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HEAT AND SALINITY INDUCED OXIDATIVE STRESS AND CHANGES IN PROTEIN PROFILE IN *AMARANTHUS LIVIDUS* L.

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SUMMARY

Treatment of *Amaranthus lividus* L. (a tropical leaf crop) seedlings separately with NaCl (50, 100 & 150 mM) and heat shock for different durations (45^{0}C for 4, 8 & 12 hours) during early germination induced oxidative stress and exhibited coinducibility of some stress proteins. Exposure of *Amaranthus* seeds to elevated temperature and NaCl salinity caused significant accumulation of reactive oxygen species such as superoxide radicals, hydrogen peroxide and TBARS contents with a reduction of membrane protein thiol level. Both forms of abiotic stress were related to significant reduction of antioxidative efficiency (viz. catalase, peroxidase, superoxide dismutase) and total thiol content. A comparative study of qualitative protein profiles of salinity stressed and heat shock raised seedlings by 8 to 15% gradient SDS-PAGE exhibited, expression of some similar polypeptides having molecular masses 90 and 110 kDa. Both forms of abiotic stress also showed coinducibility of some over expressed proteins. The results clearly suggest that heat and salinity imposed a similar kind of oxidative stress, which might induce the expression of common set of stress proteins.

***Key words*: Antioxidant enzymes, heat shock, oxidative stress, salinity, stress protein.**

INTRODUCTION

Many of the adverse effects of various environmental stresses are at least partially due to generation of ROS or oxidative stress (Bhattacharjee 2005, Shalata and Tal 1998, Bartoli *et al* 1999, Jiang and Zhang 2001). The overproduction of ROS ($O_2^{\bullet -}$, H_2O_2, $OH^{\cdot}$, $RCO^{\cdot}$ etc.) results from the exposure to the various environmental conditions like dehydration, heat, salinity etc., resulting into inactivation of enzymes and damage to membrane lipids etc. (Fadzillah *et al.* 1996, O'Kane *et al.* 1996, Bhattacharjee 2005), causing inhibition of growth and development (Navarri Izzo *et al*. 1994, Bhattacharjee and Mukherjee 2003). The present work was undertaken to evaluate separately the impact of heat shock and NaCl salinity on the induction of oxidative stress and antioxidative system during the germination of *Amaranthus lividus*. The qualitative expression of proteins under both the environmental stresses were also analysed (by gradient SDS-PAGE) to find out the coinducibility of proteins.

MATERIALS AND METHODS

Seeds of *Amaranthus lividus* L. were supplied by Sultan Seed Co., Kolkata. Seeds were washed in sterile water followed by treatment with two successive solutions of 0.1 g/dm^3 $HgCl_2$ for 5 minutes each and finally washed in sterile distilled water for 15 minutes. Seeds were then divided into different batches and kept

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at 45^0C in dark for 4, 8 and 12 hours. Seeds were then allowed to grow at 25^0C ± 2^0C under 12 hour photoperiod (irradiance level 270 mmol m^{-2} s^{-1}) and relative humidity 78 ± 2.2%. For the assessment of the parameters of oxidative stress and qualitative protein profiles, 72 hour old intact seedlings were used.

For imposing salinity stress, surface sterilized seeds were allowed to germinate in petriplates on filter paper soaked with different concentrations of NaCl, i.e. 50, 100 and 150 mM, pH 6.8 (corresponding EC was recorded as 0.69, 1.25 and 1.80 mS cm^{-1} respectively). The control experiment was done with distilled water. Seeds were grown in the same manner as described above. Five days (120 hours) old intact seedlings were used for the assessment of various parameters of oxidative stress and qualitative protein profile.

For the extraction and estimation of superoxide, the method of Chaitanya and Naithani (1994) was followed. The superoxide anion in the buffer extracted (0.2 M phosphate buffer, pH 7.2) was measured by its capacity to reduce nitrobluetetrazolium (2.5 x 10^{-4} M). The absorbance of the end product was measured at 540 nm. Superoxide formation was expressed as ΔA_{540} g^{-1} dry wt. min^{-1}. For the extraction and estimation of H_2O_2, the process of MacNevin and Uron (1953) using $Ti(SO_4)_3$ was used.

