinity-B toxicity in both the pigeonpea cultivars (Fig. 1b). More RSI was noted in ICPL 88039 than Manak H 77-216, however, maximum RSI was accrued due to S₁₀₀B₁₀ treatment. The magnitude of RSI was 62% and 69% in plumule with S₁₀₀B₁₀ treatment over S₀B₀ C in Manak and ICPL 88039, respectively. Furthermore, it was observed that application of H₂O₂, GSH and H₂O₂ + GSH partially alleviate the Salt-B toxicity effects, the order being H₂O₂ + GSH > H₂O₂ > GSH > C. The percent alleviation effect with S₁₀₀B₁₀ H + G in comparison to S₁₀₀B₁₀ C was to the tune of 18% and 25% in Manak and ICPL 88039, respectively. Above results are in conformity with Hasegawa et al. (2000) who have shown more production of reactive oxygen species (ROS) under saline conditions which mediated membrane damage leading to cellular toxicity in rice, tomato and citrus plants (Gueta-Dahan et al. 1997; Dionisio-Sese and Tobita 1998; Mittova et al. 2004).

- (iii) Lipid peroxidation (MDA content): Lipid peroxidation in terms of MDA content increased progressively with salt, B and Salt + B levels in plumule of both genotypes. ICPL 88039 depicts greater lipid peroxidation than Manak. Thus, the percent increase in MDA content in S₁₀₀B₁₀C treatment over S₀B₀C treatment was 61% and 70% in plumule of Manak and ICPL 88039, respectively (Fig. 1c). On the other hand, application of H₂O₂ caused a relative more decline in MDA content than GSH treatment, but maximum reduction was caused by H₂O₂ + GSH.

The above data on RSI and lipid peroxidation in terms of MDA content shows that B, salt and Salt + B treatment had a progressive increasing effect on these parameters. Kuiper (1980), Leopold and Willing (1980), Bliss et al. (1984) and Dat et al. (1998) have indicated that the cell membranes are the sites of primary or secondary salt/ion effects. These cause changes in permeability due to lesion formation affecting thereby the transport of both organic and inorganic solutes resulting in structural alteration and ion leakage. These effects are reflected in higher RSI. Likewise Doila et al. (1998a; b) and Bansal (1999) ascribed that higher RSI is due to leakiness of membranes along with lipid peroxidation in faba bean and wheat. Even exogenous application of H₂O₂ and GSH, particularly the former, caused a reduction in RSI and MDA in the present study which appeared intriguing at the first instance. For example, Sairam and Srivastava (2000) observed that lipid peroxida-

tion estimated as MDA content enhanced with increasing doses of exogenously applied H_2O_2 in two genotypes of wheat under water deficit stress. Membrane Stability Index (MSI) also decreased with increasing H_2O_2 concentration in both the genotypes. However in the present experimental set up the H_2O_2 (and GSH) were administered to act as an ROS scavenging system “inductive pulse” and actually caused a decline in endogenous levels of H_2O_2 , thereby reducing the observed lipid peroxidation and membrane injury.

- (iv) Osmotic potential (Ψ_s): Data illustrated in Fig. 1(d) shows that the osmotic potential of plumule of both the genotypes showed a slightly decreasing trend (more negative values) with B followed by salt and salt + B, however, Ψ_s did not differ significantly between the two pigeonpea genotypes. These results are in accord with Nandwal et al. (2000) who also reported more negative osmotic potential in various plant parts under salinity in *Vigna radiata*. Even the effect of treatment of H_2O_2 , GSH and H_2O_2 + GSH did not vary the osmotic potential significantly. Furthermore, there is no mention in the literature suggesting involvement of H_2O_2 in osmotic adjustment and resultant amelioration under salinity.
- (v) Mineral Content: The results on K content of plumule invariably remained unaffected with either B, Salt or Salt + B (Fig. 2a). Even H_2O_2 and GSH or H_2O_2 + GSH treatments did not much alter the K content of plumule. Even two cultivars did not differ in this respect. These results are more or less in conformity with Doila et al. (1998a) and Bishnoi (2003) who did not observe K content changes under B, salt and salt + B treatments in faba bean. Na levels showed a progressive increase in B, salt and salt + B treatments. Increase in Na content especially under B alone treatment could be ascribed to the nature of added B source in the medium ($Na_2B_4O_7 \cdot 10H_2O$).

