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Ameliorative effect by calcium on NaCl salinity stress related to reactive oxygen species metabolism in *Amaranthus tricolor* L.

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ABSTRACT:

Soil salinity affects plant growth and development by way of osmotic imbalance which induces oxidative damage in plant tissues. Oxidative stress is caused by increased production of Active Oxygen Species (AOS), such as O_2–, •OH, H_2O_2 and $^1O_2$ these lead to lipid peroxidation, enzyme inactivation and oxidative damage to DNA. Plants have antioxidant defense systems to protect against the production and action of the AOSs. Plants with high level of antioxidants, either constitutive or induced, have been reported to produce greater resistance to this oxidative damage in plant cells. AOS synthesis and its scavenging were investigated in the control, different concentrations of NaCl and NaCl + $CaCl_2$ stressed *Amaranthus tricolor* L. and *Phaseolus vulgaris* L.. AOS such as superoxide anion and H_2O_2 content showed a steady increase in the plants of all NaCl treated media compared to control. When the salinized media were supplemented with $CaCl_2$ the AOS level drastically decreased compared to the corresponding plants grown on salt alone. Similarly, the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase under salt stress were higher in NaCl + $CaCl_2$ supplemented media than the plants on the salinized media alone. This suggested that the alleviation effect of calcium under saline condition was through modulation of the enzyme complexes that accelerate the rate of antioxidant enzymes biosynthesis under salt stress. Similarly, the level of lipid peroxidation was found to be lower in plants of all NaCl + $CaCl_2$ media than control.

Keywords:
Amaranthus tricolor L., *Phaseolus vulgaris* L., superoxide anion, H_2O_2; NaCl, $CaCl_2$, superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase, lipid peroxidation.

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INTRODUCTION

Soil salinity is an inevitable problem for agriculture in most parts of the world. Better understanding of the mechanisms that enable plants to adapt to salt stress is necessary to make the best use of these saline soils. Cellular mechanisms are especially important to glycophytes, in which physiological and biochemical processes contribute to the adaptation to salt stress. In recent years, biochemical responses of plants to salt stress have been studied intensively. One of the biochemical changes possibly induced by salt stress is the increased production of Active Oxygen Species (AOS) (Yildirim etal., 2006; Vaidyanathan *et al.* 2003; Michaela et al., 2002). ROS are highly reactive and when the capacity of plant for scavenging is less than ROS production they can seriously disrupt normal metabolism through oxidative damages of lipids, proteins and nucleic acids. Plants posses a number of antioxidant systems that protect them from these potential cytotoxic effects. Antioxidant enzymes are the most important components in the scavenging system of ROS. Superoxide dismutase (SOD) is a major scavenger of $O_2^{\cdot-}$ and its enzymatic action results in the formation of H_2O_2. Catalase (CAT), ascorbate peroxidase (APX) and a variety of general peroxidases catalyze the breakdown of H_2O_2. Therefore, these enzymatic systems eliminate the damaging effects of toxic oxygen species. Paraquat is a redox-active compound wildly used to control existing vegetation (Suntres, 2002; Sko´rzyn ska-Polit et al., 1998). The mechanisms of paraquat toxicity involve: 1) the generation of the $O_2^{\cdot-}$ in the light, which can lead to the formation of more toxic AOS, such as •OH 2) lipid peroxidation which results in the oxidative degeneration of cellular polyunsaturated fatty acids. Therefore, the aim of this work was to evaluate the effects of NaCl stress and its alleviation by calcium on active oxygen species formation, the lipid peroxidation and the activity of antioxidative enzymes in *Amaranthus tricolor* L. and *Phaseolus vulgaris* L. in order to better understand their differences on salt stress tolerance.

Quantification of superoxide anion ($O_2^{\cdot-}$)

The $O_2^{\cdot-}$ production rate was measured as described previously (Jiang and Zhang, 2002) with some modifications. 0.2 g leaf tissue was homogenized in 1 ml of 50 mM Tris–HCl (pH 7.5) and centrifuged at 5000 × g for 10 min at 4°C. The reaction mixture (1 ml) contained 200 ml supernatant and 800 ml 0.5 mm 3- bis (2-methoxy-4 -nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilideinner salt (XTT sodium salt). The $O_2^{\cdot-}$ production rate was calculated using molar extinction coefficient 2.16×10^4 M^{-1} cm^{-1}.

