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Triadimefon pretreatment protects newly assembled membrane system and causes up-regulation of stress proteins in salinity stressed *Amaranthus lividus* L. during early germination

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Abstract: *Imposition of salinity stress during early germination imposes a secondary oxidative stress in 120 hr old Amaranthus lividus seedlings (measured in terms of accumulation of reactive oxygen species, antioxidative defense system and oxidative membrane lipid and protein damages). Seeds of Amaranthus when treated with triadimefon along with NaCl salinity, significantly enhanced the activities of catalase, peroxidase and superoxide dismutase, compared to untreated salinity stressed 5 day old seedlings. Triadimefon treatment also reduced the accumulation of both the ROS (H_2O_2 and O_2^-) in 5 day old Amaranthus seedlings. When oxidative membrane damages were estimated for triadimefon treated and salinity stressed juvenile seedlings and compared with untreated salinity stressed seedlings, it shows a clear reversal in oxidative membrane damages induced by triadimefon under salinity stress. Triadimefon treatment significantly reduces the membrane lipid peroxidation and the loss of membrane protein thiol level in salinity stressed Amaranthus seedlings. That triadimefon treatment under salinity stress restores the membrane integrity and improves the post- germinative seedling growth could be supported by the data of membrane injury index (MII), relative leakage ratio (RLR), membrane permeability status (MPS), relative growth index (RGI) and mean tolerance index (MTI). SDS–PAGE of total extractible proteins revealed that some new proteins were synthesized in triadimefon treated and salinity stressed seedlings as compared to untreated and salinity stressed one. However, the most remarkable feature is the up-regulation of some of the stress proteins in triadimefon treated and salinity stressed seedlings. So, it appears that significant extent of salinity tolerance exhibited by triadimefon pretreated Amaranthus seedlings could be related to the mitigation of oxidative damage to the newly assembled membrane system of juvenile tissues as well as synthesis and up-regulation of stress proteins that enhanced salinity tolerance.*

Key words: *Salinity, Triadimefon, Oxidative stress, Amaranthus, Membrane protein thiol, Reactive oxygen species, Stress proteins*
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Introduction

Salt stress is one of the major environmental constraints limiting agricultural productivity (Boyer, 1982). Soil salinity caused by NaCl is harmful particularly at early stages of germination, which is concentration dependent and also species specific (Levitt, 1980; Karikalan *et al*., 1999; Lakra *et al.,* 2006; Chukwu and Okpe, 2006). Crop production in saline areas largely depends upon successful germination, seedling emergence and also on post germinative development or seedling establishment.

Amaranthus lividus is one of the tropical leafy vegetable crop, acquiring increasing importance as a potential subsidiary food crop for its excellent quality of protein and micronutrients (Devdas and Saroja, 2001; Prakash and Zaidi, 2000). Presently it is also cultivated in semiarid regions, where problem of salinity is acute.

Circumstantial evidence indicates that adverse effect of salinity particularly during early germination are at least due to generation or overproduction of reactive oxygen species (ROS) or imposed oxidative stress (Bhattacharjee and Mukherjee, 2002; Jiang and Jhang, 2001). ROS can directly attack membrane lipids and enzymes (Bartoli *et al*., 1999; Navarri-Izzo *et al*., 1994) that results in the development of various injury symptoms (Fadzillah *et al*., 1996; O'kane *et al*., 1996).

Maintenance of composition and structure especially during early germination is absolutely essential for continued functions of membranes during early development that leads to seedling establishment. Indeed membranes are one of the primary targets that undergo damage under abiotic stresses including salinity (Bhattacharjee, 2005; Fletcher *et al*., 2000; Bhattacharjee and Mukherjee, 2002; Gundogdu and Alsan, 2007).

Triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-1H(1,2,4-triazol-1-Y1)-2 butanone] which is used as a fungicide belong to triazole group of compounds and has been demonstrated as "plant multiprotectant" from their ability to protect plants against various environmental stresses (Fletcher *et al*., 2000; Pinhero *et al*., 1997, 1999). The protective effects of the group against various environmental stresses are often explained in terms of shifting of balances of important plant growth regulators like gibberellic acids, abscisic acids, cytokinins *etc*. (Fletcher *et al*., 2000; Fletcher and Hofstra, 1985) and also in the biochemical adjustments (Mackey *et al*., 1990). The effect of triazole compounds on detoxification of ROS is also being shown by some authors (Upadhyaya *et al*., 1989; Paliyath and Fletcher, 1995a; Pinhero *et al*., 1999; Fletcher *et al*., 2000). But the exact mechanism of triadimefon mediated ROS detoxification processes under salinity stress especially in relation to post-germinative development and seedling establishment is very

few and deserves special attention. In the present paper an effort has been made to determine the effect of triadimefon on the mechanistic aspect of mitigation of oxidative injury under salinity stress in *Amaranthus* during early germination. An additional effort is also being made to assess the impact of triadimefon treatment on qualitative changes in protein profile under salinity stress.

Materials and Methods

Seeds of *Amaranthus lividus* L. used in the present investigation were supplied by local harvest. Seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min. (Bhattacharjee and Mukherjee, 1998) and rinsed in running water thoroughly and were allowed to germinate separately in petri plates on filter paper soaked with following solutions:

i) Distilled water (control)
ii) Triadimefon (50 mg l^{-1})
iii) 100 mM NaCl (EC 1.26 mS)
iv) 100 mM NaCl + Triadimefon (37.5 mg l^{-1})
v) 100 mM NaCl + Triadimefon (50 mg l^{-1})

The seeds were allowed to grow at 25°C ± 2 °C with 12 hr photoperiod (270 $Em^{-2}s^{-1}$) and 78 ± 2 % RH. Seedlings were taken out at the age of 5 days rinse thoroughly with distilled water and used for various analyses.

Growth performances of 120 hr old seedlings were estimated in terms of biomass accumulation, relative growth index, seedling length, mean tolerance index and relative water content. Relative growth index, mean tolerance index and relative water content were calculated following the procedure of Bhattacharjee (2006).

For all biochemical estimations, *e.g.* assessment of oxidative stress, membrane damage of root and shoot tissues and stress protein induction, 120 hr old seedlings were used. Membrane lipid peroxidation was estimated in terms of malondialdehyde (MDA) accumulation by thiobarbituric acid (TBA) test using the procedure of Heath and Packer (1968). For the study of membrane injury index and relative leakage ratio of UV-absorbing tissue leachate, the methods of Bhattacharjee and Mukherjee (1998) were followed.

For the extraction and estimation of lipoxygenase activity the method of Peterman and Siedow (1985) was used. Hydrogen peroxide was extracted and estimated by MacNevin and Uron (1953) using $Ti_2(SO_4)_3$. For the determination of superoxide, the method of Chaitanya and Naithani (1994) was followed with some necessary modifications by the present authors.

For the extractions and estimations of catalase and peroxidase activities the methods of Snell and Snell (1971) and Kar and Mishra (1976) were used and for SOD, the method of Giannopolities and Ries (1977) was followed. The enzyme activity was expressed according to Fick and Qualset (1975) and activities in all cases were expressed as enzyme unit min^{-1} g^{-1} dry matter (d.m.) of tissues.

For determination of membrane protein thiol level, the membrane was prepared according to Singh (1997) with some necessary modifications. One gram of plant tissue was homogenised in 10 ml ice-cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at 10,000 ×g at 4°C for 30 min and pellet was discarded. The membranes were sedimented at 100,000 ×g at 4°C for 3 hr and the pellet containing membrane fractions was suspended in ice-cold buffer (0.05M Tris-HCl, pH 7.0). The membrane fractions were stored under ice-cold conditions. The membrane associated protein bound thiol groups were assayed after protein precipitation with TCA (10% w/v) and quantified with DTNB following the procedures of Ellman (1959) and Dekok and Kuiper (1986).

