

Biochemical responses and proline metabolism in *Amaranthus tricolor* L. and *Phaseolus vulgaris* L. under *in vitro* NaCl Stress.

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ABSTRACT:

Salinity is currently the major factor which reduces crop yields. One of the biological approaches is to use salt tolerant plants. *Amaranthus tricolor* L. has been used as a promising plant to ameliorate the salt affected area. The objective of the study is to evaluate the effect of NaCl stress on synthesis and catabolism of proline, soluble proteins, carbohydrates and Na⁺/K⁺ ratio in *A. tricolor* and to compare with the salt-sensitive species, *Phaseolus vulgaris* L. The experiments were designed with six replications. Seedlings of both species were grown hydroponically with 0, 50, 100, 150, 200, 250 and 300 mM NaCl. The *in vitro* activity of the enzyme pyrroline 5-carboxylate synthetase under NaCl stress was higher, while, the activity of proline degrading enzyme - proline oxidase showed a reverse trend i.e., low activity in high NaCl concentrations. Soluble protein content was increased in the shoot of *A. tricolor* but decreased in *P. vulgaris*. Roots of both the species showed variation in the protein content. Proline content of shoot and roots increased significantly in all the treatments in the plants. However, *A. tricolor* showed a higher level. The total carbohydrate also showed a similar trend. High level of NaCl decreased the reduced sugar in shoots and roots of the species. Salt stress increased Na⁺ significantly and decreased the K⁺ content in both species. The biochemical variation may be interpreted as differential response of the plants to NaCl stress.

Keywords:

Amaranthus tricolor, ions, proline, salt stress, soluble proteins.

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INTRODUCTION

Agricultural productivity is severely affected by soil salinity because salt levels that are harmful to plant growth affect large terrestrial areas of the world. Efforts to improve crop performance under environmental stresses have not been fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood (Nyagah and Musyimi, 2009). Soil salinity affects plant growth in a variety of ways, reducing water uptake, causing toxic accumulation of sodium and chloride, and reducing nutrient availability. Salinity also induces water deficit even in well-watered soils by decreasing the osmotic potential of soil solutes, thus making it difficult for roots to extract water from their surrounding media (Jaleel et al., 2007e). Excessive sodium inhibits the growth of many salt-sensitive plants, which includes the most crop plants. The typical first response of all plants to salt stress is osmotic adjustment. Compatible solute accumulation in the cytoplasm is considered a mechanism to impart salt tolerance (Jaleel et al., 2007e). Complex molecular responses including the accumulation of compatible solutes, the production of stress proteins and the expression of different sets of genes are part of the plant signaling and defense system against salinity. It is well known that one of the most common responses to salinity is the overproduction and accumulation of proline, glycine betaine and total sugars. Solute accumulation by cells is one such mechanism (osmoregulation) which contributes to stabilization of enzyme/protein and turgor maintenance in growing organs and has been correlated with productivity under stress (Delauney and Verma, 1993).

The accumulation of free proline has been studied in a number of taxa subjected to hyperosmotic stress conditions for over years (Delauney and Verma, 1993). The accumulation of proline under abiotic stress conditions accounts for few millimolar concentrations, depending on the species and the extent of stress. Very high accumulation of cellular proline (up to 80% of the amino acid pool under stress and 5% under normal conditions) due to increased synthesis and decreased degradation under a variety of stress conditions such as salt and drought has been documented in many plant species. Although proline is known to confer osmotic tolerance during stress conditions, its specific role during plant growth is not completely clear. The biosynthetic pathway of proline diverged between bacteria and

higher plant systems (Kavi Kishor et al., 2005).

Amaranthus tricolor generally grows in the dry arid soils of Kerala. Due to its vigorous growth and drought tolerance, this plant has been used as a potential green manure plant. Until now, a few studies have been conducted on the mechanism of salt tolerance in this plant. The objective of this study is to obtain a better understanding on salt-tolerance mechanism in *A. tricolor* by determining the physiological and biochemical responses of the plant to NaCl in comparison with *Phaseolus vulgaris*.