Quantification of hydrogen peroxide (H_2O_2)

Horse radish POX-catalysed oxidation of TMB was used for the quantification of H_2O_2 and was measured colorimetrically at 450 nm (Gallate and Pracht, 1985),

Lipid Peroxidation

The level of lipid peroxidation in the cells was measured in terms of malondialdehyde content determined by the thiobarbituric acid reaction as described by Heath and Packer, (1968). The concentration of malondialdehyde was calculated using the extinction coefficient 155 mm/L.

Antioxidant enzyme extraction and assay

Old and young leaves (0.5 g) were homogenized in 25 mm K-phosphate buffer (pH 7.8) containing 0.4 mm EDTA, 1 mM ascorbate and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was then centrifuged at 15,000 x g for 20 min at 4°C. The filtered supernatant was used as an enzyme extract for APX, CAT and GR (Yanagida *et al.* 1999).. For SOD assay, the extract was dialyzed overnight with 10 mm K-phosphate buffer (pH 7.8) at 4°C. The dialyzed extract was centrifuged at 15,000 x g for 20 min at 4 °C.

Superoxide dismutase (SOD) assay was calculated following the formula below; (Vb/Vs)-1, where Vb is the reaction rate of the blank and Vs is the reaction rate of the sample.

Catalase (CAT) reaction mixture consisted of 50 mm K-phosphate buffer (pH 7.0) containing 10 mm H_2O_2 (0.95 ml) and enzyme extract (0.05 ml) was prepared. Immediately after adding the enzyme to the buffer, the initial rate of absorbance at 240 nm was determined. The molar absorption coefficient of H_2O_2 (0.04/ mm /cm) was used to calculate the enzyme activity.

Glutathione reductase (GR) assay mixture consisted of 100 mm K-phosphate buffer (pH 7.8) (0.25 ml), 10 mm oxidized glutathione (GSSG) (0.05 ml), distilled water (0.48 ml), 1 mm NADPH (0.12 ml) and enzyme extract (0.1 ml) was prepared. The assay was started by addition of GSSG. GR activity was determined from the rate of NADPH oxidation measuring the decrease of absorbance at 340 nm. The molar absorption coefficient of NADPH (6.1/ mm /cm) was used to calculate the enzyme activity. In ascorbate peroxidase (APX) assay, the reaction mixture consisted of 100 mm K-phosphate buffer (pH 7.0)

(0.25 ml), 1 mM ascorbate (0.25 ml), 0.4 mm EDTA (0.25 ml), distilled water (0.19 ml), 10 mm H_2O_2 (0.01 ml) and enzyme extract (0.05 ml) was used. The reaction was started by adding H_2O_2 and the oxidation rate of ascorbate was measured by the initial rate of decrease of absorbance at 290 nm. The molar absorption coefficient of ascorbate (2.8/ mm /cm) was used to calculate the enzyme activity.

Paraquat sensitivity assay

Ten discs with 4 mm diameter were excised from the NaCl and NaCl + $CaCl_2$ treated leaves and soaked into 3 ml of 0.3 µm of paraquat (1,1-dimethyl-4,4-bipiridinium dichloride salt) solution with their adaxial side up. Paraquat soaked treated leaves were preincubated in the dark for 1 h and then irradiated with fluorescent light (260-280 µE/ m^2/s) at 25°C for 48 h. Chlorophyll contents in the leaf discs were determined with the modified method described by Chappelle *et al.* (1992).

Statistical analysis

The data obtained were analyzed statistically and the results are presented as Mean ± Standard deviation.

