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ORIGINAL ARTICLE

Salt Stress responses on Protein Profile in *Vigna unguiculata* L.

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Key words: Salt stress; Protein; SDS- PAGE; Isoperoxidase; Proline.

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Environmental stress could be defined in plants as any change in growth within the plants natural habitat, which alters or disrupts its metabolic homeostasis. A few examples for plant metabolites involve in biotic/ abiotic stress response include compounds such as polyols, mannitol and sorbitol; dimethyl sulfonicum compounds such as dimethy sulfoniopropionate, glycine, betains, sugar such as sucrose, trehalose and fructose; or amino acids such as proline and protein that serve as osmolytes and osmoprotectant to protect plants under extreme salt, drought and desiccation stresses. Salination is a widespread agricultural problem affecting 20% of the world's irrigated crop plants, and many other regions of the earth designated as arid and desert lands (Yamaguchi and Blumwold, 2005). Salt stress, drought and cold elicit a broad range of physiological and gene expression responses in

plants (Zhu, 2002; Seki *et al.,* 2003; Fujita *et al*., 2006; Yamaguchi- shinozaki and Shinozaki, 2006; Liu *et al*., 2007; Siddiqui *et al.,* 2008). When plants are exposed to salinity in laboratory experiments, there is a rapid and temporary drop is growth rate followed by a gradual recovery to a new reduced rate of growth. The subsequent changes in growth rate and the underlying molecular or metabolic events are not so easily ascribed to water stress or to salt specific effects. As sessile organisms, plants are necessarily exposed to changing environmental conditions, often unfavorable. This situation has led them to develop evaluative strategies to recognize different environmental stresses and activate appropriate response.

An important adaptation found in many organisms subjected to water and other stresses is to accumulate certain organic compounds such as sucrose, amino acids (especially Proline) and several others that lower the osmotic potential and thus the water potential in cells without limiting enzyme function. Singh *et al*., (1985) and Yancey *et al*., (1982) observed the synthesis and accumulation of osmoprotective low molecular weight metabolites in plant responses to salts stress. Plant metabolic analysis has been used to examine the effects of gene deletions and transgenes (Roessner *et al.,* 2000, 2001; Weckwerth *et al.,* 2004), to elucidate the effects of physiological processes and physical stresses such as extremes of salinity or temperature (Johnson *et al.,* 2003; Kaplan *et al.,* 2004; Kim *et al.,* 2007) and to study the genetics of metabolism (Keurentjes *et al.,* 2006). Other applications include screening mutant plant populations, plant food sources and medicinal herbs or genetically manipulated (GM) crops to identify biomarkers relating to desirable or undesirable traits. Based on this background, the present investigation was initiated to extend the good work already done in our laboratory on other equally important species *Vigna unguiculata* L. The specific objectives of the investigation are to 1). Make a comparative study of the influence of NaCl on growth and development of *Vigna unguiculata* seedlings 2). Assess the relative changes of protein pattern, isoperoxidase profiles and proline content in different developmental stages 3). Elucidate the salt tolerant, salt sensitive and salt inducible proteins of *Vigna unguiculata*.

MATERIALS AND METHODS

Seeds of *Vigna unguiculata* L. were obtained from the TNAU, Coimbatore. Liquid nutrient media of two different formulations Knudson (KC) and Murashige and Skoog's medium were used for the experiments. The pH of the media were adjusted to the suitable pH (5.8) using pH meter with IN NaOH/IN Hcl before adding 0.5% (w/v) agar and dissolving it by heating at 80° C in a microwave over for solid media. The media were sterilized by autoclaving at 121° C under 1.1 kg cm-1 pressure for 15 min in an autoclave. The seeds were surface sterilized with 0.1% $HgCl_2$ & 0.1% sodium lauryl sulfate solution for 3-5 min and rinsed thrice with sterile distilled water. Influence of Nacl (0, 4%, 6%, 8%, 10%, 12%, 14% and 16%) on seedlings of *Vigna unguiculata* was analyzed. The seedlings were grown in the department for a period of 10 days. The randomly collected whole plants were used as a source for protein isolation. The soluble proteins present in the extract were precipitated with 10% trichoroacetic acid and the pellet obtained was dissolved in 0.1 N NaOH. The proteins were estimated by the method of Lowry et al. (1951). SDS – Poly Acrylamide Gel Electrophoresis was carried out at 25°C in the air conditioned room. Separation of protein was carried out at 50 v till the tracking dye

