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Effect of exogenous proline on protein pattern changes in *Citrus sinensis* (L.) Osbeck under *in vitro* salt stress

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Abstract. Effect of exogenous proline treatment on growth and protein pattern changes of plantlets of *Citrus sinensis* (L.) Osbeck cv. Local orange under NaCl stress was investigated. Under salt stress conditions, the height and leaf number of the plantlets decreased significantly, whereas the addition of proline to the culture media significantly alleviated the inhibitory effect of salinity. SDS-PAGE analyses of extracted protein revealed that plantlets grown under NaCl stress (10, 40 and 50mM) showed the presence of a high molecular weight protein (82.7, 81.3 and 81.5 kDa). The addition of proline at 50 and 75mg l^{-1} to the culture media induced the synthesis of three new proteins (20.4-21.6, 40.8-41.4 and 69.7-70.2 kDa). It is concluded that exogenous proline treatment induced the synthesis of salt responsive proteins, in addition to its well known physiological roles.
Key Words: *Citrus sinensis* (L.), Proline, SDS-PAGE, salt stress.

Introduction. Salinity is generally detrimental to plant growth, adversely affect plant metabolism and causes important modification in growth, development and gene expression of plants. Such modifications may lead to the accumulation or depletion of certain metabolites, alteration in the behavior of many enzymes, overall changes in protein synthesis, and of particular interest is the synthesis of new proteins (Dubey 1999). Several researchers have shown the synthesis of new proteins in cultured plant cells when they are subjected to salinity (Erickson & Alfinto 1984; Singh et al 1985; Bressan et al 1988; Ben-Hayyim et al 1989; Amini et al 2007). These specifically synthesized proteins under salt stress appear to have a role in providing tolerance or adaptation to the plants, although the mechanism of how such new proteins could provide adaptation to salinity is far from clear.

Compatible solutes, such as proline, are known to accumulate under salt stress in many crops (Munns & Tester 2008). The exogenous application of proline has been suggested to be an effective approach in improving salt tolerance. Although, in the past most attention has been concerned with the role of proline as compatible solute and osmoprotectant. Its further role in salinity appears to involve the induction of salt responsive genes, with the resultant formation of new proteins which may improve the adaptation to salinity stress (Khedr et al 2003).

Citrus is an important fruit crop worldwide. It is very sensitive to salinity, its critical level of salinity is 17mM NaCl, with production affected adversely with increasing salinity, up to 80Mm NaCl, where production is decreased by 50% (Storey & Walker 1999).

No information is available in the literature regarding the effect of exogenous proline application on protein pattern changes in citrus under *in vitro* salt stress. Accordingly, the present work was undertaken to study the effect of exogenous proline on protein pattern changes in plantlets of *Citrus sinensis* (L.) Osbeck cv. Local orange under NaCl stress. Such information is utmost important to understand the molecular basis of salinity tolerance in crop plants.

Material and Method

The experiment was carried out at the College of Agriculture, Basrah University, Basrah, IRAQ. Source of plant material, preparation of explants, callus induction, formation of the primary callus, organogenic callus induction, adventitious shoot proliferation and plantlet regeneration have been described in detail by Al-Taha (2008).

Effect of NaCl, proline and their interaction on growth of the plantlets. Uniform regenerated plantlets via indirect organogenesis, were selected and cultured on basal medium consisted of Murashige & Skoog (1962), salts supplemented with sucrose 25g, 100mg myoinositol, 2mg glycine, 200mg glutamine, 1mg nicotinic acid, 1mg biotin and 50g agar (per liter). Activated charcoal was added at $500mg^{-1}$. NaCl was added to the 0, 40, and 50mM, while proline was used at 0, 25, 50 and 75 mg l^{-1}. The culture tubes were then incubated at 27±2.0°C and light intensity of 50µmol $m^{-2}S^{-1}$ provided by a cool white fluorescent lamp for 4 weeks, at the end of which, plantlet height and leaf number were determined (Figure 1).