Proteins from salinity stressed (50, 100 and 150 mM NaCl) and salinity stressed-triadimefon treated seedlings were analyzed by 8-15% SDS-PAGE (Bhattacharjee, 2006). For electrophoretic analysis, seedling proteins were extracted in extraction buffer [200 mM Tris-HCl, pH 7.5, 2% (w/v) SDS, 0.05% (w/v) β-mercaptoethanol, 2.5 mM p-hydro mercuricbenzoate, 1mM PMSF and one of antifoam C emulsion]. Coarse debris were removed through centrifugation at 3000 ×g for 15 min. Five volumes of cold acetone were added to the decanted supernatant and the mixture was stored overnight at –20°C. Resulting precipitate was collected by centrifugation (12000 ×g for 10 min). Pellet was washed twice with cold acetone and protein content was determined by Bradford's dye binding method (Bradford, 1976). Proteins were then analysed using Lemmli (1970) one-dimensional 8-15% gradient SDS-PAGE. After electrophoresis the proteins were stained following the procedure of Paliynth and Fletcher (1995a).

Results and Discussion

Salinity during early germination elevated the level of ROS (Table 1 and Fig. 2) with concomitant reduction in the activities of free radical scavenging enzymes (catalase, peroxidase and superoxide dismutase) in 120 hr old *Amaranthus* seedlings (Table 1), hinting at imposition of a secondary oxidative stress. The imposition of secondary oxidative stress by NaCl salinity during early germination could be further substantiated from the data of enzymatic membrane lipid peroxidation (showing accumulation of thiobarbituric acid reactive substances and elevated lipoxygenase activity, Table 2) and reduced membrane protein thiol level in seedlings (Fig. 1).

Triadimefon treatment (37.5 and 50 mgl^{-1}) in salinity imposed (100 mM NaCl) *Amaranthus* seedlings enhanced the activities of free radical scavenging enzymes (Table 1). Seedlings derived from

Table - 1: Effect of triadimefon on NaCl-salinity (100 mM) induced changes in activities of free radical scavengers (peroxidase, POD, catalase, CAT, superoxide dismutase, SOD) and H_2O_2 content in *A. lividus* seedlings (120 hr old). Values are mean of three replicates (± SE)

Treatments	Free radical scavengers (U g^{-1} d.m. min^{-1})						H_2O_2 (µ mol g^{-1} d.m.)	
	POD		CAT		SOD			
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated	9.8 (0.11)	7.8 (0.01)	4.5 (0.01)	3.9 (0.02)	5.9 (0.07)	5.1 (0.02)	82.5 (0.41)	94.6 (0.14)
Triadimefon (50 mg l^{-1})	7.9 (0.11)	7.0 (0.08)	4.5 (0.01)	3.9 (0.01)	5.9 (0.02)	5.2 (0.03)	81.4 (0.31)	92.2 (0.1)
NaCl	6.1 (0.08)	6.6 (0.06)	3.2 (0.01)	3.0 (0.02)	5.0 (0.01)	4.7 (0.04)	146.2 (0.61)	141.7 (0.41)
NaCl + Triadimefon (37.5 mg l^{-1})	6.5 (0.09)	7.0 (0.01)	3.9 (0.01)	3.2 (0.01)	5.1 (0.02)	4.9 (0.01)	131.6 (0.21)	130.0 (0.31)
NaCl + Triadimefon (50 mg l^{-1})	6.7 (0.03)	6.9 (0.02)	4.0 (0.02)	3.7 (0.01)	5.5 (0.01)	4.9 (0.02)	128.5 (0.21)	127.5 (0.21)

d.m. = Dry mass

Table - 2: Effect of triadimefon on NaCl-salinity (100 mM) induced changes in membrane permeability status, membrane lipid peroxidation (in terms of malondialdehyde, MDA) and activity of lipoxygenase (LOX) in *A. lividus* seedlings (120 hr old). Values are mean of three replicates (± SE)

