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SHORT COMMUNICATION

PROTEIN PROFILE CHANGES UNDER SALT-BORON TOXICITY AND ITS REGULATION BY HYDROGEN PEROXIDE AND GLUTATHIONE IN PIGEONPEA (*CAJANUS CAJAN* L.)

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Two genotypes of pigeon pea (*Cajanus cajan* L. Millsp.), salt tolerant Manak, (H77-216) and salt sensitive ICPL 88039 were subjected to treatment solutions with distilled water (C), H_2O_2 (100 µM; H), Glutathione, GSH (500 µM; G), H_2O_2 (100 µM) + GSH (500 µM) superimposed with S_0B_0 (DW), S_0B_{10} (0 mM NaCl + 10 mM B as $Na_2B_4O_7.10H_2O$, S_{100} + B_0 (100 mM NaCl + 0 mM B) and $S_{100}B_{10}$ (100 mM NaCl + 10 mM B) at the seedling stage in a BOD incubator at 27 ± 1^{0}C in dark for 96 h. Proteins from coleoptiles and radicles were resolved by SDS-PAGE. In tolerant cultivar Manak two proteins of molecular weight 67.6 and 81.1 kDa disappeared in S_0B_{10} C, however 67.6 kDa reappeared with S_0B_{10} H and S_0B_{10} H +G treatments. Similarly two bands of 51.2 and 87.0 kDa disappeared due to S_{100} treatment but only 51.2 kDa reappeared with $S_{100}B_0$H and $S_{100}B_0$ H +G treatments. Likewise in radicle of Manak a 66.6 kDa protein disappeared with S_0B_{10} C but same protein reappeared with H_2O_2 , Glutathione, H_2O_2 + GSH treatments. Even $S_{100}B_{10}$ C treatment repressed 79.8 kDa protein in ICPL 88039 but got recovered with H_2O_2, Glutathione and H_2O_2 + GSH treatments. These observations accord credence to step-up regulation of protein synthetic machinery in pigeonpea by H_2O_2 and glutathione under salt-B toxicity conditions.

Key words: **Boron, glutathione, H_2O_2, pigeonpea, proteins, regulation, salt, toxicity**

Salinity and boron co-exist naturally in soil and ground waters of semi-arid and arid conditions (Tanji 1990). The toxic effects of salinity are invariably accentuated in the presence of B particularly in cereals and leguminous crops (Manchanda and Sharma 1991, Alpaslan and Gunes 2001, Ismail 2004, Lee 2006). While the mechanism of salinity effects on crop plants are well documented but that of salinity in conjuction with boron are not well understood (Wimmer *et al.* 2003). Both H_2O_2 and glutathione play an important role in salt stress signaling (Singha and Choudhary, 1990, Hernandez *et al.* 2001) and glutathione is a major biomolecule scavenging the reactive oxygen species via ascorbate-glutathione cycle (Foyer *et al.* 1997). 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). 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). Therefore, it has been considered worthwhile not only to investigate the toxic effects of B alone and in interaction with salinity, but also to study the extent of removal of their adverse

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effects on proteins through treatments with H_2O_2 and glutathione in two cultivars of pigeon pea (*Cajanus cajan*L.Millsp.) possessing a varying salt resistance.

Seeds of two genotypes of pigeon pea (*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 ± 1^{0}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 daily for 3 days continuously.

Thereafter, the partially germinated seedlings were transferred to petriplates 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 (C), S_0B_{10} (0 mM NaCl + 10 mM B as $Na_2B_4O_7.10H_2O$) S_{100} + B_0 (100 mM NaCl + 0 mM B) and $S_{100}B_{10}$ (100 mM NaCl + 10 mM B) so as to have a total of 16 treatments each with 4 replications of the two aforementioned pigeonpea cultivars in a BOD incubator at 27 ± 1^{0}C in dark for 96 h. Therefore, samplings to observe changes in isozymic activity in protein profile of both plumule and radicle were studied for different treatments using standard procedure for SDS-PAGE under non-reduced, non-denatured conditions at 4^{0}C (Laemmli 1970 and Bradford 1976 as detailed on similar lines by Bishnoi et al. 2006). Specific conditions were maintained for keeping native proteins intact.