RESULTS AND DISCUSSION

Application of external NaCl + Calcium (5 mM $CaCl_2$) elevated greatly FW and DW compared to NaCl alone in both species, but differently i.e., FW of root and shoot in *A.tricolor* ranged between 0.4 to 1.88; 0.58 – 2 fold respectively whereas, in *P.vulgaris* it was 0.68 - 0.83; 0.43 – 0.58 fold. Similarly the DW of root and shoot in *A.tricolor* was 0.74-1.47 and 0.63 – 1.6 fold. However, in *P.vulgaris* external calcium treatment had less significant difference on FW and DW of shoot in comparison with the same parameters in the control (**Table 1 a & b**).

External Ca^{2+} on O_2^- and H_2O_2 Content

The trend of O_2^- and H_2O_2 content was remarkably different between treatments containing external calcium (**Table 2 & 3**). O_2^- and H_2O_2 content reduced marginally in stress media containing only external calcium (5 mm $CaCl_2$), in both the species (data not shown). But NaCl + $CaCl_2$ treated plants shows a significant reduction in O_2^- and H_2O_2 content upto 200 mm NaCl in *A.tricolor* and thereafter the free radical content slightly increased. In *P.vulgaris,* external Ca^{2+} did not significantly reduced O_2^- and H_2O_2 content after 100 mm NaCl concentration.

Ca^{2+} treatment on MDA Content

Application of external Ca^{2+} + NaCl could reduce MDA content considerably, compared with NaCl stress media in *A.tricolor*, where as marginally in *P.vulgaris*, compared with those of non-external calcium medium contain only NaCl (150 mm) (Table 4). However, in control, application of external calcium made no significant difference to MDA content compared with non-external Ca^{2+} media.

Paraquat assay

Effect of paraquat on leaf discs preparing from the seedlings treated with NaCl + Ca^{2+} were determined by measuring chlorophyll contents (Fig. 1 A & B). Chlorophyll contents in the young and old leaf discs of *A.tricolor* both of nontreated and treated with 0.3 µM paraquat slightly increased with increasing NaCl + Ca^{2+}concentration. In contrast, chlorophyll contents in the young and old leaf discs of *P. vulgaris* were greatly reduced with increasing NaCl + Ca^{2+}concentration.

Chlorophyll contents in the disks were measured to determine the decomposition of the pigment caused by generated AOS. With increasing NaCl concentrations + 5 mm Ca^{2+}, chlorophyll contents in the discs from young and old leaves of

Table 1 b. Effect of NaCl + $CaCl_2$ (5 mM) on fresh and dry weights (g/plant) of 14 days old *P.vulgaris* seedlings (D= dead).

NaCl Conc. (mM)		0	50	100	150
	Fresh weight	8.2 ±0.3	6.8 ±0.2	5.6 ±0.4	D
Root	Dry weight	0.6 ±0.89	0.45 ±0.3	0.4 ±0.2	D
Shoot	Fresh weight	7.2 ±0.6	4.2 ±0.2	3 ±0.2	D
	Dry weight	0.86 ±3	0.58 ±0.1	0.4 ±1	D

Table 1 a. Effect of NaCl + $CaCl_2$ (5 mM) on fresh and dry weights (g/plant) of 14 days old *A.tricolor* seedlings.

NaCl Conc. (mM)		0	50	100	150	200	250
Root	Fresh weight	4.1 ±0.6	6.9 ±0.3	4.2 ±0.2	3.4 ±0.2	2.4 ±0.3	1.6 ±0.2
	Dry weight	0.38 ±0.04	0.56 ±0.7	0.42 ±0.1	0.42 ±0.1	0.35 ±2	0.28 ±0.1
Shoot	Fresh weight	3.8±0.4	7.6 ±0.2	5.04 ±0.2	5.4 ±0.1	3.1 ±0.6	2.2 ±0.3
	Dry weight	0.76±0.1	1.2 ±0.4	0.64 ±0.3	0.61 ±0.1	0.53 ±1	0.48 ±0.2