Treatments	Membrane permeability status						MDA (n mol g^{-1} d.m.)		LOX (U g^{-1} d.m. min^{-1})	
	Injury index (%)		RLR (A_{280} /A'_{280})		Electrical conductivity (mS cm^{-1} g^{-1} d.m.)					
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated	-	-	0.15 (0.01)	0.14 (0.01)	1.71 (0.09)	1.3 (0.01)	113 (0.31)	97 (0.18)	1.72 (0.01)	2.11 (0.02)
Triadimefon (50 mg l^{-1})	-	-	0.15 (0.01)	0.14 (0.01)	1.74 (0.09)	1.3 (0.02)	112 (0.21)	97 (0.21)	1.72 (0.01)	2.11 (0.02)
NaCl	51.4 (0.1)	44.6 (0.16)	0.44 (0.01)	0.32 (0.02)	16.17 (0.1)	15.0 (0.08)	163 (0.23)	129 (0.61)	3.76 (0.02)	3.95 (0.04)
NaCl + Triadimefon (37.5 mg l^{-1})	44.4 (0.12)	41.2 (0.12)	0.40 (0.01)	0.30 (0.01)	14.40 (0.09)	12.8 (0.07)	157 (0.2)	123 (0.24)	3.58 (0.01)	3.9 (0.04)
NaCl + Triadimefon (50 mg l^{-1})	42.1 (0.12)	41.6 (0.17)	0.40 (0.01)	0.29 (0.01)	13.44 (0.08)	12.1 (0.1)	156 (0.41)	121 (0.22)	3.47 (0.01)	3.7 (0.01)

RLR = Relative leakage ratio, d.m. = Dry mass

Table - 3: Effect of triadimefon (37.5 mg l^{-1} and 50 mg l^{-1}) on NaCl-salinity (100 mM) induced germination behavior and growth status (in terms of seedling biomass, length, relative growth index (RGI), mean tolerance index (MTI) and relative water content (RWC) in *A. lividus* seedlings (120 hr old). Values are mean of three replicates (± SE)

Treatments	Percent germination	Growth status					
		Seedling growth (mm)		Seedling biomass (mg)			
		Root	Shoot		RGI (%)	MTI (%)	RWC (%)
Untreated	90.0 (0.3)	26.1 (0.17)	27.8 (0.18)	7.0 (0.02)	--	--	--
Triadimefon (50 mg l^{-1})	88.0 (0.28)	28.0 (0.12)	27.0 (0.16)	7.2 (0.02)	99.6 (0.22)	99.6 (0.31)	91.3 (0.31)
NaCl	52.0 (0.17)	8.6 (0.1)	10.9 (0.1)	3.0 (0.01)	42.8 (0.13)	32.9 (0.17)	66.4 (0.21)
NaCl + Triadimefon (37.5 mg l^{-1})	54.0 (0.12)	11.5 (0.12)	11.0 (0.08)	3.3 (0.01)	47.2 (0.17)	44.1 (0.2)	70.0 (0.3)
NaCl + Triadimefon (50 mg l^{-1})	54.0 (0.1)	11.9 (0.09)	11.3 (0.1)	3.4 (0.01)	48.3 (0.12)	45.6 (0.22)	70.8 (0.22)

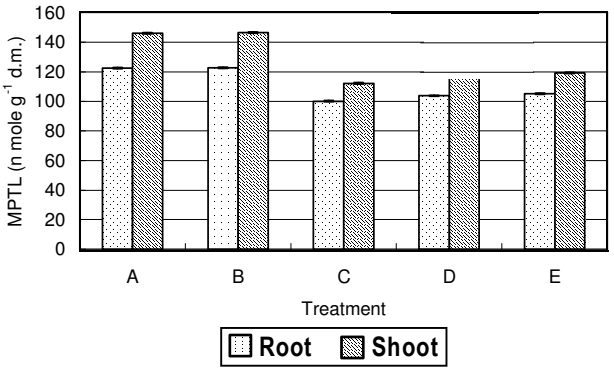

Fig. 1: Effect of triadimefon on NaCl salinity (100 mM) induced changes in membrane protein thiol level (MPTL) in *Amaranthus lividus* seedlings (120 hr old) A = Untreated, B = Triadimefon (50 mg l^{-1}), C = NaCl, D = NaCl + triadimefon (37.5 mg l^{-1}), E = NaCl + triadimefon (50 mg l^{-1})