On the other hand, H_2O_2 , GSH and H_2O_2 + GSH appeared to have no significant effect in Na levels in plumule (Fig. 2b). Similarly Cl content was moderately present in S_0B_0C treatment and also in $S_0B_{10}C$ treatment, but increased manifolds in the presence of salt i.e. $S_{100}B_0C$ and $S_{100}B_{10}C$ treatments in plumule of both Manak and ICPL 88039 (Fig. 2c). Furthermore, a slight synergism between B and salt was noticed especially in the Manak genotype. Genotypic differences were significant with Manak maintaining slightly less Cl in plumule particularly in $S_{100}B_0C$ and $S_{100}B_{10}C$ treatments. Application of H_2O_2 or GSH alone or in combination with each other had no consistent effect on Cl content in both the genotypes.

Fig. 2 Effect of H_2O_2 and GSH on (a) Potassium content (b) Sodium content (c) Chloride content and (d) Boron content of relatively tolerant (Manak) and sensitive (ICPL 88039) genotypes of pigeonpea. Bars on the histograms show the standard error mean

Likewise, the B content was at its optimal low in S_0B_0C treatment, increased manifold with the addition of B in the medium i.e. in $S_0B_{10}C$ in plumule (Fig. 2d). Again it recorded a low in $S_{100}B_0C$ comparable to S_0B_0C treatment in plumule of both genotypes. In $S_{100}B_{10}C$ again a significant multiple increase in B content was noted as compared to S_0B_0C and $S_{100}B_0C$ in all cases. Manak, in general, maintained a lower B levels as compared to the sensitive ICPL 88039. Even chemical treatment of H_2O_2 , GSH and H_2O_2 + GSH did not have any significant effect on B levels. Overall it was brought

forth that salt tolerant Manak registered low levels of Na, Cl and B than salt sensitive ICPL 88039.

However the worthwhile point to be noted in the present context is that, in general, in all the cases H_2O_2 and GSH either alone or in combination with each other did not have any specific effect on the mineral ion accumulation. It would be logical to conclude, therefore, that the ROS scavenging system has no direct bearing on mineral ion uptake.

(vi) Antioxidant defense system:

Superoxide dismutase (SOD): Fig. 3(a) represents the activity of SOD which declined progressively with B, salt and salt + B treatments in plumule of both Manak and ICPL 88039, respectively. Genotypic differences were significant, containing slightly higher SOD activity in plumule of Manak. Magnitude of decrease in enzyme activity with stress level was greater in ICPL 88039 as compared to relatively tolerant Manak being 52.7% and 48.7% in plumule in the stress treatment $S_{100}B_{10}C$ over S_0B_0C . It was interesting to note that in general, H_2O_2 caused a slight decline in the activity of SOD in contrast to GSH, which caused a general increase in SOD activity over their respective controls in plumule of both the cultivars, combination of both $H_2O_2 + GSH$ also caused an increase in activity of SOD. So overall effect was in order of $GSH > H_2O_2 + GSH > C > H_2O_2$.

Catalase (CAT): Imposition of salt, B and salt + B caused reduction in CAT activity (Fig. 3b) being maximum in the latter case. Magnitude of decrease in CAT activity in the maximum stress treatment $S_{100}B_{10}C$ over S_0B_0C was 26% and 51.4% in the plumule of Manak and ICPL 88039 respectively. Addition of $H_2O_2 + GSH$ in the medium also caused well marked rise in the CAT activity as compared to control in both the genotypes. Although, magnitude of increase in case of GSH was less than H_2O_2 . The combination treatment of $H_2O_2 + GSH$ in general, caused maximum increase in CAT activity. Thus, the overall trend of stimulation of CAT activity in plumule of both genotypes would appear to be $H_2O_2 + GSH > H_2O_2 > GSH > C$. The decrease in CAT activity under NaCl and B stress could be attributed to CAT being a H_2O_2 detoxifying enzyme and mostly associated with peroxisomes where it removes H_2O_2 formed during photorespiration (Apel and Hirt 2004). Even, Corpas et al. 1993 reported a significant decrease in the enzyme activity during salt stress in both salt tolerant and salt sensitive cultivars of pea leaves.