MATERIALS AND METHODS

Seeds of *A. tricolor* (obtained from Agricultural University, Trichur, Kerala) were soaked in concentrated H₂SO₄ for 30 min to break dormancy. The seeds were surface-sterilized with NaClO (1.25% active chlorine) under vacuum for 15 min and rinsed several times with sterile distilled water and soaked in it for 12 h at 25°C. Fifteen seeds were placed in a petri dish with two layer filter papers. Ten ml of 0, 50, 100, 150, 200, 250 and 300 mM NaCl solution was sprayed into the into petri dish. They were then kept at room temperature. Germination rate were determined seven days after treatment. The experiment was designed with six replications.

Seeds of *A. tricolor* were broken their dormancy and then surface sterilized as described above. They were then soaked in sterile water at 25°C for one night. *Phaseolus vulgaris* L. seeds were incubated on moist filter paper at 30°C for 2 days in darkness. Germinated seeds were planted in a container containing vermiculite and grown at 25°C for 2 days in darkness with aluminum foil cover. Then a half strength of MS medium (1962) was added to the vermiculite. After 5 days, seedlings were transferred to a hydroponic culture with the same nutrient solution. *A. tricolor* and *P. vulgaris* were grown to the 3rd to 4th and to the 1st to 2nd leaf stage respectively. Salt treatment was given by adding 50 mM NaCl to the solution. For the treatments with higher concentrations, plants were transferred to 100, 150, 200, 250 and 300 mM at two-day intervals. The NaCl solution containing nutrient was renewed every 4 days. Plants were grown in a growth chamber at 25/20°C day/night temperatures, with a 14 h photoperiod at 280- 290 μE/m²/s, at a relative humidity of 70-80%. Seedlings were harvested 14 days after starting NaCl treatment. All experiments were designed with six replications.



Shoots and root were dried at 70°C for 24 h and weighed. Sodium, chloride and potassium were extracted from each sample in a mixture of HNO₃, HClO₄, and distilled water (1: 5: 2.5). The solution was kept for 12 h at 100°C, diluted with 100 mM HCl, and analyzed by atomic emission spectrometry. Soluble proteins were extracted from young shoots and roots in an extraction buffer (0.01 M Tris-HCl, 10% glycerol, 5% PVP, 1% Triton X 100, pH = 6.8) and protein assay was carried out according to the method of Bradford, (1976).

For proline determination, 10 mL of 3% (W/V) aqueous sulfosalicylic acid solution was added to 1 g of fresh shoot and root samples and was homogenized, filtered through one layer of Whatmann, No. 1, filter paper, then proline was assayed according to the method of Bates *et al.*, 1973. Total free amino acids were extracted and estimated as per the method of Walter Troll and Keith Cannan (1952).

Carbohydrates were extracted from dry shoots and roots of plantlets in warm water. Concentration of total and reduced sugars were determined based on the methods of Dubois, (1956) and Jeffries, (1998) respectively.

Enzyme extraction was based on the method of Chen *et al.*, (2001). Seedlings were homogenized in prechilled mortar and pestle in the extraction medium at 4° C. The extraction medium contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 10 mM 2-mercaptoethanol, 1% (w/v) PVP, 5 mM MgCl₂, and 0.6 M KCl. The homogenate was centrifuged at 12000 g for 20 min at 4°C, the resulting supernatant was kept at -20°C and used for enzyme assaying.

P5CS activity was assayed according to the method of modified Vogel and Kopac, (1960) as follows. 0.7 mL reaction medium containing 50 mM Tris (pH 7.5), 2 mM MgCl₂, 10 mM ATP, 1.0 mM NADH, 50 mM glutamic acid, and 0.1 mL enzyme extract was incubated at 37°C for 30 min. The reaction was then stopped by adding 0.3 mL of 10% (w/v) trichloroacetic acid. Color reaction was developed by incubating with 0.1 mL of 0.5% (w/v) *o*-aminobenzaldehyde for 1 h. After centrifugation at 12000 g for 10 min, the clear supernatant fraction was taken to measure the absorbance at 440 nm. Enzyme activity was calculated using the extinction coefficient of 2.68 / (mM cm).

Proline oxidase (PO) activity was determined according to the method outlined by Huang and Cavalieri, (1979). Plant samples (1 g) were

extracted with 5 mL of Tris-HCl buffer (pH 8.5) grinding medium and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was again centrifuged at 25,000 g at 20 min at 4°C. A 3-mL assay mixture was prepared by taking 0.1 mL of extract, 1.2 mL of 50 mM Tris HCl buffer (pH 8.5), 1.2 mL of 5 mM MgCl₂, 0.1 mL of 0.5 mM NADP, 0.1 mL of 1 mM KCN, 2 0.1 mL of 1 mM phenazine methosulphate (PMS), 0.1 mL of 0.06 mM 2, 6-dichlorophenol indophenol (DCPIP) and 0.1 mL distilled water instead of PRO. The reaction was monitored at 600 nm at 25°C using PRO to initiate reaction; the increase in OD values increase were noted at 0, 1, 2, 3, 4 and 5 min. PO activity was expressed in U (one U = mM DCPIP reduced min⁻¹ mg⁻¹ protein).