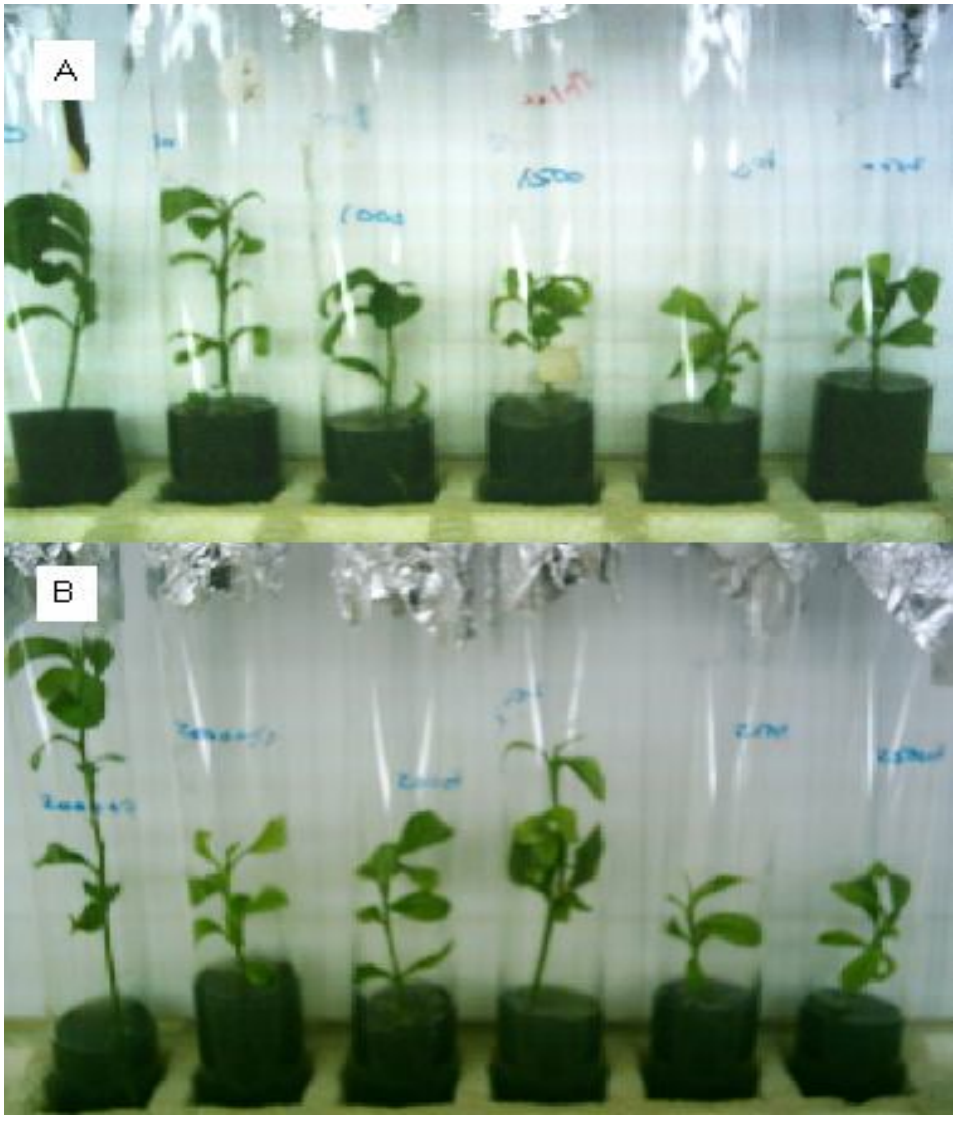

Figure 1. Effect of NaCl, and proline on growth of regenerated plantlets of C*itrus sinensis* cv. Local orange A, from left to right control 10, 20, 30, 40 and 50mM NaCl; B, from left to right 40mM NaCl+ 25mg l^{-1} proline, 40mM NaCl+ 50mg l^{-1} proline, 40mM NaCl+ 75mg l^{-1} proline; 50mM NaCl+ 25mg l^{-1} proline, 50mM NaCl+ 50mg l^{-1} proline , 50mM NaCl+ 75mg l^{-1} proline.