Ascorbate peroxidase (APX): Likewise, the APX activity (Fig. 3c) was drastically decreased in response to NaCl stress over unstressed control in both the cultivars. Extent of decline in APX activity in the

Fig. 3 Effect of H_2O_2 and GSH on (a) SOD activity (b) Catalase activity (c) APX activity in plumules of relatively tolerant (Manak) and sensitive (ICPL 88039) genotypes of pigeonpea. Bars on the histograms show the standard error mean

$S_{100}B_{10}C$ treatment over S_0B_0C treatment was 46.8% and 49.5% in the plumule of Manak and ICPL 88039 respectively. H_2O_2 and GSH and their combinations caused an activation of APX in control in both the cultivars as well as under salt, B and salt + B treatments. For example percent increase in Manak was 15% in $S_{100}B_{10}$ $H_2O_2 + GSH$, 10% in $S_{100}B_{10}$ H_2O_2 and 6% in $S_{100}B_{10}$ GSH over the control $S_{100}B_{10}$ C. These results are in consonance with the results of Comba et al. (1998) who observed more APX activity at 50 than 200 mmol/L NaCl in soybean root nodules. It was suggested that decreased enzymes activity might be due to imbalance between the production of active oxygen species and the quenching ability of antioxidants that got upset and resulted in oxidative damage.

Peroxidase (POX) and Glutathione peroxidase (GPX): Similarly, both POX and GPX activity exactly followed the same pattern (Fig. 4a and b) and both of these mimic the effect of CAT activity (Fig. 3b). Furthermore, APX activity (Fig. 3c) also more or less showed the same trend including efficacy in the order

Fig. 4 Effect of H_2O_2 and GSH on (a) POX activity (b) GPX activity (c) GR activity in plumules of relatively tolerant (Manak) and sensitive (ICPL 88039) genotypes of pigeonpea. Bars on the histograms show the standard error mean

of $GSH + H_2O_2 > H_2O_2 > GSH > C$. The resultant analysis of all the activities of the above enzymes clearly showed that the decline in the activity was in the order of $S_{100}B_{10} > S_{100}B_0 > S_0B_{10} > C$.

Glutathione reductase (GR): Another interesting point that emerges from these studies was that although H_2O_2 stimulated GR activity to a considerable extent, GSH caused a decline in GR activity vis-à-vis the control (Fig. 4c). Combination treatment of H_2O_2 and GSH alleviated the inhibitory effect of GSH to some extent but it was still below the respective control values (Fig. 4c). In addition GR activity was prominently higher in Manak than ICPL-88039 irrespective of stress and treatment levels. The overall effect was in the order of $H_2O_2 > C > GSH + H_2O_2 > GSH$.

The overall crux of the above results point out that the increase in the enzymes activity of CAT, APX, GPX, POX and even GR by both H_2O_2 and GSH could be explained by a precocious signaling and/or deactivation of the Asada-Halliwell pathway of H_2O_2 scavenging and ascorbic acid regeneration involving various antioxidant enzymes (See Arora et al. 2002).

Levine (1999) attributed a cardinal signaling role to H_2O_2 in plant defense response. He opined that perception of pathogens or stress elicitors by plant cells causes an immediate activation of NADH oxidase that generates superoxide anions. The acidic pH in the apoplast stimulates rapid distribution of $O_2^{\cdot -}$ to the membrane permeable species H_2O_2 . Since H_2O_2 is an endogenous oxidant which accumulates in many stress conditions (stressed with B, Salt or B + Salt in our case), a central role for this metabolite as a diffusible signal for selective induction of defense genes has been envisaged (Levine et al. 1994; Prasad et al. 1994). The relative stability and higher concentrations of H_2O_2 in plant cells could point to the fact that H_2O_2 plays a key role as a signal transduction factor. However, signal molecules are usually present in cells in very low concentrations (Hung et al. 2005); the relatively high levels of H_2O_2 in plant tissues support the assumption that H_2O_2 is not only a signaling molecule, but also plays a key role in primary plant metabolism (Slesak et al. 2007). Moreover, H_2O_2 regulates the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production (Neill et al. 2002a; Neill et al. 2002b; Geisler et al. 2006). Similarly, rather both H_2O_2 and GSH is the product of SOD and GR, respectively. Again, the exogenous treatment of GSH caused an increase in the activity of SOD, CAT, APX, GPX, POX over their respective controls. Conversely, GSH decreased the activity of GR which could be ascribed to more involvement of