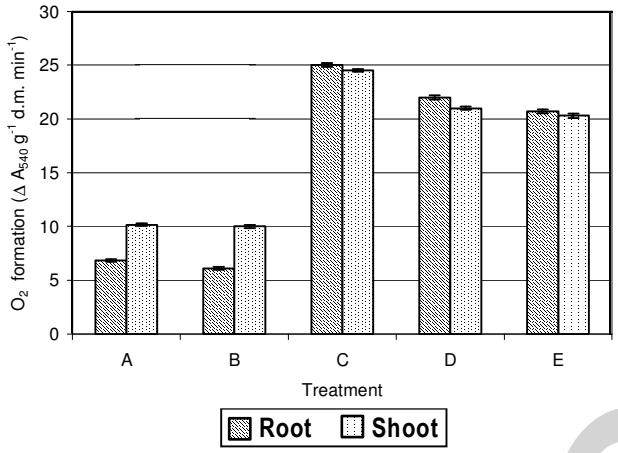

Fig. 2: Effect of triadimefon on NaCl salinity (100 mM) induced changes on superoxide ($O_2^{\cdot-}$) formation in *Amaranthus lividus* seedlings (120 hours old) A = Untreated, B = Triadimefon (50 mg l^{-1}), C = NaCl, D = NaCl + triadimefon (37.5 mg l^{-1}), E = NaCl + triadimefon (50 mg l^{-1})

triadimefon treated seeds under salinity stress also showed significant reductions in the accumulation of reactive oxygen species like $O_2^{\cdot-}$ and H_2O_2 (Fig. 2 and Table 1).

Triadimefon (50 mgl^{-1}) treatment reduced the accumulation of $O_2^{\cdot-}$ and H_2O_2 by 17% and 10%, respectively in salinity stressed *Amaranthus* shoot tissues when compared with untreated salinity stressed seedlings. That triadimefon treatment significantly reduced oxidative membrane damage could be strongly substantiated from the data of membrane protein thiol level (Fig. 1), which showed significant restoration of membrane protein thiol content under triadimefon treatment in salinity stressed seedlings. The data of membrane lipid peroxidation (thiobarbituric acid reactive substances) and lipoxygenase activity of the triadimefon treated salinity stressed seedlings (Table 2) further strengthened the fact that triadimefon treatment could significantly reduce salinity induced oxidative membrane damage.

That triadimefon treatment could significantly protect integrity of newly assembled membrane system could be further substantiated from the data of membrane injury index and relative leakage ratios, data in both the cases exhibited significant reduction while treated with retardant during salinity stress (Table 2). In a study of growth parameters, triadimefon treatment (both 37.5 and 50 mgl^{-1}) under NaCl salinity stress improved germination performances and seedling establishment. This observation received support from the data (increased values) of percent germination, relative growth index, relative water content and mean tolerance index (Table 3). In all cases, 50 mgl^{-1} concentration of triadimefon had been found to be more effective in mitigating oxidative membrane damage and restoration of membrane protein thiols thereby improving germination performance and seedling establishment.

Plants are subjected to various forms of abiotic stresses during early growth and development; and depending on the severity of the stress, metabolic processes could be adversely affected (Bhattacharjee,2005). Germination and early growth performances of seedlings are severely affected by NaCl salinity (Karikalan *et al*., 1999) in a concentration guided manner. Previous workers (Fletcher and Arnold, 1986; Pinhero *et al*., 1999; Karikalan *et al*., 1999; Fletcher *et al*., 2000) have demonstrated the protective roles of triazole type plant growth retardants (like triadimefon) from stress. These compounds are inhibitors of Cyt. P-450 mediated conversion of ent-kaurene to GA_{12} – aldehyde, leading to the formation of gibberellic acids (Fletcher *et al*., 2000; Rademacher, 1992).