Changes in protein profile with respect to control (S_0 B_0 C), Boron (S_0B_{10} C), salt ($S_{100}B_{10}$ C) and salt +B ($S_{100}B_{10}$ C) alone as well as with H_2O_2 , glutathione, H_2O_2 + glutathione were studied in plumule and radicle in Manak and ICPL 88039 genotypes of pigeonpea (Figs. 1,2,3 and 4). Results illustrated in Fig. 1 depict that in plumule of Manak a maximum number of 24 protein bands were resolved with the molecular weight having range between 14.0 to 93.3 kDa in all the treatments. As a result of B treatment S_0B_{10} C, 2 bands of molecular weight 81.1 and 67.6 kDa were found to disappear. Recovery of one such band of 67.6 kDa was observed with S_0B_{10} H, S_0B_{10} G and S_0B_{10} H+G treatments only. In the salt treatment ($S_{100}B_0$ C) 22 bands were recorded and here 2 bands of 87.0 kDa and 51.2 kDa molecular weight reappeared with $S_{100}B_0$ H, $S_{100}B_0$ H+G treatments. In $S_{100}B_0$ C treatment, 9 bands of molecular weight 93.3, 81.1, 79.8, 67.6, 62.3, 55.0, 53.7, 52.4, 51.2 kDa were found to be less expressed than the control S_0B_0 C. Further, in salt + B treatments of $S_{100}B_{10}$ C,

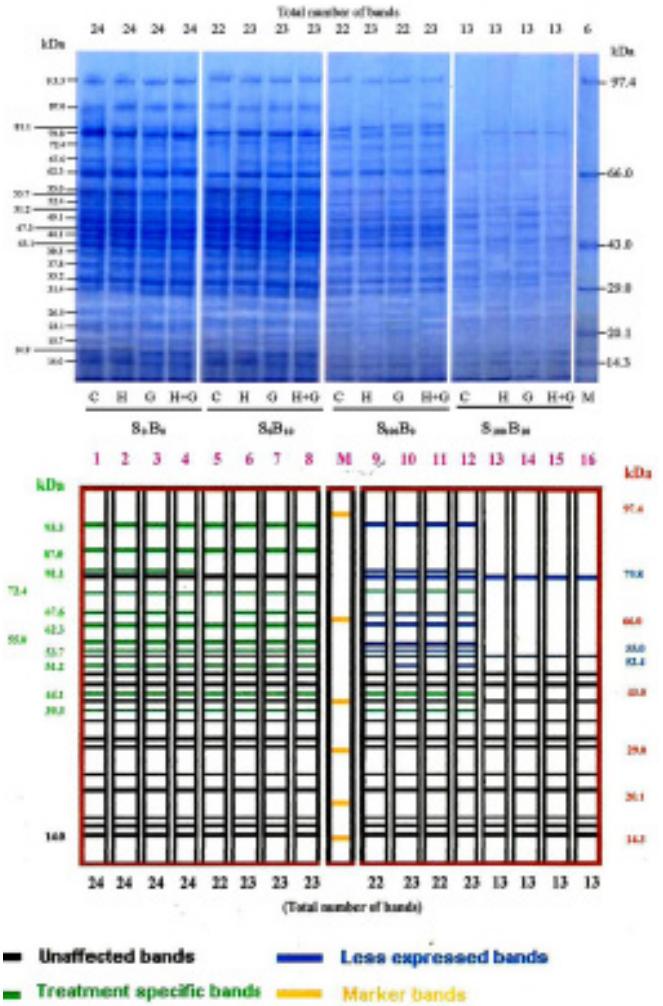

Fig. 1. Effect of H_2O_2 and glutathione on protein profile in plumule of genotype Manak of pigeonpea under boron-salt toxicity [SDS PAGE (10%) CBBR staining]

$S_{100}B_{10}H$, $S_{100}B_{10}G$ and $S_{100}B_{10}H+G$, only 13 bands of the total number of bands showed no recovery with H_2O_2 and glutathione, thus 11 bands of molecular weight 93.3, 87.0, 81.1, 72.4, 67.6, 62.3, 55.0, 53.7, 51.2, 44.1, 39.3 kDa disappeared in salt +B treatments. Two bands of molecular weight 79.8, 52.4 kDa showed less expression in salt +B treatments.