Fig. 5 Effect of H_2O_2 and GSH on (a) Hydrogen peroxide content (b) Ascorbate content in plumules of relatively tolerant (Manak) and sensitive (ICPL 88039) genotypes of pigeonpea. Bars on the histograms show the standard error mean

GSH in homeostasis in plants having implication for environmental stress sensing and its effect on plant growth and development (Komives et al. 1998; May et al. 1998).

- (vii) H_2O_2 content: The contents of H_2O_2 (Fig. 5a) showed a progressive and significant increase in plumule of both genotypes with imposition of B, salt and salt + B levels. The maximum increase was noticed in the latter case. The magnitude of increase in H_2O_2 content at the maximum stress treatment of $\text{S}_{100}\text{B}_{10}\text{C}$ over $\text{S}_0\text{B}_0\text{C}$ (control) was 72% and 92% in Manak and ICPL-88039, respectively. One of the most interesting observations was that exogenous application of H_2O_2 in general decreases its endogenous contents. The decrease was more marked in $\text{S}_0\text{B}_0\text{C}$, $\text{S}_{100}\text{B}_0\text{C}$ and to a maximum extent in $\text{S}_{100}\text{B}_{10}\text{C}$ treatments. Treatment of GSH also caused a decrease in H_2O_2 level, but magnitude of this decrease was less as compared to H_2O_2 treatment. Interestingly, the maximum decrease in H_2O_2 content was observed in the H_2O_2 + GSH treatment thereby showing positive synergism between H_2O_2 and GSH. The magnitude of decrease of H_2O_2 with different treatments would, therefore, appear to be in the order of $\text{C} < \text{GSH} < \text{H}_2\text{O}_2 < \text{H}_2\text{O}_2 + \text{GSH}$. For example, in $\text{S}_{100}\text{B}_{10}$ stress levels the reduction in H_2O_2 content was 14.5% and 10.2% in $\text{S}_{100}\text{B}_{10}\text{G}$ treatments, 21.2% and 14.4% in $\text{S}_{100}\text{B}_{10}\text{H}$ treatments and 27.2% and 17.5% in $\text{S}_{100}\text{B}_{10}\text{H} + \text{G}$ treatments in Manak and ICPL 88039, respectively. Agarwal et al. (2005) also found that H_2O_2 effectively increased the activities of H_2O_2 scavenging enzymes in addition to stimulation of growth as has also been observed in our present study.

The present experimental scenario definitely envisages antioxidant defense system signaling and inductive role through exogenously applied H_2O_2 and GSH. Therefore, not only H_2O_2 but also GSH had a similar effect, so much so that the two acted synergistically i.e. $\text{H}_2\text{O}_2 + \text{GSH} > \text{H}_2\text{O}_2 > \text{GSH}$ in decreasing endogenous H_2O_2 levels. Moreover, GSH and H_2O_2 are known to act alone or in unison as multifunctional triggers modulating metabolism and gene expression, in intercellular and systemic signaling systems to achieve acclimation/tolerance to abiotic/biotic stresses (May et al. 1998; Karpinski et al. 1999; Levine 1999; Neill et al. 1999).