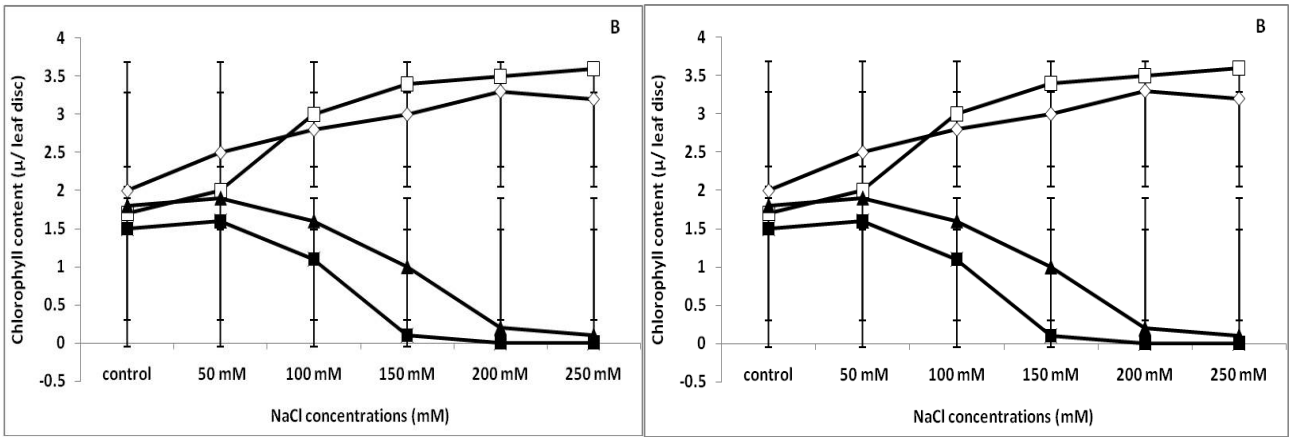

Fig. 1 A & B. Effects of paraquat on chlorophyll contents in leaf discs from young leaves (A) and old leaves (B) of *A.tricolor* and *P. vulgaris* treated by 0, 50, 100, 150, 2000 and 250 mM NaCl + 5 mm $CaCl_2$ for 12 days. Vertical bars indicate standard errors (n=3). 0 µm paraquat; 0.3 µm paraquat.

A.tricolor did not decrease. On the contrary, tolerance to paraquat was slightly higher in NaCl + Ca^{2+} treated plants. This finding indicates that this species has greater tolerance to the toxicity of AOS. Antioxidative enzyme system in *A.tricolor* seemed to be effective for elimination of AOS which arises from salt stress. In case of *P. vulgaris*, it was difficult to determine whether the treated paraquat cause reduction of chlorophyll content with the 50 and 100 mM NaCl treatment, because the leaves showed chlorosis and necrosis.

Ca^{2+} treatment on APX, SOD, CAT and GR

The antioxidant enzyme activity trends in the species were similar to control plants containing only external calcium ($CaCl_2$). However, a paramount increase in activities of antioxidant machinery was observed in the species when treated with external calcium + NaCl i.e., SOD, CAT, and APX activities were higher than in media contain only NaCl (Table 5 a & b). GR showed steady activity in all the experimental conditions irrespective of treatments.

In recent years, it has been widely noted that Ca^{2+} plays an important role in the adaptation of plants to adverse environments (Bowler and Fluhr, 2000). It was indicated that Ca^{2+} had a pertaining role in preventing cell membrane injury and leakage as well as stabilizing cell membrane structure under adverse environmental conditions (Pie *et al.,* 2000). *In vitro* studies on the cell wall formation in spruce hypocotyl cuttings indicated that Ca^{2+} was essential and its absence made the wall more susceptible to injury (Price *et al.,* 1994). Shabala *et al.,* (1997) observed that higher Ca^{2+} concentration was important in maintaining cell membrane integrity under water stressed conditions and that was a function specific to Ca^{2+}. In this study, application of external calcium resulted in higher FW and DW content and lower MDA content in cells compared with the contents in media with only NaCl. The data suggested that more concentrated $CaCl_2$ treatment could significantly mitigate the damage of NaCl stress, and the effect was specific to Ca^{2+} modulated proteins.