In plumule of ICPL 88039, a total of 20 bands were obtained with molecular weight ranging from 19.7 to 93.3 kDa in S_0B_0C, S_0B_0H, S_0B_0G and S_0B_0H+G treatment (Fig. 2). With the B treatment ($S_0B_{10}C$), 2 bands of 81.1 kDa and 67.6 kDa molecular weight disappeared. It is worthwhile to recall that such bands were also found to disappear in plumule of Manak. Likewise, here also 67.6 kDa band was recovered with $S_0B_{10}H$, $S_0B_{10}G$, $S_0B_{10}H+G$ treatment. In the salt treatments $S_{100}B_0C$ reduction in number of bands was not observed but out of 20 bands, 12 bands of molecular weight 93.3, 81.1, 79.8, 72.4, 67.6, 62.3, 55.0, 53.7, 52.4, 51.2, 22.3, 19.7 kDa showed reduced expression. It was worthwhile to note that with salt + B ($S_{100}B_{10}C$) treatment only 9 bands were resolved. However, with $S_{100}B_{10}H$, $S_{100}B_{10}G$ and $S_{100}B_{10}H+G$ treatments, one band of 79.8 kDa reappeared.

Fig. 3 shows the protein profile of radicle of Manak genotype. It is seen that a maximum number of 17 bands were detected with molecular weight ranging from 20.3 to 93.3 kDa. It was interesting to note that protein with

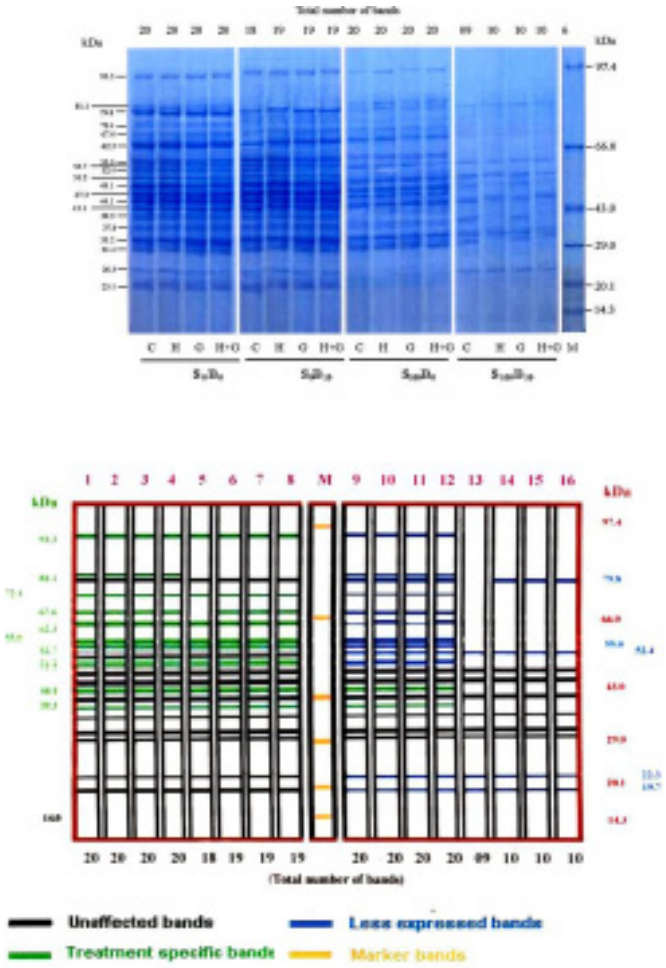

Fig. 2. Effect of H_2O_2 and glutathione on protein profile in plumule of genotype ICPL 88039 of pigeonpea under boron-salt toxicity [SDS PAGE (10%) CBBR staining]