- (viii) Ascorbic acid content: Fig. 5b illustrates a declining trend in ascorbic acid content with increasing levels of stress of salt, B and salt + B in both the genotypes. Genotypic differences were clearly significant and magnitude of decline in the maximum stress levels of $\text{S}_{100}\text{B}_{10}\text{C}$ over the $\text{S}_0\text{B}_0\text{C}$ was 37.6%

and 42.3% in Manak and ICPL-88039, respectively. Treatment of H_2O_2 caused an increase in the ascorbate content more particularly as shown by the higher $\text{S}_{100}\text{B}_{10}$ stress levels. Glutathione (GSH) also caused an increase in the ascorbate content but to a lesser extent than H_2O_2 . Combination treatment of $\text{H}_2\text{O}_2 + \text{GSH}$ recorded a maximum ascorbate levels indicating a synergism between H_2O_2 and GSH. The general trend of increase in ascorbate content was $\text{H}_2\text{O}_2 + \text{GSH} > \text{H}_2\text{O}_2 > \text{GSH} > \text{C}$. These observations corroborated the findings of Hernandez et al. (2000) who observed high levels of ascorbate in salt tolerant and lower levels in salt sensitive cultivars e.g. pea and foxtail millet in response to salt stress (Sreenivasulu et al. 2000). Ascorbate is known to be oxidized by a direct reaction with superoxide or by serving as a reductant of α -chromoxyl radical of oxidized α -tocopherol, which in turns disrupts lipid peroxidation reactions by reacting with superoxide and results in scavenging hydroxyl, peroxy and alkoxy radicals (Halliwell and Gutteridge 1989).

Conclusively, above observations prove that H_2O_2 and GSH have versatile functions in plant defense mechanism (i) as a signaling agent and inducer/reactant of antioxidant enzyme system and (ii) as an oxidant/reductant responsible for damaging/preventing activities like cellular oxidation, lipid peroxidation, membrane injury, pigment bleaching and protein enzyme inactivation. Even various workers like Elstner and Oßwald 1994, Levine 1999, Sairam and Srivastava 2000 and Pnueli et al. 2003 have supported the above contention. The lower H_2O_2 levels resulting from H_2O_2 application or GSH could be due to the high activity of APX and CAT as has been observed earlier by Venacker et al. 1998 who showed that apoplast of barley leaves have shown substantial amounts of SOD, APX and other enzymes required for scavenging/destruction of O_2^- and H_2O_2 .

Acknowledgements The authors are grateful to ICAR, New Delhi for financial assistance in the form of an adhoc research project entitled, "Physiological and biochemical basis of boron toxicity in important crops under salinity stress."

References

- Aebi HE (1983) Catalase. In: Bergmeyer HU (ed) Methods of enzyme analysis. Verlag Chemie Weinheim, pp 273–286
 Agarwal S, Sairam RK, Srivastava GC, Tyagi A, Meena RC (2005) Role of ABA, salicylic acid, calcium and hydrogen peroxide on