Effect of NaCl, proline and their interaction on protein pattern changes of the plantlets. Protein extraction and SDS- PAGE. Complete plantlet obtained from the previous step were frozen immediately and freeze-dried. The freeze-dried samples were ground just before extraction.

400 mg of the ground tissues were mixed with 1ml of extraction buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS). The extract was centrifuged at 10000 rpm for 6 minutes. Characterization of proteins was carried out using one dimensional sodum dodecyl sulphate polyacrymide as described by Laemmli (1970). Protein samples were prepared by mixing clear super ant with sample buffer (0.12 M Tris-HCl, pH 6.8, 10% SDS, 10% sucrose and 0.1% mercaptoethanol) and denatured by heating at 90°C for 5 minutes, then loaded in equal amounts. Protein bands were separated at constant current, 20 mA for 10 minutes and the current increased up to 144 mA until the tracking dye (Bromophenol blue 0.05%), reached the end of the gel. Protein bands were visualized by staining the gels with 0.1% Comassie Brilliant blue R-250. Relative molecular weight of proteins was determined using standard curve generated from the standard proteins. The standard proteins were as follows: Bovine serum albumin (67kDa), Ovalbumin (43kDa), Pepsin (3kDa), Trypsin (23kDa) and Lysozyme (14.4kDa).

Experimental design and analysis. For the effect of proline, NaCl and their interaction on certain vegetative characteristics of the plantlets, a completely randomized design was used with ten replicates. Revised LSD at 5% level was used to compare mean values (Gomez & Gomez 1984).

Results and Discussion. Table 1 A and B, shows the main effect of NaCl, proline and their interaction on height and leaf number of regenerated plantlets.

Table1

The effect of NaCl (mM), proline and their interaction on the height (cm) (A) and leaf number (B) of *Citrus sinensis* (L.) Osbeck plantlet

A

NaCl concentration (mM)	*Proline concentration (mg l^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	6.38	13.50	11.0	10.50	10.43
40	3.16	9.75	5.16	4.50	5.64
50	3.00	7.83	4.16	3.50	4.62
Mean	4.18	10.63	6.77	6.16	-

RLSD p=0.05 for NaCl=0.29
Proline=0.34
For the interaction=1.62

B

NaCl concentration (mM)	*Proline concentration (mg l^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	7.83	14.00	12.00	11.00	11.20
40	7.16	10.66	8.66	8.33	8.70
50	6.50	9.66	7.33	7.00	7.62
Mean	7.16	11.44	9.33	8.77	-

RLSD p=0.05 for NaCl=0.41
Proline=0.48
For the interaction=0.93

It is clear, that NaCl at both concentrations significantly decreased plantlet height and leaf number. Such results are due to the known inhibitory effects of salinity on growth and physiological presses. Furthermore, it has been suggested, that the reduction of growth in response to salinity is the result of the respiratory energy being diverted toward processes resulting in salt tolerance, rather than growth (Munns & Tester 2008).

The main effect of proline is to increase significantly plantlet height and leaf number, in particular at 25mg^{-1}. Such results are due to the fact that proline is a nitrogen source, respiratory substrate generate ATP, as well as its role in increasing cell division and osmosis. As for the interaction, it was also significant, in that exogenous application of proline significantly alleviated the inhibitory effects of salinity. Similar results were reported by El-Hammady et al (1999) for grape plantlets.