Five independent determinants from individual plants were used for statistical analysis. Student's *t* test and analysis of variance (ANOVA) were used for analyzing significant differences between the control and treated plants ($P < 0.05$).

RESULTS AND DISCUSSION

Total protein and proline content of *A. tricolor* and *P. vulgaris* shoots were increased moderately by salt treatment up to 150 mM NaCl and thereafter declined sharply at higher NaCl concentrations (Data not shown). In the roots the protein and proline content increased at all NaCl concentrations tested, although a decline was apparent at 300 mM (**Table 1 a and b**). Nevertheless, the protein and proline contents of severely stressed roots were lower than those of moderately stressed ones, but still higher than the control. However, seedlings of *P. vulgaris* wilted or died at higher NaCl concentrations (100 mM and above). Significant positive relationship was examined between salinity and total protein and proline content ($P < 0.05$).

The amount of soluble amino acids increased in both plants with increasing NaCl concentration. At the highest concentration, although the total amino acid was clearly built up in both the shoots and roots of *A. tricolor*, their accumulation was more obvious in shoots (Table 1a and b). Similarly, the free amino acids accumulation was also found in *P. vulgaris* at the higher concentration (100 and 150 mM). The data was statistically significant at 1% level.

We found significant difference in total carbohydrate and reduced sugar between the two species under NaCl treatment. For example, at 150

Table 1a and b. Amino acid contents (mg/g DW) in NaCl treated *A.tricolor* and *P. vulgaris* 14 days after starting NaCl treatment.
Table 1a

NaCl (Mm)	Roots			Shoots		
	Free aminoacids	Total proteins	Proline	Free aminoacids	Total proteins	Proline
0	2.5 ±0.9	3.6±0.5	0.4±1	3.8.2±1.9	4.1±0.3	0.53±0.01
50	5.9±0.29	4.0±0.4	0.52±0.29	12.7±2	5.2±0.09	1.2±0.03
100	9.2±0.35	4.8±0.25	1.5±0.38	16.9±3.2	6.5±0.26	1.5±1.9
150	11.4±2.9	5.7±0.11	2.2±0.7	23±0.99	7.8±0.38	2.9±2.3
200	12±2.6	6.3±0.23	2.2±0.5	27.8±0.65	8.5±0.4	3.3±3.9

Table 1b

NaCl (Mm)	Roots			Shoots		
	Free aminoacids	Total proteins	Proline	Free aminoacids	Total proteins	Proline
0	3.5 ±0.91	4.0±0.5	0.2±0.45	8.8±1	4.8±2.1	0.5±0.2
50	8.3±0.8	5.4±1.5	0.3±0.12	11.3±2.1	5.6±0.7	0.8±0.34
100	13.1±0.45	6.9±2	0.81±0.33	19.2±2.7	7.0±0.9	0.6±0.5
150	22.4±0.78	7.2±1.7	0.6±0.65	23±2.9	8.0±2.8	0.5±0.8

The data are the mean of six replicates ± S.E. Significant at 0.01 levels. $P < 0.05$.

mM NaCl total carbohydrate in *A.tricolor* 18.6 mg g⁻¹ DW while in *P. vulgaris* was only 8.4 mg g⁻¹ DW. A similar but moderate pattern of carbohydrate changes was observed in roots. Reduced sugar of shoot and root in both the species was decreased when plants are exposed to NaCl stress (Fig. 1 and 2).

Na⁺ and Cl⁻ contents were enhanced with increasing salinity in roots and shoot of *A.tricolor* and *P. vulgaris* (Fig. 3 and 4) However, their accumulation was greater in shoots than in roots in *A.tricolor*. In the case of *P. vulgaris* the pattern of accumulation was different i.e. higher in roots than in shoots. K⁺ content on the other hand has been reduced markedly in both the species. Instability of Potassium (K⁺) content in shoots and roots was

observed in *P. vulgaris*, while it slightly reduced in *A.tricolor* (Fig. 5). The ion analyses in individual leaf showed that Na⁺ were tended to accumulate in the older leaves with higher concentration, but roots in *P. vulgaris* (Fig. 6 a and b).