reaches the separating gel and at 100 v thereafter for 3-5 hours or until the tracking dye had migrated to the bottom of the gel. After electrophoresis, the gels were carefully removed from the mold and subjected to activity staining (Anbalagan, 1999). For peroxidase (EC 1.11.1.7), 500 to 1000 mg of freshly harvested young leaves were taken and homogenized with 3.5 ml of ice-cold 0.1M phosphate buffer (pH 7.0) in a pre-chilled pestle and mortar and centrifuged at 12,000 rpm for 10 min and the supernatant was collected and used for iso enzyme (peroxidase) analysis (Smila *et al.,* 2007). The Poly acrylamide gel electrophoresis was performed by Anbalagan (1999) method. The staining and fixation of the enzyme was performed by the Sadasivam and Manickam (1992) method. For the quantitative estimation of proline the standard procedure was followed (Bates *et al.,* 1973).

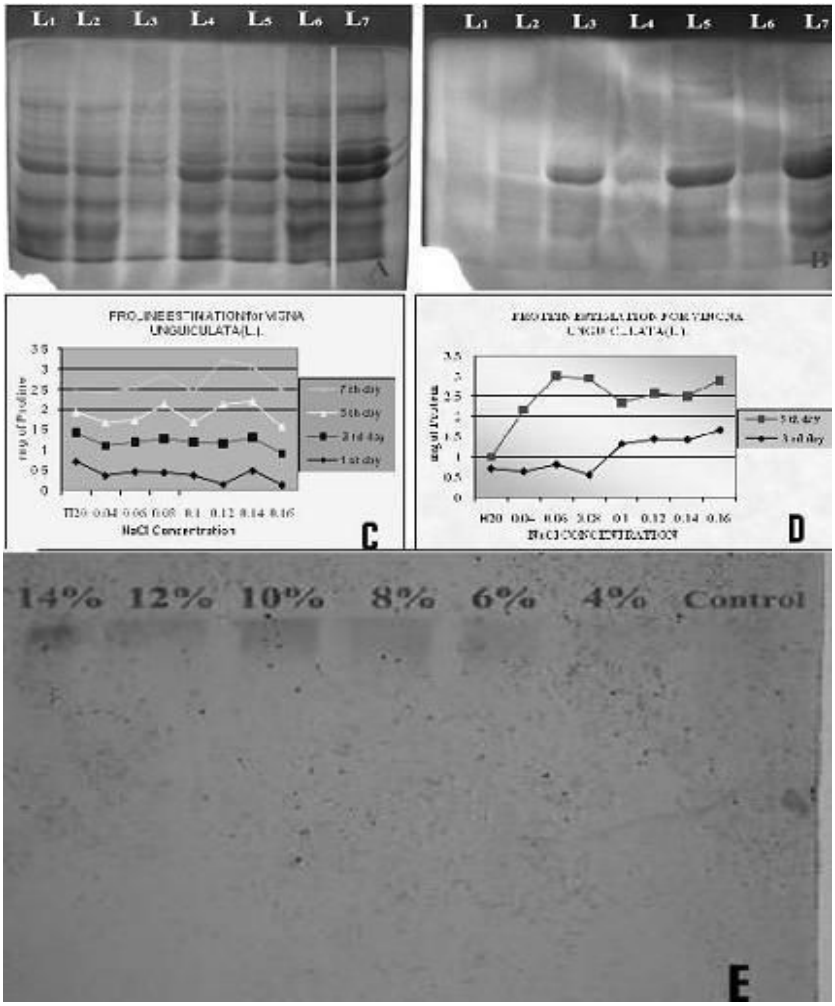

Figure 1 Salt Stress responses on Protein Profile in *Vigna unguiculata* L.