- antioxidant enzymes induction in wheat seedlings. *Plant Sci* 169:559–570
- Alpaslan M, Gunes A (2001) Interactive effects of boron and salinity stress on the growth, membrane permeability and mineral composition of tomato and cucumber plants. *Plant Soil* 236:123–128
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Ann Rev Plant Biol* 55:373–399
- Arora A, Sairam RK, Srivastava GC (2002) Oxidative stress and antioxidative system in plants. *Curr Sci* 82:1227–1238
- Bansal S (1999) Physiological changes in wheat (*Triticum aestivum* L.) under salinity-boron interaction. M.Sc. Thesis, CCS Haryana Agricultural University, Hisar, India
- Bingham FT (1982) Boron. In: Page AL (ed) *Methods of soil analysis*, Part 2. *Agronomy* 9: 431–447
- Bingham FT, Strong JE, Rhoades JD, Karen R (1987) Effect of salinity and varying boron concentrations on boron uptake and growth of wheat. *Plant Soil* 97:345–351
- Bishnoi S (2003) Physiological and biochemical investigations on boron toxicity under salt stress and its alleviation by calcium in pigeonpea (*Cajanus cajan* L. Millsp.). Ph.D. Thesis, CCS Haryana Agricultural University, Hisar, India
- Bliss RD, Platt-Aloia KA, Thomson WW (1984) Effect of salt on cell membranes of germinating seeds. *California Agric* 34:24–25
- Bowler C, Van Montagu M, Inze D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43:83–116
- Cartwright B, Zarcinas BA, Mayfield AH (1984) Toxic concentration of boron in a red-brown earth at Gladstone, South Australia. *Aust J Soil Res* 22:261–272
- Comba ME, Benavides MP, Tomaro ML (1998) Effect of salt stress on antioxidant defense system in soybean root nodules. *Aus J Plant Physiol* 25:665–671
- Corpas F, Gomez JM, Hernandez JA, Del Rio JA (1993) Metabolism of activated oxygen in leaf peroxisomes from two *Pisum sativum* L. cultivars with different sensitivity to sodium chloride. *J Plant Physiol* 141:160–165
- Dat JF, Lopez-Delgado H, Foyer CH, Scott IM (1998) Parallel changes in H_2O_2 and catalase during thermo-tolerance induced by salicylic acid or heat acclimation in mustard seedlings. *Plant Physiol* 116:1351–1357
- De Gara L, De Pinto MC, Tommasi F (2003) The antioxidant system vis-à-vis reactive oxygen species during plant pathogen interaction. *Plant Physiol Biochem* 41:863–870
- Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Sci Limerick* 135:1–9
- Doila MYAA, Datta KS, Angrish R, Dayal J, Kumari P, Kumar B (1998a) Mineral ion uptake characteristics during boron toxicity under saline conditions in broad bean (*Vicia faba* L.). *Plant Physiol Biochem* 25:1–8
- Doila MYAA, Kumar B, Dayal J, Angrish R, Datta KS (1998b) Effect of salinity and boron on germination and early seedling growth of broad bean *Vicia faba* L. *Haryana Agric Univ J Res* 28:63–72
- Elstner EF, Obwald WF (1994) Mechanism of oxygen activation in plant stress. *Proc R Acad Sci Edinburgh* 102b:131–154
- Evans WR, Fuchsman DE, Calver HE, Pyati PV, Alter GM, Subha Rao NS (1999) Bacteriochlorophyll and photosynthetic reaction centres in Rhizobium strain BTA II. *Appl Environ Microbiol* 56:3445–3449
- Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol Plant* 119:355–364
- Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866–1875
- Foyer CH, Lopen-Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide and glutathione associated mechanisms of acclimatory stress tolerance and signaling. *Physiol Plant* 100:241–254