The present study provides an in-depth look at the aspects of preservation of newly assembled membrane systems of Triadimefon treated, *Amaranthus* seedlings under salinity stress. To study the mechanisms underlying salinity tolerance, we investigated ROS mediated oxidative damages, membrane protein thiol contents, antioxidative defense systems and expression of salt induced proteins.

Triadimefon treatment incurred significant enhancement of free radical scavenging enzymes like POD and CAT in NaCl salinity imposed seedlings. The activity of SOD, on the other hand, increased slightly due to triadimefon treatment. The level of ROS such as H_2O_2 and $O_2^{\cdot-}$ in salinity stressed tissues of *Amaranthus* seedlings also decreased significantly by triadimefon treatment corroborating further evidences of strengthening antioxidative defense system. Triadimefon treated *Amaranthus* seedlings under salinity might help in the detoxification of reactive oxygen species, as evident from the higher free radical scavenging enzyme activities and reduced accumulation of $O_2^{\cdot-}$ and H_2O_2, thus mitigating the secondary oxidative stress imposed by NaCl during early germination. The higher activities of catalase and peroxidase in triadimefon treated salinity stressed seedlings indicate that, it could be a stress responsive mechanism caused by excess production of H_2O_2. These results are consistent with the previous observations of elevated antioxidant activities conferred by triazole compounds under abiotic stresses (Pinhero *et al*., 1997, 1999; Karikalan *et al.,* 1999).

Mitigation of oxidative membrane injury and preservation of structural integrity of membranes in salinity stressed *Amaranthus* seedlings by triadimefon is clearly evident from the data of membrane protein thiol content. The elevated level of membrane protein thiol

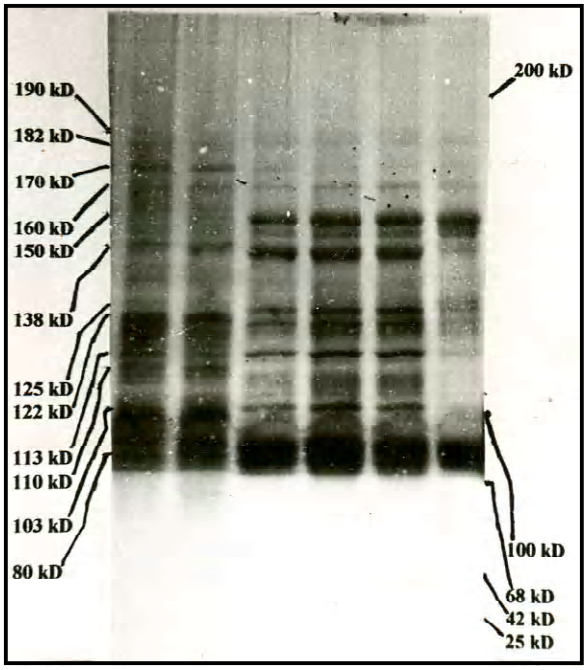

Fig. 3: 8-15% SDS-PAGE of protein samples from untreated, triadimefon treated – salinity stressed and salinity stressed *Amaranthus* seedlings (120 hours old). Mol. wt. (kDa) of marker proteins are on the right side and of polypeptides that deviated in regard to untreated seedlings are on the left side. 20 mg of protein sample was loaded in each lane. Lane A = Untreated control, Lane B = Treated with 50 mM NaCl, Lane C = Treated with 100 mM NaCl, Lane D = Treated with 150 mM NaCl, Lane E = Triadimefon (50 mgl^{-1}) treated without NaCl treatment, Lane F = Triadimefon (50 mgl^{-1}) treated and salinity stressed (100 mM NaCl)

level in triadimefon treated salinity stressed seedlings over untreated salinity stressed seedlings might be a plausible explanation of triazole compound-mediated architectural stability and oxidative stress mitigation under salinity stress. Paliyath and Fletcher (1995b) showed that triazole treatment alone could increase significantly the membrane protein content as well as non- sedimentable vehicles.