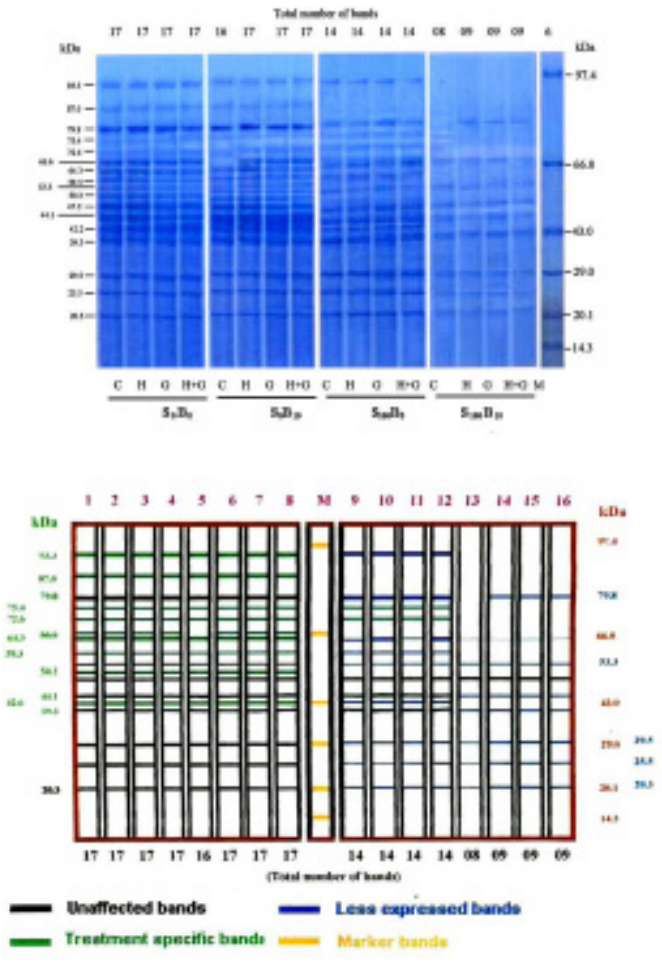

Fig. 3. Effect of H_2O_2 and glutathione on protein profile in radicle of genotype Manak of pigeonpea under boron-salt toxicity [SDS PAGE (10%) CBBR staining]

molecular weight 66.6. kDa disappeared with B treatment ($S_0 B_{10} C$), but same protein reappeared with $H_2 O_2$ ($S_0 B_{10} H$), glutathione ($S_0 B_{10} G$) and $H_2 O_2$ + Glutathione ($S_0 B_{10} H + G$) treatments. In the salt ($S_{100} B_{10} C$) treatment, only 14 bands were observed. Three bands of 87.0, 66.6, 50.2 kDa disappeared. Also 9 bands of molecular weight 93.3, 79.8, 64.3, 59.3, 53.3, 42.6, 29.5, 25.5 and 20.8 kDa showed less expression. Further, combination treatment of salt and B ($S_{100} B_{10} C$) only 8 bands of molecular weight 93.3, 87.0, 79.8, 75.4, 72.6, 66.6 59.3, 42.6 kDa bands were found to disappear. Out of these, a band of 79.8 kDa was observed with H_2O_2 ($S_{100} B_{10} H$), glutathione ($S_{100} B_{10} G$) and H_2O_2 + glutathione ($S_{100} B_{10} H + G$) treatments. The other 7 bands of molecular weight 64.3, 53.3, 44.1, 39.3, 29.5, 25.5, 20.3 kDa showed less expression.

The protein profile of radicle of the genotype ICPL 88039 as shown in Fig. 4 depicted the presence of a total of 15 bands with molecular weight ranging from 19.2 kDa to 93.3 kDa in ($S_0 B_0 C$) control treatment. With the further addition of H_2O_2 or glutathione i.e. $S_0 B_{10} H$, $S_0 B_{10} G$ and $S_0 B_{10} H + G$ treatments the same number of 15 bands were resolved. One band of 62.3 kDa molecular weight was found to be absent in salt treatments alone or in presence of H_2O_2 or glutathione. Five bands of 93.3, 79.8, 67.6, 59.0 and 53.7 kDa molecular weight showed less expression in all salt treatments. Salt +B treatments further showed only a total of 7 bands out of which 4 bands of 53.7, 30.2, 25.5, 19.2 kDa molecular weight were found to be less expressed. Again a total of 8 bands were observed with $S_{100} B_{10} H$, $S_{100} B_{10} G$ and $S_{100} B_{10} H$+G treatments with recovery of one band of 79.8 kDa but this also showed less expression.