- Geisler M, Kleczkowski LA, Karpinski S (2006) A universal algorithm for genome-wide *in silico* identification of biologically significant gene promoter putative cis-regulatory-elements; identification of new elements for reactive oxygen species and sucrose signaling in *Arabidopsis*. *Plant J* 45:384–398
- Goldberg DM, Spooner RJ (1983) Glutathione reductase. In: Bergmeyer HU, Bergmeyer J, Grabi M (eds) *Methods of enzymatic analysis*, vol III. pp 258–265
- Gorham G, Mc Donnell E, Wyn Jones RG (1984) Salt tolerance in triticeae. I. *Leymus sabulosus*. *J Exp Bot* 35:1200–1209
- Grill D, Tausz M, De Kok LJ (2001) Significance of glutathione to plant adaptation to the environment. Kluwer Academic Publishers, Dordrecht, ISBN 1-4020-0178-9
- Gueta-Dahan Y, Yanin Z, Zilinskas BA, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. *Planta* 203:460–469
- Gupta UC, James YM, Campbell CA, Leyshon AJ, Nicholaichuk W (1985) Boron toxicity and deficiency: a review. *Can J Soil Sci* 65:381–409
- Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. Clarendon Press, Oxford, pp 446–489
- Hasegawa PM, Bressan RA, Zhu JK, Bohnert H (2000) Plant cellular and molecular responses to high salinity. *Ann Rev Plant Mol Biol* 51:463–469
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125:189–198
- Hernandez JA, Jimenez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. *Plant Cell Environ* 23:853–862
- Hernandez JA, Ferrer MA, Jimenez A, Barcelo AR, Sevilla F (2001) Antioxidant systems and O_2^-/H_2O_2 production in the apoplast of pea leaves, its relation with salt induced necrotic lesions in minor veins. *Plant Physiol* 127:817–831
- Hung SH, Yu CW, Lin CH (2005) Hydrogen peroxide function as a stress signal in plants. *Bot Bull Acad Sin* 46:1–10
- Jiang Y, Huang B (2001) Drought and heat stress injury to two cool season turfgrasses in relation to antioxidant metabolisms and lipid peroxidation. *Crop Sci* 41:436–442
- Karpinska B, Wingsle G, Karpinski S (2000) Antagonistic effects of hydrogen peroxide and glutathione on acclimation to excess excitation energy in *Arabidopsis*. *IUBMB Life* 50:21–26
- Karpinski S, Reynolds H, Karpinska V, Wingsle G, Greissen G, Mullineaux P (1999) The role of hydrogen peroxide and antioxidants in systemic acclimation to photo-oxidative stress in *Arabidopsis*. In: *Text book-Plant Responses to Environmental Stresses*, pp 25–32
- Keren R (1990) Reclamation of saline, sodic and boron-affected soils. In: Tanji KK (ed) *Agricultural salinity assessment and management*. American Society of Civil Engineers, New York, pp 410–431
- Komives T, Gullner G, Kiraly Z (1998) Role of glutathione and glutathione-related enzymes in response of plants to environmental stress. *Ann NY Acad Sci* 851:251–258
- Kuiper PJC (1980) Functioning of plant cell membranes under saline conditions: membrane lipid composition and ATPases. In: Staples RC, Toenniessen GH (eds) *Salinity tolerance in plants—strategies for crop improvement*. John Wiley Co. New York, pp. 77–91.
- Lauchli A (2002) Functions of boron in higher plants: recent advances and open questions. *Plant Biol* 4:190–192
- Lee SKD (2006) Hot pepper response to interactive effects of salinity and boron. *Plant Soil Environ* 52(5):227–233