Activities of catalase, peroxidase and superoxide dismutase were assayed following the methods of Snell and Snell (1971), Kar and Mishra (1976) and Giannopolities and Ries (1977) respectively. The enzyme activities in all the cases was expressed as enzyme unit g^{-1} dry wt. min^{-1} (Fick and Qualset 1975).

To study membrane injury index the process of Bhattacharjee and Mukherjee (1998) was followed. Root and shoot tissues of seedlings (200 mg each) from each treatment were placed in vials containing in 15 cm^3 of deionised water and incubated at 25^0C for 24 hours. Electrical conductivity of the bathing medium was measured at 25^0C. The tissue with leachate was then autoclaved at 15 lb/cm^2 for 15 minutes and brought to 25^0C and EC was measured again. Membrane injury index was calculated as

M II = [1 - (T_1/T_2) / 1 – (C_1/C_2)] x 100, where C_1 and C_2 are Ecs of the untreated control sample before and after autoclaving and T_1 and T_2 are the Ecs of the heat stressed sample before and after autoclaving.

Oxidative stress index was evaluated in terms of relative H_2O_2 accumulation and relative membrane lipid peroxidation as

$$\text{Relative } H_2O_2 \text{ accumulation (\%)} = \frac{H_2O_2 \text{ accumulated under treatment}}{H_2O_2 \text{ accumulated in untreated control}} \times 100$$

$$\text{Relative membrane lipid peroxidation (\%)} = \frac{\text{Accumulation of TBARS under treatment}}{\text{Accumulation of TBARS under untreated control}} \times 100$$

Total thiol content was assayed in acid-soluble extracts (0.2 g fw ml^{-1}) as described by Tietze (1969). Thiol content was determined by measuring absorbance at 412 nm in presence of 0.5 mM 5,5/ dithiobis 2 - nitrobenzoic acid (DTNB), 0.5 U ml^{-1} glutathione reductase and 0.2 mM NADPH.

For the determination of membrane protein thiol content, the membrane protein was prepared following the procedure of Singh (1997). The membrane protein bound thiol groups were assayed after protein precipitation with 10% TCA and quantified by DTNB following the procedure of Ellman (1959) and Dekok and Kuiper (1986).

Soluble proteins for electrophoresis was extracted by the process of Bhattacharjee (2001) using extraction buffer [200 mM Tris-HCl, pH 7.5, 2% (w/v) SDS, 0.05% (w/v) β-mercaptoethanol, 2.5 mM p - hydromercuric - benzoate, 1 mM PMSF and one antifoam C emulsion]. The protein was analysed by 8 to 15% gradient SDS-PAGE using the procedure of Laemmli (1970). CBB-G250 staining of protein bands was done following the process of Bradford (1976).

RESULTS AND DISCUSSION

With the increase in duration of heat stress and magnitude of NaCl-salinity stress there was a general increase in the contents of ROS (superoxide and hydrogen peroxide) in 72 and 120 hours old juvenile intact *Amaranthus* seedlings (Table 1). The extent of

Table 1. Influence of elevated temperature and NaCl salinity on the formation of reactive oxygen species ($O_2^{\bullet-}$ and H_2O_2) and thiobarbituric acid reactive substances in *Amaranthus* intact seedlings (72 & 120 hours old). Values are mean of four replicates (± SE).

Treatment	Reactive oxygen species		
	O_2^- content (ΔA min^{-1} g^{-1} dry wt.)	H_2O_2 content (mmol g^{-1} dry wt.)	TBARS content (nmol g^{-1} dry wt.)
Untreated (72 hours old)	12.50(0.11)	91.00(0.20)	58.00(0.14)
Heat Shock			
45^0C for 4 hours	30.50(0.17)	151.50(0.28)	89.00(0.13)
45^0C for 8 hours	60.20(0.20)	186.50(0.28)	102.00(0.12)
45^0C for 12 hours	95.50(0.20)	223.00(0.17)	147.00(0.17)
Untreated (120 hours old)	9.80(0.17)	88.50(0.23)	71.00(0.11)
NaCl salinity			
50 mM	10.30(0.13)	86.90(0.27)	120.00(0.23)
100 mM	26.90(0.20)	144.00(0.34)	132.00(0.14)
150 mM	46.50(0.33)	197.00(0.47)	186.00(0.22)