Δ¹-Pyrroline-5- carboxylate synthetase (P5CS) shows a positive correlation with NaCl concentrations up to 150 mM in shoot and root of *A.tricolor*, but *P. vulgaris* showed decrease in the activity of P5CS at 100 mM NaCl concentration ($P < 0.05$) (Fig. 7). The trend of change in the activity of P5CS corroborates with the proline accumulation in different NaCl treatments in the species. The level of proline degrading enzyme, proline oxidase (PO) activity was inhibited to a large extent in both shoot and roots of *A.tricolor* by

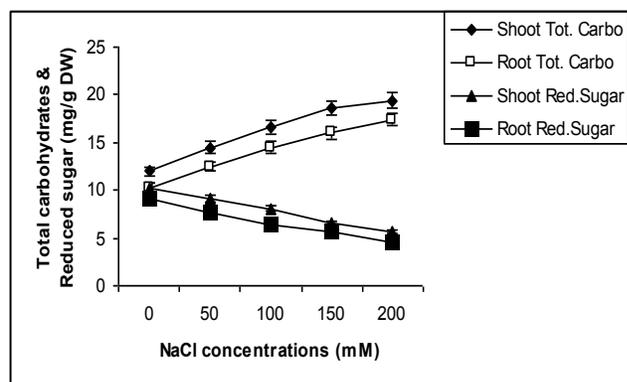


Figure 1. Total carbohydrates and reduced sugar in shoots and roots from NaCl-treated *A.tricolor* 18 days after starting NaCl treatment.

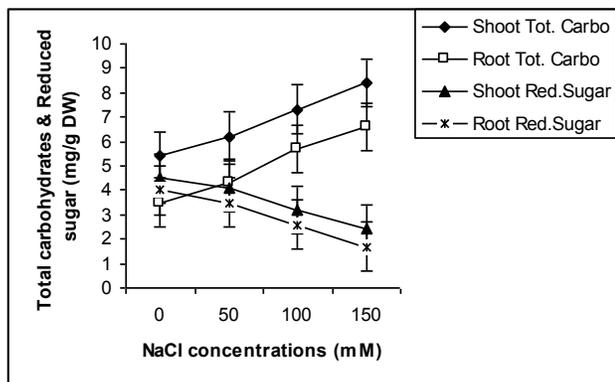


Figure 2. Total carbohydrates and reduced sugar in shoots and roots from NaCl-treated *P.vulgaris* 18 days after starting NaCl treatment.

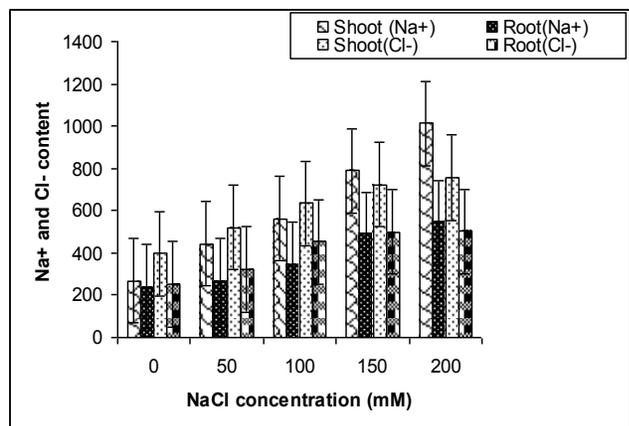


Figure 3. Ions distribution (Na^+ and Cl^- content $\mu\text{mol/g DW}$) in NaCl -treated *A.tricolor* 18 days after starting NaCl treatment.

NaCl stress (Figure 8), with the lowest activity was recorded at 150 mM of NaCl concentrations (50 mM in *P.vulgaris*) compared with control.