Figure 2 and Table 2, shows SDS-PSGE of proteins extracted from orange plantlets subjected to various concentrations of NaCl. It is obvious, that extracts from control plantlets showed the presence of two proteins with molecular weights of 32.6 and 62.7kDa respectively. However, when the plantlets were subjected to NaCl at concentrations of 10, 40 and 50mM, only one additional novel protein appeared on the gel with molecular weights of 82.7, 81.3 and 81.5kDa for the above concentrations of NaCl respectively. The addition of proline to the culture media of the plantlets induced the formation of three novel proteins. For the treatment of 40Mm NaCl+50mg l^{-1} proline, the following proteins appeared on the gel: 21.6, 30.1, 40.8, 61.3, 69.9 and 81.6kDa. Similarly, for the treatment of 40Mm NaCl+75mg l^{-1} proline, the following proteins appeared on the gel: 20.4, 34.5, 41.4, 62.5, 70.2 and 82.2kDa.

Table 2

Molecular weight of proteins extracted from *Citrus sinensis* (L.) Osbeck cv. Local orange planlet subjected to various NaCl levels in the presence or absence of proline

Treatments	*Molecular weight, kDa*
Control	32.61, 62.75
10mM NaCl	37.0, 61.8, 82.7
40mM NaCl	30.9, 61.5, 81.3
50mM NaCl	34.0, 61.5, 81.5
40mM NaCl+ 50mg l^{-1} poline	21.6, 30.1, 40.8, 61.3, 69.7, 81.6
40mM NaCl+75 mg l^{-1} proline	20.4, 34.5, 41.4, 62.2, 70.2, 83.1

Results presented in this paper shows, that subjecting local orange plantlets to various concentrations of NaCl in the presence or absence of proline induced the formation of a high molecular weight proteins (82.7, 81.3, 81.5, 81.5 and 82.2kDa). This high molecular weight protein is considered as a unique protein associated with NaCl adapted plantlets cells, and it is specific for such cells, as it was not present in plantlets growing without NaCl. It is possible, that this unique protein is synthesized in salt adapted cells of local orange plantlets, and may be involved in the salt adaptation process. Furthermore, this process of cellular adaptation of the plantlets to salinity stress, involves the specific alteration of gene expression of NaCl adapted cells leading to the synthesis of this unique protein.

The high molecular protein found in the present work, is different from those reported by other workers. Thus, Ericson & Alfinto (1984) examined the protein pattern of NaCl adapted and NaCl non-adapted cell lines of tobacco and found that cells adapted to a medium containing NaCl, showed two protein bands with molecular weights of 32.0 and 20.0kDa. Furthermore, in the salt-adapted cells, a unique protein of 26.0kDa was found. Similar to tobacco cells, the synthesis of salt induced proteins has also been shown in various plant species.