- Leopold AC, Willing RP (1980) Evidence for toxicity effects of salt on membranes. In: Staples RC, Toenniessen M (eds) Salinity tolerance in plants—strategies for crop improvement. John Wiley Co. New York, pp. 67–76
- Levine A (1999) Oxidative stress as a regulator of environmental responses in plants. In: Lerner HR (ed) Plant responses to environmental stresses from phytohormones to genome reorganization. Marcel Dekker, Inc., New York, Basel
- Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitivedisease resistance. *Annu Rev Plant Physiol* 121:245–257
- Manchanda HR, Sharma SK (1991) Boron tolerance in wheat in relation to soil salinity. *J Agric Sci* 116:17–21
- Manchanda HR, Karwasra SP, Sharma HC (1993) Saline water, soil salinity and crop growth. In: Salinity Research in HAU. CCS Haryana Agricultural University, Hisar
- Martínez-Ballesta MDC, Bastías E, Carvajal M (2008) Combined effect of boron and salinity on water transport: the role of aquaporins. *Plant Signal Behav* 3(10):844–845
- May MJ, Vernoux T, Leaver C, Montagu MV, Inze D (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J Exp Bot* 49:649–667
- Mehta N, Bharti S (1983) Growth and metabolism of gram seedling under salt stress. *Indian J Plant Physiol* 26:322–325
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410
- Mittova V, Guy M, Volokita M (2004) Salinity up-regulates the anti-oxidative system in root mitochondria and peroxisomes of the wild salt tolerant tomato species *Lycopersicon pennelli*. *J Exp Bot* 55:1105–1113
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880
- Nandwal AS, Godara M, Kamboj DV, Kundu BS, Mann A, Kumar B, Sharma SK (2000) Nodule functioning in trifoliate and pentafo- liate mungbean genotypes as influenced by salinity. *Biol Plant* 43 (3):459–462
- Neill S, Desiken R, Clarke A, Hancock J (1999) H_2O_2 signaling in plant cells. In: Smallwood MF, Calvert CM, Bowlers DJ (eds) Text book. Plant responses to environmental stresses. pp 59–64
- Neill S, Desikan R, Hancock J (2002a) Hydrogen peroxide signalling. *Curr Opin Plant Biol* 5:388–395
- Neill S, Desikan R, Clarke A, Hurst RD, Hancock J (2002b) Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J Exp Bot* 53:1237–1247
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Ann Rev Plant Physiol Plant Mol Biol* 49:249–279
- Ogawa K, Iwabuchi M (2001) A mechanism for promoting the germination of *Zinia elegans* seeds by hydrogen peroxide. *Plant Cell Physiol* 42:286–291
- Pnueli L, Liang H, Rozenberg M, Mittler R (2003) Growth suppression altered stomatal responses and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (APX 1)—deficient *Arabidopsis* plants. *Plant J* 34:187–203
- Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and regulatory role of hydrogen peroxide. *Plant Cell* 6:65–74
- Rao KVGK, Agarwal MC, Singh OP, Oosterbaan RJ (1994) Reclamation and management of waterlogged saline soil. In: Rao KVGK (ed) Central Soil Salinity Research Institute, Karnal and CCS Haryana Agricultural University, Hisar, India
- Rao MV, Paliyath G, Ormrod DP (1996) Ultraviolet-B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol* 110:125–136
- Reid R (2007) Update on boron toxicity and tolerance in plants. In: Xu F, Goldbach HE, Brown PH, Bell RW, Fujiwara T, Hunt CD, Goldberg S, Shi L (eds) Advances in plant and animal boron nutrition. Springer, Dordrecht, The Netherlands, pp 83–90
- Rennenberg H (1982) Glutathione metabolism and possible biological roles in higher plants. *Phytochemistry* 21:2771–2781
- Sagi M, Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. *Plant Physiol* 141:336–340
- Sairam RK, Srivastava GC (2000) Induction of oxidative stress and antioxidant activity of hydrogen peroxide treatment in tolerant and susceptible wheat genotype. *Biol Plant* 43:381–386
- Sairam RK, Srivastava GC (2001) Water stress tolerance of wheat (*Triticum aestivum* L.): variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. *J Agric Crop Sci* 186:63–70
- Schopfer P (1966) Der Birfluss von Phytochrom auf die sationharen Kouzeutration en Von Ascorbin Saure und Lehydmas corbiusaurz beim Senkeinling (*Sinspio albg* L.). *Planta* 69:158–177
- Sheoran IS (1975) Effect of salinity on some important aspects of plant metabolism. Ph.D. Thesis, Haryana Agricultural University, Hisar, India
- Slesak I, Karpinska B, Surówka E, Misalski Z, Karpinski S (2003) Redox changes in the chloroplast and hydrogen peroxide are essential for regulation of C3-CAM transition and photooxidative stress responses in the facultative CAM plant *Mesembryanthemum crystallinum* L. *Plant Cell Physiol* 44:573–581
- Slesak I, Libik M, Karpinska B, Karpinski S, Misalsk Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta Biochim Pol* 54:39–50
- Sreenivasulu N, Grimm B, Wobus U, Weschke W (2000) Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). *Physiol Plant* 109:435–442
- Srivalli B, Chinnusamy V, Khanna-Chopra R (2003) Antioxidant defense in response to abiotic stresses in plants. *J Plant Biol* 30:121–139
- Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses; a delicate balance between signaling destruction. *Physiol Plant* 126:45–51
- USDA (1954) Diagonosis and improvement iof saline and alkali soils. Agriculture handbook no. 60. US Govt. Printing office, Washington DC
- Vaidyanathan H, Shivakumar P, Chakrabarty R, Thomas G (2003) Scavenging of reactive oxygen species in NaCl stressed rice (*Oryza sativa* L.)—differential response in salt tolerant and sensitive varieties. *Plant Sci* 165:1411–1418
- Venacker H, Carver TLW, Foyer CH (1998) Pathogen induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiol* 117:1103–1114
- Vranová E, Inzé D, Van Breusegem F (2002) Signal transduction during oxidative stress. *J Exp Bot* 53:1227–1236
- Yadav OP, Manchanda HR (1982) Effect of boron applications on mustard on a sierozem sandy soil. *J Indian Soc Soil Sci* 30:408–410
- Yu C-W, Murphy TM, Lin C-H (2003) Hydrogen peroxide-induced chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. *Funct Plant Biol* 30:955–963