In plants, higher accumulation of Na^+ and Cl^- requires compatible solutes for osmotic adjustment under salinity stress (Kavi Kishor *et al.*, 2005). In this study, accumulation of Na^+ and Cl^- found in the species as a response to changing salinity stress levels. However, a different pattern of Na^+ and Cl^- distribution in each part of the two species was clearly seen with increasing salinity. *A.tricolor* has a higher accumulation of the ions in the shoots, but *P.vulgaris* in roots. The ability to translocate Na^+ and Cl^- from roots and transfer them to shoots is considered to be one of the mechanisms of salt tolerance in *A.tricolor*. High accumulation of the excessive salts in shoots indicates there is some mechanism to reduce detrimental effect of the ions in the shoot cells. Localizing Na^+ and Cl^- in the

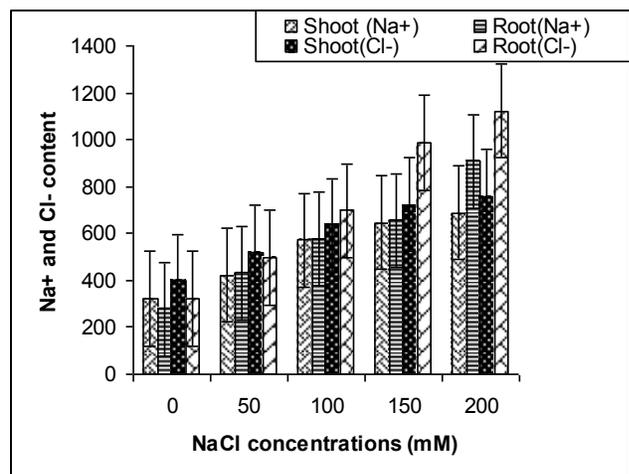


Figure 4. Ions distribution (Na^+ and Cl^- content $\mu\text{mol/g DW}$) in NaCl -treated *P.vulgaris* 18 days after starting NaCl treatment.

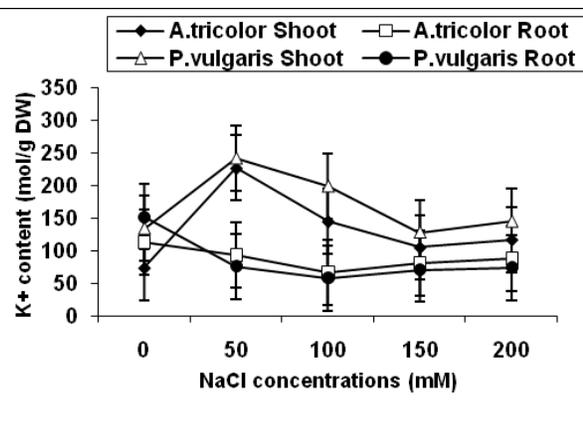
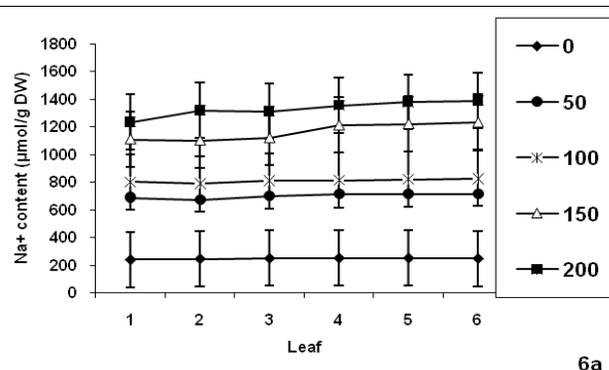
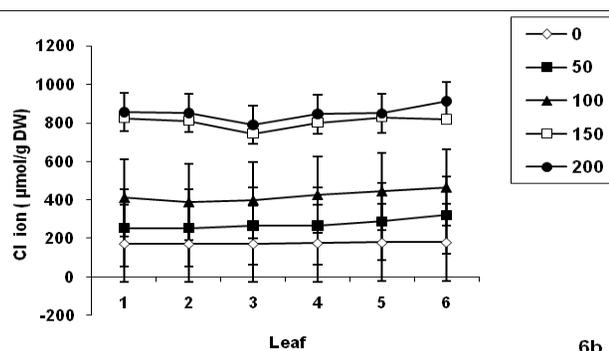


Figure 5. Ions distribution (K^+ mol/g DW) in NaCl -treated *A.tricolor* and *P.vulgaris* 18 days after starting NaCl treatment.

vacuole and balancing by compatible solutes within the cytoplasm was reported as the important salt tolerant mechanisms at cellular level (Bremberger and Lutge 1992). It may likely indicate that *A.tricolor* has an ability to translocate the ions and hold them in the leaves. This physiological process may be important to reduce the salt toxicity in and away from the root cells. As a result the plant could



6a



6b

Figure 6 a and b. Distribution of Na^+ and Cl^- in leaves (a and b), and 1st – 6th leaves of *A.tricolor* 14 days after starting NaCl treatment. \diamond 0 mM, \blacksquare 50 mM, \blacktriangle 100 mM, \square 150 mM, \bullet 200 mM of NaCl . Vertical bars indicate standard errors ($n = 3$).