accumulation of $O_2^{\bullet-}$ was more than H_2O_2, especially in case of heat shock raised seedlings. Assessments of one of the products of lipid peroxidation, TBARS, revealed its augmentation under heat and NaCl-salinity stress (Table 1). Assessment of antioxidative enzymes SOD, CAT and POX under both heat shock and salinity stress showed a gradual decline with increasing duration (heat shock) and magnitude (NaCl-salinity) of stress (Table 2). Water soluble antioxidants and total thiol content also exhibited significant decline under the influence of both the stresses (Table 2). Estimation of membrane protein thiol level further showed vulnerability of newly assembled membrane systems to oxidative damage due to stress (Table 2). Further, oxidative stress index [measured in terms of relative H_2O_2 accumulation (%) and relative lipid peroxidation (%)] showed significant increase due to heat shock and salinity stress (Table 3).

The results presented here clearly indicate that *Amaranthus lividus*, when subjected to heat shock and salinity stress during early phases of germination, encountered a significant oxidative stress, assessed in terms of accumulation of $O_2^{\bullet-}$, H_2O_2, TBARS and reduced efficiency of both enzymatic (CAT, POX and SOD) and non-enzymatic (total thiol content) antioxidative systems. Accumulation of $O_2^{\bullet-}$ and H_2O_2 induced similar kind of oxidative stress in both heat shock and salinity stressed seedlings that increased the degradation of membrane lipid via peroxidation and also increases vulnerability of oxidation of membrane protein bound thiol groups (Fadzillah *et al.* 1996, O'Kane *et al.* 1996, Shalata and Tal 1998, Bhattacharjee and Mukherjee 2003/2004). Thus toxicity caused by excess generation of ROS ($O_2^{\bullet-}$ and H_2O_2) both under imbibitional heat shock and salinity stress aggravate membrane lipid peroxidation and oxidation of membrane protein bound thiol groups which ultimately causes membrane injuries (as revealed from the data of membrane injury index). This is in agreement with the results of Jiang and Zhang (2001), Bartoli *et al.* (1999), Bhattacharjee and Mukherjee (2003/2004). However, transient increase in H_2O_2 content under heat shock and salinity stress might induce a signal transduction mechanism of acclimation, so the significance of accumulation of H_2O_2 may not be simply an injury symptom (Smirnoff 1998, Neill *et al.* 1999).

SDS-PAGE analysis of total extractable soluble proteins from the intact heat shock and salinity stressed *Amaranthus* seedlings (Fig.1 and 2) revealed coinducibility of polypeptide bands of 110 and 90 kDa. Over expression of polypeptide band of 125 kDa were

Table 2. Influence of elevated temperature and NaCl salinity on the activities of antioxidative enzymes (superoxide dismutase, catalase and peroxidase), total thiol content and membrane protein thiol level (MPTL) in intact *Amaranthus* seedlings (72 hours and 120 hours old). Values are mean of four replicates (± SE).

Treatment	Antioxidative enzyme (U min^{-1} g^{-1} dry wt.)			Total thiol content (µmol g^{-1} dry wt.)	MPTL (nmol g^{-1} dry wt.)
	SOD	CAT	POX		
Untreated (72 hours old)	5.53(0.04)	3.92(0.03)	8.55(0.02)	1.31(0.02)	137.40(0.41)
Heat Shock					
45^{0}C for 4 hours	4.45(0.05)	3.27(0.03)	8.50(0.06)	1.28(0.02)	118.25(0.30)
45^{0}C for 8 hours	3.48(0.02)	3.00(0.01)	7.55(0.03)	0.97(.004)	70.40(0.18)
45^{0}C for 12 hours	3.42(0.07)	2.72(0.02)	5.78(0.02)	0.81(0.03)	39.00(0.16)
Untreated (120 hours old)	5.50(0.03)	4.22(0.02)	8.80(0.04)	1.57(0.07)	134.00(0.30)
NaCl salinity					
50 mM	5.66(0.08)	4.41(0.01)	9.66(0.02)	1.40(0.03)	131.50(0.38)
100 mM	4.85(0.06)	3.11(0.02)	6.38(0.04)	1.33(0.01)	79.80(0.04)
150 mM	3.77(0.03)	1.91(0.01)	4.88(0.04)	1.02(0.07)	46.61(0.21)