The preservation of membrane structure and mitigation of oxidative membrane damage by triadimefon treatment were also evident from the data of lipoxygenase mediated membrane lipid peroxidation. Triadimefon mediated reduction in the MDA level in salinity stressed *Amaranthus* seedlings might be due to increased activities of ROS scavenging enzymes and reduced level of ROS (Shankhla *et al*., 1992). That triadimefon treatment under salinity stress causes significant reduction in membrane damage could be further supported by the data of membrane injury index and relative leakage ratio. Although some authors (Asare Bomah and Fletcher, 1986) proposed lower accumulation of Na^+ and increased level of cytokinin as cause of triadimefon mediated membrane stabilization, present study strongly supports the view of marginal but significant reduction of oxidative stress and restoration of membrane protein thiol level as key factors for maintenance of newly assembled membrane architecture of juvenile seedlings.

To examine whether triadimefon treatment caused any alterations in protein composition, qualitative analyses of extractible soluble proteins by 8-15% gradient SDS-PAGE (Fig. 3) of triadimefon treated (50 mgl^{-1})and untreated salinity stressed (50, 100 and 150 mM NaCl) seedlings (120 hr old) were performed. Triadimefon treatment alone (without salinity stress), expressed some polypeptide bands similar to salinity influenced (100 mM NaCl treated) *Amaranthus* seedlings, with molecular masses of 90, 120 and 136 kDa. Triadimefon treated salinity imposed seedlings showed appearance of some new high molecular weight protein bands (170 and 182 kDa). The band intensity of 110-120 kDa polypeptide also increased significantly in triadimefon treated salinity stressed seedlings.

In response to environmental stresses, increased gene expression have been noticed which results in the synthesis of proteins, collectively called stress proteins, having putative role in stress protection. It has been reported that salinity adversely affects protein metabolism due to decrease in protein synthesis, accelerated proteolysis, decreased availability of amino acids and denaturation of enzymes associated with protein synthesis (Levitt, 1980; Muthukumarasamy and Panneerselvam, 1997; El-Masud and Kamat, 2000). Variations in banding pattern of proteins by SDS-PAGE, extracted from NaCl salinity stressed, triadimefon treated salinity stressed and triadimefon treated unstressed *Amaranthus* seedlings have been noticed. Triadimefon treatment alone (without salinity stress) caused expression of some polypeptide bands similar to salinity influenced seedlings with molecular masses of 90, 120 and 136 kDa. The polypeptide pattern of triadimefon treated salinity imposed seedlings showed appearance of some new high molecular mass protein bands (170 and 182 kDa). The band intensity of 110-120 kDa polypeptides also increased significantly in triadimefon treated salinity stressed seedlings. Thus, triadimefon treatment results in the expression of a number of proteins whose molecular masses indicate them to be stress-related proteins, even without subjecting it to NaCl salinity. If these proteins have any putative role in stress protection, it might be said that triadimefon treated plants have already expressed these proteins and were ready to withstand salinity stress. Not only the triadimefon treatment led to over-expression of a number of polypeptides, those might have protective roles and some of them might be already present prior to salinity stress. Paliyath and Fletcher (1995a), Pinhero *et al*. (1999) and Kraus *et al*. (1995) also showed the over expression of several proteins in response to paclobutrazole under abiotic stresses.

So, our results conclusively suggest that triadimefon mediated salinity tolerance in *Amaranthus* during early germination involves not only the mitigation of oxidative stresses and membrane stabilization through restoration of membrane protein thiols and lipids but also expression of stress proteins that could provide additional tolerance to salinity.

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