Above results on the resolution of protein profile in plumule and radicle of Manak and ICPL 88039 brings forth some interesting points. At the outset, it has been seen that number of proteins resolved differed constitutively within the genotypes as well as in tissues. Thus, in plumule of Manak (Fig. 1) 24 bands were resolved as compared to only 20 bands in ICPL 88039 (Fig. 2). Again in radicle of Manak a maximum of 17 bands were resolved (Fig. 3), whereas, in ICPL 88039 (Fig. 4), only 15 bands were resolved. A large number of these bands were also expressed in all the stress (B, salt and salt +B) treatments and chemicals ($H_2 O_2$, glutathione and $H_2 O_2$ + glutathione) treatments as well. Rani *et al.* (2007) also reported that in wheat, maximum of 22 and 20 bands were resolved in coleoptile and radicle, respectively, of salt resistant cultivar KRL 1-4 and 19 and 24 bands in coleoptile and radicle, respectively, of sensitive HD 2329 cultivar in response to various salt, B and salt +B treatments. Grover *et al* (2001) attributed the constitutive expression of such proteins as "house keeping proteins" responsible for vital cellular functions of routine nature.

Another important feature was the disappearance of certain bands with B, salt and salt + B treatments. Thus,

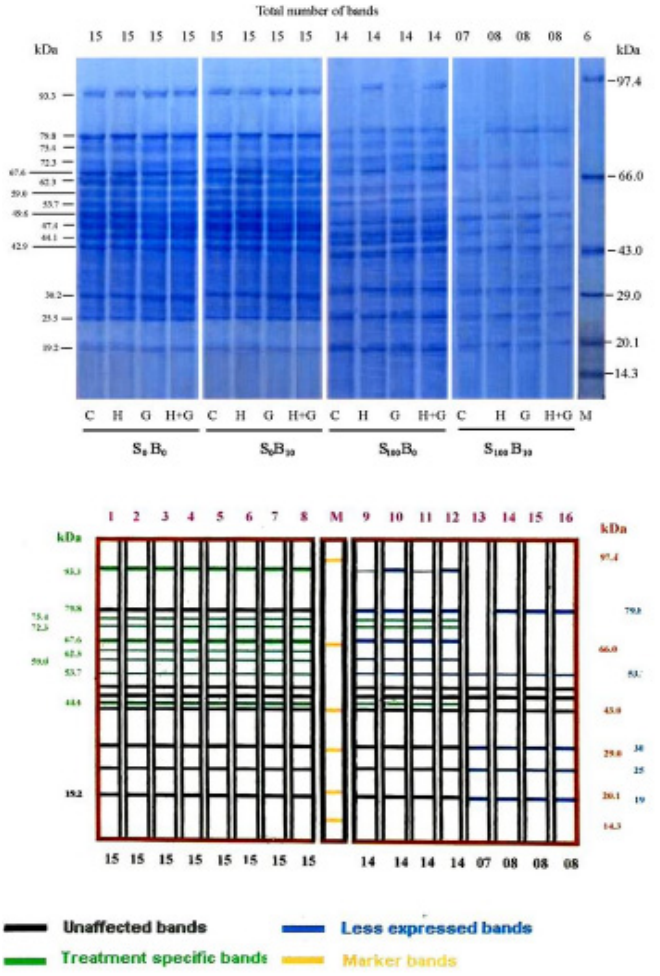

Fig. 4. Effect of H_2O_2 and glutathione on protein profile in radicle of genotype ICPL 88039 of pigeonpea under boron-salt toxicity [SDS PAGE (10%) CBBR staining]