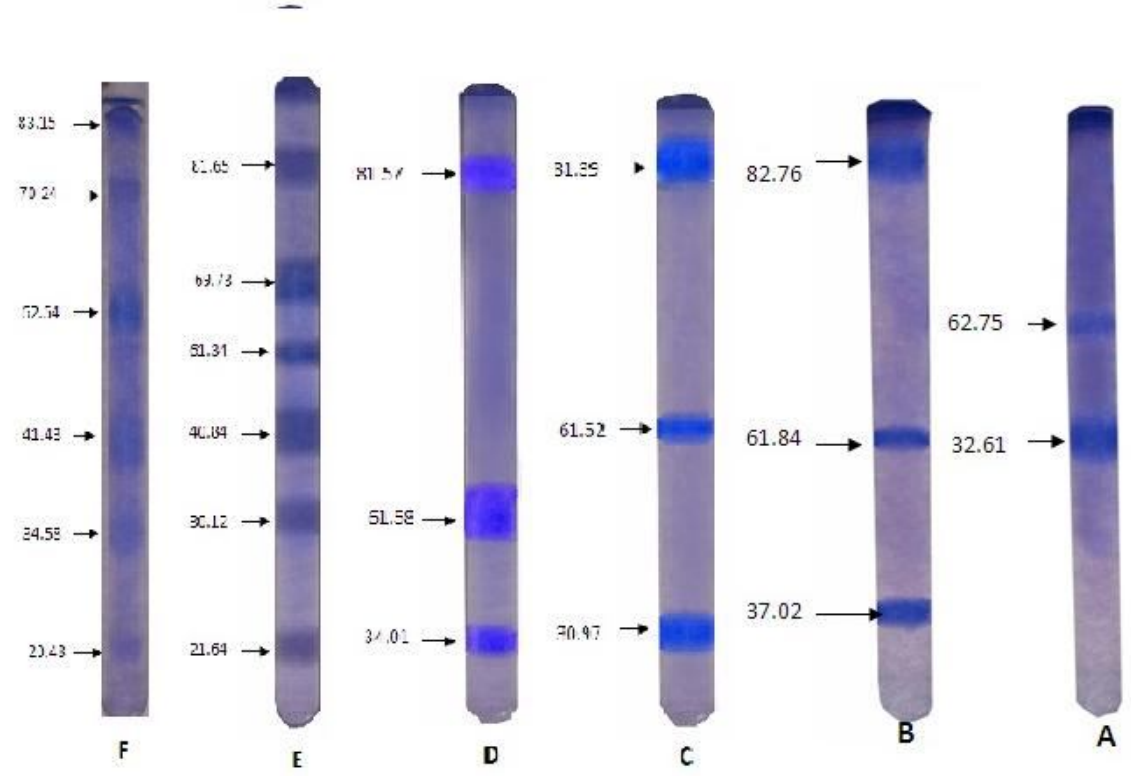

Figure 2. SDS-PAGE of proteins extracted from plantlets of Citrus sinensis (L.) Osbeck cv. Local orange subjected to various concentrations of NaCl in the presence or absence of proline, A: Control; B: 10mM NaCl; C: 40mM NaCl; D: 50mM NaCl; E: 40mM NaCl+ 50mg l^{-1} poline; F: 40mM NaCl+75 mg l^{-1} proline.

In *C. sinensis* Osbeck cv. Shamouti a 26 kDa protein has been shown to be associated with salt tolerance (Ben-Hayyim et al 1989). With olive callus tissues, two new proteins with molecular weights of 24.0 and 40.0kDa accumulated under NaCl stress were reported (Garcia et al 2008). This indicates, that salt-induced changes in proteins are species specific and that different proteins are associated with salt tolerance in different species. It is also clear from Figure 2 and Table 2 that those orange plantlets when grown in a medium with 40mM NaCl and proline, they synthesized extra poly peptides. Thus, when proline was added at 50mg l^{-1}, the following polypeptides appeared on at the gel: 21.6, 40.8 and 69.7kDa. The addition of proline at 75mg l^{-1} to the culture medium with 40Mm NaCl, the following new poly peptides appeared on the gel: 20.4, 41.4 and 70.2kDa. It is possible, that the addition of proline induced the synthesis of these new proteins via its effect on the signal transduction pathways, which regulate salinity stress responsive genes (Khedr et al 2003). Such effects of proline in the induction of synthesis of new proteins, in addition to its previously known physiological roles, are probably, responsible for the improvement of tolerance to salt stress found in the present work. Although salinity-specific proteins are thought to lend a protective role to the tissues against the deleterious effects of salinity, the exact physiological functions of many these proteins are not very clear. Proteins induced under salinity are thought to act as osmoprotectants, as regulatory proteins, or as enzymes of biosynthetic pathways for osmolytes. However, the functions of many proteins are still unknown (Dubey 1999).

Conclusions. In conclusion, the results presented in this communication showed, clearly that salinity inhibited the growth of orange plantlets and that the addition of proline alleviated the inhibitory effects of salinity and promoted plantlet growth. SDS-PAGE of

extracted proteins revealed that salinity induced the formation of a high molecular weight unique protein and the addition of proline to the culture media containing NaCl, induced the synthesis of three new additional proteins. It is very likely, that salinity induced proteins appear to endow plants with the capacity to adapt to salinity by physiological and biochemical adjustments. As for proline, it probably plays an important role in plant adaptation to salinity via its effects on protein synthesis, as well as its known physiological roles.

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