A) SDS – PAGE Profile of 3rd day seedlings treated with different concentrations of NaCl
B) SDS – PAGE Profile of 5th day seedlings treated with different concentrations of NaCl
C) Graphical view of the protein level at various stress conditions
D) Graphical view of the proline level at various stress conditions
E) Isoperoxidase expression of salt treated and control seedlings

RESULTS

Seeds of *V. unguigulata* soaked for 24h, 48h and 72h in 1% Nacl, showed different percentage of seed germination and seedlings growth. The seeds soaked for more than 24 h showed very less percentage of germination and growth. The germinated seedlings were cultured on MS medium augmented with various percentage viz., 0, 2, 4, 6, 8, 10, 12 and 14 of salt (Nacl). The seedlings showed the maximum tolerance up to 10% of Nacl. The exomorphic character such as shoot length, fresh weight and dry weight were also affected differently at diverse concentrations. The Nacl treated plants showed that the reduction in height was progressively and consistently increased with increasing Nacl concentrations. The graphical view of the protein at different stage of the seedlings was shown in Fig 1C. The prolines of seedlings at different developmental stages were illustrated in Fig. 1 D.

Table 1 : SDS – PAGE banding pattern of Different concentrations of NaCl treated *Vigna unguiculata* L. seedlings on third day

MW - Rf	Different Concentration of Nacl percentage						
	Control (0%)	4%	6%	8%	10%	12%	14%
0.012	+	-	-	-	-	-	-
0.192	-	-	-	+	+	-	-
0.200	+	-	-	-	-	-	-
0.204	-	-	-	-	-	+	+
0.253	-	-	-	+	-	-	-
0.265	+	-	-	-	+	+	+
0.289	+	+	+	+	-	+	+
0.304	-	-	-	-	+	-	-
0.313	+	+	+	+	+	+	+
0.326	-	-	+	-	+	-	-
0.337	+	+	-	-	-	+	+
0.385	+	-	-	-	-	-	-
0.409	-	-	-	-	-	+	+
0.434	+	-	-	-	-	-	-
0.445	-	-	+	+	+	+	+
0.457	-	+	-	-	-	-	-
0.481	-	+	+	+	+	+	+
0.518	+	+	+	-	-	-	-
0.542	-	-	-	+	+	+	+
0.602	-	-	-	-	+	+	+
0.638	-	-	-	-	-	+	+
0.650	+	-	-	-	-	-	-
0.662	-	+	+	+	+	+	+
0.746	-	-	-	-	-	+	+
0.783	-	-	-	+	+	+	+
0.795	+	+	-	-	-	-	-
0.831	-	-	-	-	+	-	-
0.843	-	-	-	+	-	-	-
0.867	+	-	-	+	+	+	-
0.891	+	+	+	-	-	-	-

Proteome analyses have identified many proteins that are inducible by salt stress including the metabolism related genes. The relative positions of the protein bands as revealed by SDS- PAGE of the *V. unguiculata* seedlings under different stress conditions are shown in Fig 1A and 1B. The

seedlings treated with various concentrations of Nacl at third day showed maximum of 85 bands, with nine active regions and their MW-Rf values ranged from 0.012 to 0.891. The seedlings treated with same experimental set up at fifth day showed maximum number of 63 bands with eight active regions and their MW-Rf values ranged from 0.108 to 0.891. Fig 1A and B illustrated the banding profile of salt stressed and control seedling of *Vigna unguiculata*. On the 3rd day the isoperoxidase expression was not obtained. On 5th day seedlings showed the isoperoxidase expression with various sizes of bands (Fig. 1 E). Maximum size of band was observed in the seedlings treated with 8 percentage of Nacl. The isoperoxidase expression was observed in the seedlings treated with 6 percentages of Nacl and above.

Table 2: SDS – PAGE banding pattern of Different concentrations of NaCl treated Vigna unguiculata L. seedlings on fifth day

MW - Rf	Different Concentration of Nacl percentage						
	0%	4%	6%	8%	10%	12%	14%
0.108	-	-	-	+	-	-	-
0.152	+	-	-	-	-	-	-
0.163	-	+	-	-	-	-	-
0.173	+	+	+	-	-	-	-
0.195	+	-	+	-	-	-	-
0.217	-	-	+	-	-	-	-
0.239	+	+	+	-	-	-	+
0.260	-	-	-	-	+	+	+
0.271	-	+	-	+	-	-	-
0.282	-	-	+	-	-	-	-
0.304	-	-	-	+	-	-	-
0.326	-	-	-	-	-	-	+
0.358	-	-	-	-	+	+	-
0.380	+	+	-	-	-	-	-
0.413	-	-	+	+	+	+	+
0.434	+	-	-	-	-	-	-
0.456	-	-	-	-	-	+	+
0.467	-	+	+	+	+	-	-
0.489	+	-	-	-	-	-	-
0.500	-	-	-	-	+	-	-
0.505	-	-	-	-	+	+	-
0.565	-	-	-	-	+	+	+
0.597	+	+	+	-	-	-	-
0.619	-	-	+	+	-	-	-
0.706	+	-	-	-	-	-	-
0.765	+	+	-	-	-	-	-
0.717	-	+	-	-	-	-	-
0.760	-	-	+	-	-	-	-
0.782	-	-	-	+	-	-	-
0.836	+	-	-	-	-	-	-
0.869	-	+	+	+	+	-	-
0.891	-	-	-	+	+	+	+