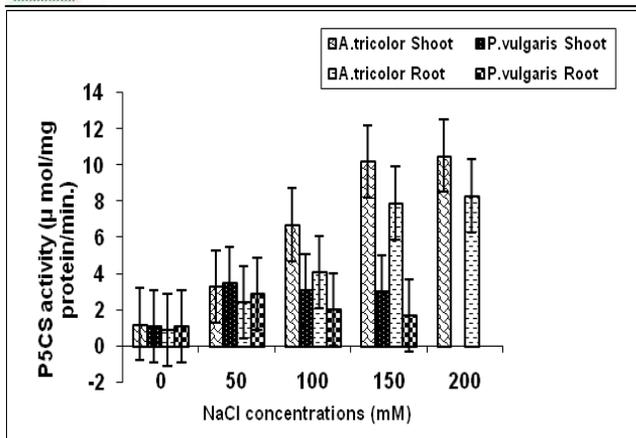


Figure 7. Pyrroline 5-carboxylate synthetase (P5CS) activity in shoot and roots of *A.tricolor* and *P.vulgaris* 18 days after starting NaCl treatments.

survive even at the higher salinity levels to which were subjected during this study.

In the present study, accumulation of total free amino acids, proteins and proline was observed in the roots and shoots of both species. However, at higher NaCl concentrations seedlings of *P. vulgaris* died; suggesting that total protein or proline does not help in reducing salt damage in this plant. On the contrary, these concentrations had no detrimental effect on the seedlings of *A.tricolor*; this indicates that higher proline accumulation may contribute to the alleviation of NaCl stress in the plant. Our results indicating that increasing or decreasing of protein content in plants exposed to salt stress is relatively genotype dependent. The effect of salt-stress on protein content depended on the concentration of NaCl. At lower levels of NaCl, there was an increase in protein content, but higher concentrations caused it to decline in both shoot and root (above 250 mM). This suggests that the initial response to salt stress involves increased

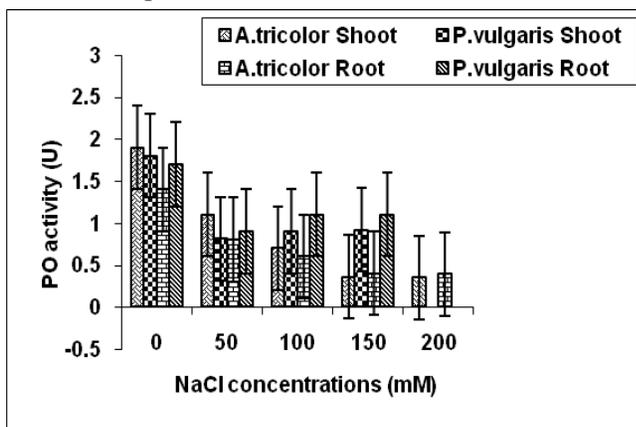


Figure 8. Proline oxidase (PO) activity in shoot and roots of *A.tricolor* and *P.vulgaris* 18 days after starting NaCl treatment.

protein synthesis that is prevented when the stress becomes too severe. It would be predicted that plants under stress would have a powerful protein turnover machinery to degrade stress damaged and environmentally-regulated proteins. Proline generally serves as a physiologically compatible solute that increases in order to maintain a favorable osmotic potential between the cell and its surroundings. Proline accumulation under stress conditions may either be caused by induction or activation of enzymes of proline biosynthesis or a decreased proline oxidation to glutamate or decreased utilisation of proline in protein synthesis, or enhanced protein turnover (Delauney, and Verna, 1993). The expression of genes encoding key enzymes of proline synthesis (P5C synthase, P5C reductase,) and proline oxidation (proline dehydrogenase) is controlled by osmotic and salinity stress and precedes the increase or decrease of proline concentration in plant tissue (Claussen, 2005). With increasing of NaCl, the proline content of two species increased significantly. Proline accumulation in shoots and, mainly, in roots is considered as a marker to salt stress that may be used to select plants with different degrees of tolerance. Proline may also act as a signaling/regulatory molecule able to activate multiple responses that are component of the adaptation process (Sankar Ganesh et al., 2009).