Table 3. Effect of elevated temperature and NaCl-salinity on oxidative stress index (in terms of relative accumulation of H_2O_2 & relative membrane lipid peroxidation), membrane injury index and expression of inducible proteins and over-expressed proteins in intact *Amaranthus* seedlings (72 & 120 hours old). Values are mean of four replicates (± SE).

Treatment	Oxidative stress index			Mol. wt. (kDa) of newly appeared proteins	Mol. wt. of over-expressed proteins
	Relative H_2O_2 accumulation (%)	Relative MLP (%)	M\II (%)		
Heat Shock					
45^{0}C for 8 hours	204.94	175.80	28.40	197, 193, 190, 170, 138, 130, 110, 90	125, 119, 70-68, 54-52
45^{0}C for 12 hours	245.05	253.00	39.60	same as above	same as above
NaCl salinity					
100 mM	162.70	185.90	34.40	125, 110, 90	160, 138-136, 125, 120, 106, 112, 96-92, 76
150 mM	222.50	261.90	58.20	same as above	same as above

also noticed under both heat and salinity stress. Abundant evidences, however, have shown that although oxidative stress is a lethal situation for cell by ROS (especially H_2O_2 and $O_2^{\cdot-}$), it may be involved in cellular signalling procedure as second messenger to induce a large number of genes and produce proteins involved in stress defenses (Rao *et al.* 1997, Dat *et al.* 1998, Guan *et al.* 2000, Jiang and Zhang 2001, Bhattacharjee, 2005). Our

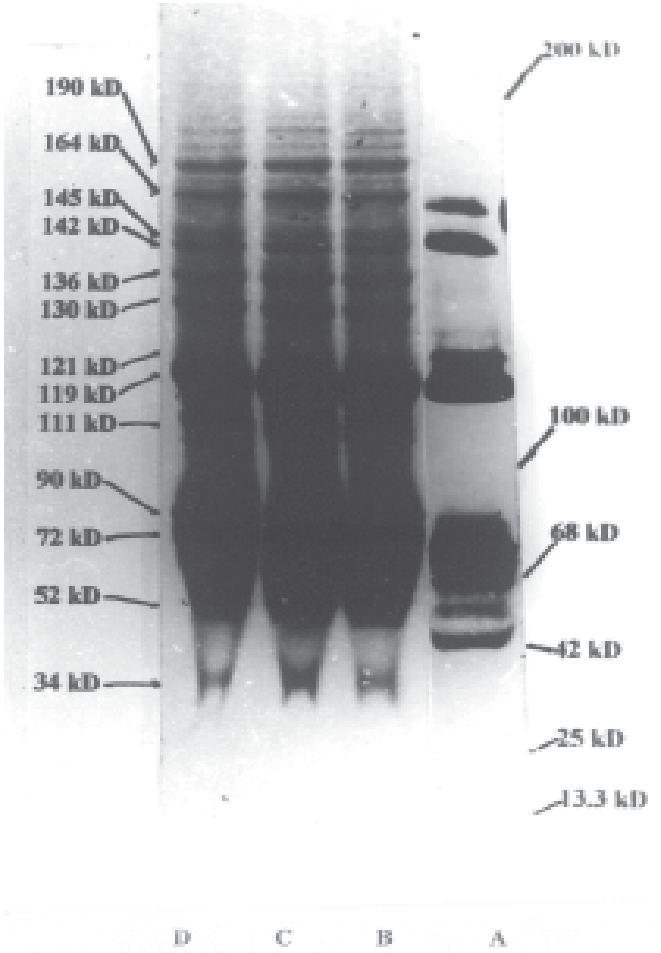

Fig. 1. SDS-PAGE (8-15%) of protein samples from untreated and heat shock raised *Amaranthus* seedlings (72 hours old). Mol. wt. (kDa) of marker proteins are on the right side and of the polypeptides that deviated from untreated control are on the left side. 20 µg of protein sample was loaded in each lane. Lane A - untreated, lane B - imbibitionally heat shocked at 45^{0}C for 4 hours, lane C - imbibitionally heat shocked at 45^{0}C for 8 hours, lane D - imbibitionally heat shocked at 45^{0}C for 12 hours