Our data showed that the activities of SOD, CAT and APX in cells remained relatively steady under control conditions compared with those under NaCl stress conditions. This suggests that SOD, CAT and APX participate in the regulating mechanism of cells against salt stress and was consistent with the result of Shabala *et al.,* (1997). The result indicated that external calcium could elevate the activities of SOD, CAT, and APX in cells under NaCl stress conditions. This increase is regarded as an adaptive signal to trigger gene expression and activate some unknown biochemical

Table 2. Effect of NaCl + $CaCl_2$ (5 mM) on Super oxide anion (O_2^-) (µmolmin^{-1}g^{-1}) of 14 days old seedlings. (D= dead).

NaCl Conc. (mM)	0	50	100	150	200	250
A.tricolor	0.29±0.08	0.45±0.3	1.3±0.3	1.9±0.2	2.3±0.3	3.2±0.11
P.vulgaris	0.15±0.4	1±0.05	2.4±0.15	4±0.52	D	D

Table 3. Effect of NaCl + $CaCl_2$ (5 mM) on hydrogen peroxide (µmolmin^{-1}g^{-1}) of 14 days old seedlings.

NaCl Conc. (mM)	0	50	100	150	200	250
A.tricolor	0.7±0.01	1 ±0.22	1.7±0.6	2.4±0.6	2.9±0.7	3.9±0.2
P.vulgaris	0.4±0.13	2.2±0.12	5.9±0.23	12.4±0.2	D	D

(D=dead)

events to enable plants to adapt to environmental stress (Rengel, 1992). In addition, the calcium receptor protein calmodulin and other calcium-binding proteins have been found to be involved in the regulation of plant responses to environmental stress (Ishitani *et al*. 2000).

Ca^{2+} is also a primary second messenger in signal transduction and regulates physiological and biochemical processes in the responses of plants to extracellular adverse abiotic environments (Bowler and Fluhr, 2000). The important role of calcium signals in the transduction of environmental change into plant response has been documented over a wide range of stimuli (Shabala et al., 1997; Romano *et al.,* 1998). Much of the evidence has shown that external calcium treatment can increase tolerance capacity to adverse environments involving drought (Bowler and Fluhr, 2000), cold (Shabala and Newman, 2000), heat, and salt stress (White and Broadley, 2003).

Ca^{2+} signal patterns can occur as single transients or repetitive oscillations (Demidchick *et al.,* 2002 a; Elphick *et al.,* 2001), and also different signaling patterns, such as oscillations and waves, may arise from the selective activation of transcriptional regulators. Special calcium signaling coming from different stimuli/conditions should be a mechanism that perceives and transudes different signal's stimuli and causes different physiological responses in plants. In this investigation, external calcium treatment increased total calcium content in cells. For determining the signal role of Ca^{2+}, intracellular free calcium ion content and subcellular locations of Ca^{2+} will be measured in our next investigation.

AOSs such as O_2.- and H_2O_2 content in NaCl stressed *A.tricolor* was comparatively more than control but its increase was more obvious in *P.vulgaris*. Thus an unambiguous correlation was noticed between salt concentrations and ROSs content (Table 3 & 4). Interestingly, the magnitude of ROS content in *A.tricolor* was decreased by supplementing the salinized media with $CaCl_2$ compared to the plants with corresponding NaCl concentrations and was significant at 200 mM NaCl + $CaCl_2$. The results are comparable with that of *Phyllanthus amarus* and *Catharanthus roseus* under water and salt stress (Jaleel *et al.,* 2008; 2007 b). The increased ROS accumulation in *Arabidopsis* root and leaf cells has been related to the maintenance of a more favourable K^+/ Na^+ ratio in the NaCl stressed roots supplied with calcium and it was suggested that the failure to maintain a favourable K^+/ Na^+ ratio can inhibit enzyme functions (Shabala *et al.,* 2006). Studies on salt tolerance in peanut reveals that the K^+/ Na^+ ratio increased at all levels of external salinity (Girija *et al.,* 2002). The mechanism leading to the increase in antioxidant enzyme levels in salinized plants by calcium is not clear still. However, it is evident from the present study that the AOX enzymes synthesis is accelerated by some Ca^{++} dependent mechanisms, may be by the formation of Ca^{++} modulated protein complexes in the cytosol, which might have activated enzymes; leading to scavenging of ROSs (Jaleel et al., 2008). Ca^{++} is reported to reduce the toxic effect of NaCl salinity. Ca^{++} supplementations apparently alleviate NaCl stress, at least in part, by modulating the enzyme protein and a greater reduction in lipid peroxidation to maintain low ROS levels which can be expected to contribute for an ideal level osmoregulation. Osmotic adjustment is an important mechanism of a plant's tolerance to a stress environment (Halfter *et al*., 2000). Osmotic adjustment and osmotic potential increased slowly in cells under prolonged stress, but were not affected significantly by external Ca^{2+} treatment under salt stress conditions. The results indicate that the effect of external Ca^{2+} on cells was not due to the regulation of osmotic potential or osmotic adjustment. Thus, the present study suggests that *A.tricolor* has higher levels of antioxidative enzymes both constitutive and induced resulting in greater resistance to oxidative damage caused by NaCl stress and is mitigated efficiently by calcium salts.