Change in soluble sugars content under salt stress has already been reported for a number of species (Nyagah and Musyimi, 2009). Higher amount of carbohydrates may be one of the reasons responsible for higher salt tolerance in *A.tricolor*. In the preliminary part of this study we have found out that *A.tricolor* has higher seed germination and better growth under salt stress conditions.

Some halophytes are known to accumulate salts in their leaves under salinity stress (Greenway and Munns, 1980). Glycophytes, on the other hand, respond to salinity basically by ion inclusion. The majority of these species accumulate high levels of Na^+ in their roots and stems (Flower, 1991). At lower NaCl concentration, an ability to localize Na^+ and Cl^- ions in root cells seemed to be important to alleviate salinity stress in *P. vulgaris*. At higher doses of NaCl, however, they could not resist toxicity of salt resulting in wilting and death of the plant. The higher Na^+ and Cl^- accumulation in leaves of *A.tricolor* than *P. vulgaris* may indicate that leaf cells of *A.tricolor* has mechanism to tolerate higher ions concentration. The replacement of K^+ by Na^+ observed in *P. vulgaris* was



considered to be similar with the previous report in tomato in which the replacement occurred progressively with increasing absorption of Na^+ , and the absorbed Na^+ was transported to the stem (Besford, 1978). The high concentration of NaCl (150 mM) is not a lethal dose of *A.tricolor*, while the lowest concentration (50 mM) proved too strong dose for the shoots of *P. vulgaris*. Greater accumulation of these ions in the older leaves has been reported in several other plant species and this function is considered to be effective in avoiding huge accumulations of the toxic salts in the growing young leaves (Wahome et al, 2001).

Under salt stress one of the mechanisms of salt tolerance is accomplished by uptake and accumulation of inorganic ions, mainly Na^+ , K^+ and Cl^- (Dionisio-Sese and Tobita, 2000). In our study under salinity, *A.tricolor* accumulated high level of Na^+ in shoots in comparison with *P.vulgaris*. The rise in Na^+ concentrations in the shoots lower the osmotic potential, so contributing to the maintenance of water potential difference between the shoots and the medium required to obtain water from the saline solution (Dionisio-Sese and Tobita, 2000). Na^+/K^+ ratio may serve as an indicator of crop tolerance to stress as the increase of Na^+ in salt tolerant species is generally associated with a decrease in K^+ (Dionisio-Sese and Tobita, 2000). The increase in Na^+/K^+ ratio in leaf and root up to 150mM NaCl and decrease at higher salt concentrations in *A.tricolor* suggests that *A.tricolor* is relatively more salt tolerant or they may have defense for salt adaptation.

Enhancement of proline under stress may be due to increase in the activity of P5CS. The enzyme P5CS is known to catalyze the rate limiting step reaction in proline synthesis (Song et al., 2005). Transgenic plants over expressing P5CS gene show increased proline levels due to increased P5CS activity. Such plants were reported to possess greater tolerance to osmotic stress (Song et al., 2005). Similarly, proline oxidase oxidise the proline to glutamate and the determinant enzyme in proline accumulation (Kavi Kishor et al., 1995). The relationships found between the PO activity and the proline concentrations ($r = -0.83^{**}$) suggest that proline accumulation depends on the reduction or inhibition of PO activity (Jaleel et al., 2007e). The decrease in proline oxidase activity with concomitant increase in P5CS activity might be the reason for higher proline accumulation in *A.tricolor* than *P.vulgaris* during NaCl stress.

CONCLUSION:

The results obtained showed that two genotypes studied, *A.tricolor* was the salt-tolerant and *P.vulgaris* the salt-sensitive species. Sodium and Cl^- accumulation in the roots as a result of salt stress appeared to play an important role in the acclimation of the genotype to salt stress, suggesting that they could be used as physiological markers during the screening for salt tolerance. Total protein, carbohydrates, proline and PRO and P5CS enzymes responded distinctly to NaCl stress, suggesting divergent of response mechanisms in the species. Genetic evaluation of genotypes based on salt tolerance indices could be exploited in the breeding of salt tolerant genotypes.

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