as compared with the $S_0 B_0 C$ control to the maximum salt +B stress level, the number of bands were reduced from 24 to 13 (Fig .1) and from 17 to 8 (Fig. 3) in plumule and radicle of Manak, and 20 to 9 (Fig. 2) and 15 to 7 (Fig. 4) in plumule and radicle of ICPL 88039, respectively. Disappearance of the protein bands may be interpreted as the "tuning off" of protein synthetic genetic machinery (genes?) in response to salt and / or B treatments. It is more likely, however, that the "disappeared" proteins in response to stress are a result of the denaturation, depressed protein synthesis and acceleration in its degradation in plants in response to salt stress (Garg and Garg 1982, Pareek *et al.* 1997). Dell'Aquila and Spada (1993) reported that expression of "salt stress" proteins is related to the adaptation process of seeds to salinity as well as genetic contribution of the genotype. Grover *et al*. (1998, 2001) emphasized importance of proteins, which are up regulated in response to stress in relation to salt tolerance.

The most important feature of the present experimentation is the role of H_2O_2 / glutathione in the up-regulation of the protein synthetic machinery. For example, in the plumule of Manak (Fig. 1), as a result of B treatment ($S_0 B_{10} C$), two bands of molecular weight 67.6 and 81.1 kDa were found to disappear. However, 67.6 kDa band recovered with $S_0 B_{10} H$, $S_0 B_{10} G$ and $S_0 B_{10} H + G$ treatments. Likewise in salt treatment, $S_{100} B_0 C$, 2 bands of molecular weight of 51.2 and 87.0 kDa disappeared but only 51.2 kDa band reappeared with H_2O_2 i.e. $S_{100} B_0 H$, $S_{100} B_0 H+G$ treatments. Again, in plumule of ICPL 88039, a similar disappearance of bands 81.1 and 67.6 kDa with B or salt treatments and the reappearance of 67.6 kDa with H_2O_2, glutathione or H_2O_2 + glutathione treatments was noteworthy. Also, in radicle of Manak, a 66.6 kDa protein disappeared with the $S_0 B_{10} C$ treatment of B but the same protein reappeared when H_2O_2 - $S_0 B_{10} H$, glutathione- $S_0 B_{10} G$ or H_2O_2 + glutathione - $S_0 B_{10} H$ +G treatments were given. A band of 79.8 kDa which disappeared with $S_{100} B_{10} C$ reappeared in H_2O_2 ($S_{100} B_{10} H$), glutathione (($S_{100} B_{10} G$) and H_2O_2 + glutathione ($S_{100} B_{10} H + G$) treatments. In radicle of genotype ICPL 88039, a band of 79.8 kDa showed similar trend of disappearance and recovery (Fig. 4). The latter observations may become understandable in light of reports which advocated that the expression of stress proteins is related to the adaptation / acclimation process to salinity as well as constitutional contribution of the genotypes. Grover *et al.* (1998, 2001) highlighted the importance of the proteins which are up- regulated during stress tolerance. Bishnoi *et al.* (2006) reported partial alleviation of the inhibitory effects of B and salt with calcium and also reported that 75.8 kDa protein in plumule of Manak which was present in the water treated controls disappeared with treatments of salt and B. Wingate *et al*. (1987) reported that exogenous application of glutathione induced production of transcripts and proteins, which resembled those produced by biotic (fungal) stress elicitor. These authors opined that GSH has possible role in stress signaling.

Desikan *et al*. (1998 ab) reported the expression of biotic stress defense related genes induced by harpin in *Arabidopsis* suspension cultures. Such genes include PAL, encoding phenylalanine ammonia lyase, a key enzyme of phenyl propanoid metabolism and GST, encoding glutathione -S- transferase, required for detoxification of rapid hydro peroxides generated during oxidative stress as well as the g p ^{91}homologue. The expression of these genes can also be induced by H_2O_2. It may be therefore, concluded that as reported in our research step up-regulation of certain proteins repressed or disintegrated by salt and / or B stress through favourable regulation with H_2O_2, glutathione or H_2O_2 + glutathione treatments in terms of their reappearance, would be on the same line. This phenomenon is obviously correlated with pepped up ROS system actively by H_2O_2 and/or glutathione under stress tolerance (Foyer *et al.* 1997, Sairam & Srivastava, 2000, Hung *et al.* 2005).

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