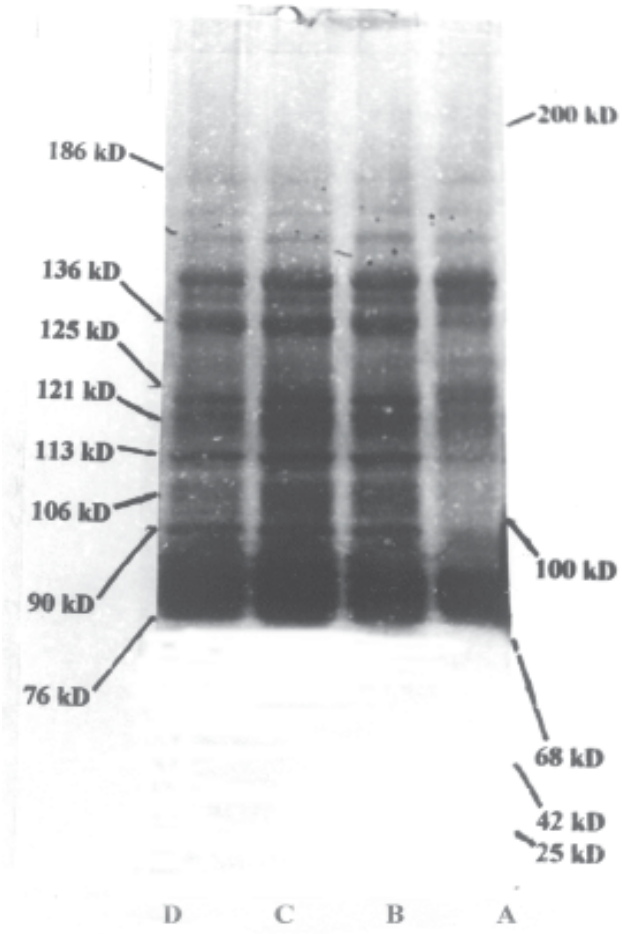

Fig. 2. SDS-PAGE (8-15%) of protein samples from untreated and NaCl-salinity stressed *Amaranthus* seedlings (120 hours old). Mol. wt. (kDa) of marker proteins are on the right side and of the polypeptides that deviated in regard to untreated seedlings are on the left side. 20 µg of protein sample was loaded in each lane. Lane A - untreated, lane B - seedlings treated with 50 mM NaCl, lane C - seedlings treated with 100 mM NaCl, lane D - seedlings treated with 150 mM NaCl

result showed that a significant increase in the levels of $O_2^{\bullet-}$ and H_2O_2 occurred during early germination under the influence of heat and salinity stress might cause the expression of some important stress proteins having molecular mass 90 and 110 kDa and also caused over-expression of 125 kDa proteins.

So, abiotic stresses like heat and salinity during early germination may result in the induction of oxidative stress in germinating tissues which increases the vulnerability of newly assembled membrane systems to oxidative damage. However, the imposed oxidative stress might have dual effects both as toxins and triggers. Although it is not clear from the experiments the exact concentration range of ROS as triggers or if there exists other mediators of coinducibility of stress proteins. However, it is clear that significant increase in the levels of ROS induced by different stress factors may be necessary for the induction of stress proteins and survival of the plants exposed to environmental stress (Jiang and

Zhang 2001, Karpinski *et al.* 1999, Bhattacharjee, 2005). So, we have some preliminary evidence correlating the fact that different abiotic stresses (heat and salinity) may increase the production of ROS or impose oxidative stress that may trigger coinducibility of stress proteins required for cross tolerance.

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