Table 4. Effect of NaCl + $CaCl_2$ (5 mM) on Lipid peroxidation (nmolg^{-1}) in NaCl + $CaCl_2$ (5 mM) treated *A.tricolor* and *P. vulgaris* 14 days after starting NaCl treatments (D= dead).

NaCl Conc.	0 mM	50 mM	100 mM	150 mM	200mM	250mM
A.tricolor	1±0.01	1.3±0.22	2±0.6	2.8±0.04	3.2±0.9	4.2±0.25
P.vulgaris	0.5±0.1	2.7±0.3	6±0.65	9.5±0.4	D	D

Table 5 a & b. Antioxidant enzymes such as superoxide dismutase (SOD, U/g), Ascorbate peroxidase (APX, µmol AsA decomposed/gFW/min), Guaiacol peroxidase (POX), Catalase (CAT, µmol H2O2 decomposed/gFW/ min) and Glutathione reductase (GR, µmol NADPH oxidized/gFW/min) in NaCl + $CaCl_2$ (5 mM) treated *A.tricolor* and *P. vulgaris* 14 days after starting treatments.

Table 5 a	Young leaves *A.tricolor*			
NaCl conc. (mm)	**SOD**	**APX**	**CAT**	**GR**
0	672 ±0.2	4.9±0.7	442±0.2	2.4±0. 09
50	1289±0.6	9 ±0.8	698±0.8	3.9±0.6
100	1406±0.4	16±0.5	744±0.6	4.5±0. 9
150	1498±0.2	18 ±0.4	924±0.9	4.6±0.8
200	1522±0.3	19.7±1	947±0.3	4.7±0.2
NaCl conc. (mm)	**Young leaves *P.vulgaris***			
0	207 ±0.1	3±0.03	158±0.27	1.4±0. 09
50	388±0.07	8.6 ±0.07	231±0.11	1.9±0.15
100	223±0.04	11.5±0.06	201±0.12	1.6±0. 43
150	156±0.01	6 ±0.09	196±0.12	1±0.24
200	D	D	D	D

Table 5b.	Old leaves *A.tricolor*			
NaCl conc. (mm)	**SOD**	**APX**	**CAT**	**GR**
0	332 ±0.9	4.3±0.17	369±0.22	3±0. 9
50	512±0.06	5.9 ±02.8	481±0.28	4±0.05
100	699±0.34	8±0.35	514±02.6	4.8±0. 06
150	803±0.22	9.8 ±0.44	567±0.29	3.9±0.07
200	820±0.23	10.4±0.81	577±0.33	4±0.02
NaCl conc. (mm)	**Old leaves *P.vulgaris***			
0	197 ±42	2.4±0.17	119±0.12	1.8±0. 9
50	249±0.26	5 ±0.81	141±0.28	2.3±0.26
100	198±0.64	5.4.9±0.25	114±0.16	1.8±0.1 9
150	184±0.82	4.7 ±0.24	96.4±0.39	1.4±0.38
200	D	D	D	D

The data are the means of four replicates ± S.E.

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