The protein profiles were classified in to three categories based on their expression viz., salt tolerant protein, salt inducible protein and salt sensitive protein. The present study elicited following salt inducible protein viz., MW- Rf 0.192, 0.204, 0.253, 0.301, 0.325, 0.406, 0.445, 0.457,

0.542, 0.602. 0.662. 0.746, 0.783, 0.831, 0.843 and 0.867 on the third day seedling of *Vigna unguicualta*. The following salt inducible proteins MW – Rf 0.108, 0.163, 0.217, 0.260, 0.271, 0.282, 0.304, 0.326, 0.358, 0.413, 0.456, 0.467, 0.500, 0.505, 0.565, 0.619, 0.717, 0.760, 0.782, 0.869 and 0.891 were identified on the 5th day salt stressed seedling of *Vigna unguiculata*. The following proteins MW – Rf. 0.173, 0.195, 0.239, 0.265, 0.289, 0.313, 0.337, 0.380, 0.518, 0.597, 0.765, 0.795 and 0.891 were expressed in the control and salt stressed seedling, they are called as salt tolerant proteins. The following proteins MW-Rf. 0.012, 0.152, 0.200, 0.385, 0.433, 0.489, 0.650, 0.706 and 0.836 failed to express in the salt treated seedling, they are called salt sensitive proteins.

DISSUSSION

Growth of any organ is associated with an additional synthesis of protein which are building blocks of protoplasm and are again the resultant on inter- mediatory metabolism. The soluble protein showed a gradual increase during the initial stage and after fifth day there was gradual decrease in their content. This may be due to reduction in chlorophyll contents. The leaves were turned pale yellowish green. This is a symptom of chlorosis. The chlorophylls in photosynthetic pigments established a complex with protein and when chlorophyll was decreased, there was decrease in protein content also.

Proline generally alleviated the inhibitory effect of salinity on the studied parameters. This alleviation was generally associated with K+/Na+ ratio of shoots and roots. Hegde and Joshi (1974) found that K+ / Na+ ratio was higher in salt tolerance than sensitive cultivars and recommended it as a suitable selection criterion for salt tolerance. It is clear from Fig 1 D that proline is very high at every period of salt stress. Our result depicting proline accumulation is in agreement with other reports (Routely, 1966). The greater accumulation of proline in these plants, presumably render them drought tolerant. A possible reason for this increased level of proline during the salt stress could be an alteration in the activities of the enzymes involved in the biosynthesis and degradation of proline. Proline is thought to play a multifunctional role in the defense mechanisms. It acts as a mediator of osmotic adjustment, a stabilizer of subcellular structure, a scavenger of free radicals, an energy salt and stress related signal (Nanjo *et al.,* 2003).

A strong correlation between the accumulation of proline tolerance of drought stress has been demonstrated by over expression of the Δ' pyrroline- 5- carboxylatesynthesis gene p5cs or by anti-sense suppression of the proline dehydrogenase (proDH) gene in various plants. Seedlings of *Vigna unguiculata* exposed to different environmental conditions exhibited a plethora of physio-chemical responses. Salt stress causes the production of reactive oxygen radicals or species (ROS). Mechanisms of ROS detoxification exist in all plants and can be categorized as enzymatic (SOD, APX, POX, GR, etc) and non-enzymatic (AA, flavanones, anthocyanins, etc) (Shao *et al.,* 2008). The results of the present study also confirm the Shao et al., observation. Results of the present study also showed the isoperxidase content acceleration in accordance with the salt stress. In conclusion, the data presented here revealed that salinity induced changes in protein and isoperoxidase profiles in seeds and seedling of *Vigna unguiculata.* It is necessary to make further study on the structural and functional roles of these salt tress responsive polypeptide to enhance our understanding of the salt stress responses in *Vigna unguiculata*.

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