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Effect of proline and salt stress on organogenesis, growth, proline and carbohydrate content of regenerated plantlets in *Citrus sinensis* (L.) Osbeck cv. Local orange

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Abstract. The effect of exogenous proline and NaCl stress on organogenesis, growth and certain chemical constituents (soluble carbohydrates and free proline) in *Citrus sinensis* (L.) Osbeck cv. Local orange in vitro was investigated. Callus cultures of nucleus tissues derived from under developed ovule of immature fruits were used. Primary callus were cultured on different levels of NaCl in the culture media (0, 10, 20, 30, 40 and 50 mM). For interaction experiments, proline was used at 0, 25, 50 and 75 mg L^{-1}, whereas NaCl concentrations were 0, 40 and 50 mM. Organogenic callus was obtained, when this primary callus was cultured on MS medium supplemented with 5.0 mg L^{-1} BA, adventitious shoots were obtained when the organogenic callus was incubated on MS medium supplemented with BA at 1.0 mg L^{-1} and NAA at 0.1 mg L^{-1}. The addition of NaCl at 10 mM significantly increased the number and length of adventitious shoots, but high concentration of NaCl significantly reduced the number and length, this effect was alleviated by the exogenous application of proline, in particular at 25mg L^{-1}. Complete plantlets were developed when the adventitious shoots were transferred to half strength MS medium supplemented with 0.1mg L^{-1} NAA. The addition of NaCl at 10 mM significantly increased plantlet height, leaf number per plantlet, fresh weight of the shoot and root system. However at higher concentration (20 – 50 mM), NaCl significantly decreased all the growth parameters of the plantlets. Proline on its own significantly increased organogenesis, and growth parameters of the plantlets. NaCl significantly decreased soluble carbohydrates of the plantlets and this effect was alleviated by the addition of proline. Proline alone significantly increased carbohydrate levels. NaCl significantly increased free proline of the regenerated plants, and so does exogenous proline treatment. Salt-tolerant plantlets were successfully regenerated, which were transferred to a potting mix containing sand and peat moss (2:1) and grown for 8 months, with a survival rate of 100 %.

Key Words: Soluble carbohydrates, callus cultures, plantlets, salt stress, tissue culture.

Introduction. Salinity is one of the major abiotic stresses, which adversely affect crop productivity and quality, especially in arid and semi arid regions of the world. The problem of soil salinity is further increasing because of the use of poor quality water for irrigation and poor drainage. Adverse effects of salinity on plant growth may be due to ion cytotoxicity, and osmotic stress (Zhu 2003). Most crop plants are susceptible to salinity even when ECe is less then 3.0 ds m^{-1}. At these salinity levels, the predominant cause of crop susceptibility appears to be ion toxicity rather then osmotic stress (Chinnusamy et al 2005).

Citrus is considered as salt sensitive crop, and the critical level of salinity for vegetative growth is 17 mM NaCl, and productivity of the trees decreased by 50 %, when the salinity level is 80 mM NaCl (Storey & Walker 1999).

Plant tissue culture techniques have been used to produce salt tolerance cell lines and plants in several species (Munns & Tester 2008). In citrus, callus cultures have been used by some workers to produce salt tolerant plantlets in *Citrus sinensis* (L.) Osbeck cv. Shamouti (Ben-Hayyim & Kochba 1983). Furthermore, plants subjected to salinity are known to accumulate many organic compounds at high concentration, such as the amino acid proline. There are several report in the literature which showed, that exogenous application proline to cell and callus cultures under salt stress increased salinity tolerance

in tomato (El- Enany 1995), grape (El-Hammady et al 1999), tobacco (Okuma et al 2000) ground nut (Jain et al 2001) and date palm (Jasim et al 2010).

Orange (*Citrus sinensis* (L.) Osbeck cv. Local) is the most important citrus cultivar in Iraq, but it is adversely affected by salinity which accounts for the large decrease in its yield all over the country. Accordingly, the present work was undertaken to regenerate salt tolerance orange trees cv. Local, using plant tissue culture, as well as exogenous application of the amino acid proline. In this communication, the effect of exogenous proline treatment on organogenesis, growth and certain chemical constituents of regenerated plantlets of *Citrus sinensis* (L.) Osbeek, cv. Local orange under NaCl stress is presented.

Material and Method

The experiment was carried out at the plant Tissue Culture Laboratories, Date Palm Research Centre, Basrah University, Basrah, Iraq.

Source of plant materials, preparation of the explants, callus induction and proliferation, organogenic callus induction has been described in details elsewhere (Al-Taha 2013).

Adventitious shoot proliferation. The organogenic callus was incubated on half strength MS medium supplemented with 1.0mg L^{-1} BA (6-Benzyl aminopurine) and 0.1 mg L^{-1} NAA (Naphthalene acetic acid), for the induction of adventitious shoots on the surface of the callus. To the culture tubes, NaCl was added at (0, 10, 20, 30, 40, and 50 mM). The replication was 10-fold. The culture tubes were then incubated at 27 ± 2.0 °C and light intensity of 50 µmol m^{-2} s^{-1} provided by a cool white fluorescent lamp for 8 week, with sub culturing every 4 week, and at the end of which number and length of adventitious shoots were recorded.

The effect of NaCl, proline and their interaction on indirect organogenesis. The organogenic callus grown on 0, 40 and 50 mM NaCl was used for the interaction experiments. Proline was added at 0, 25, 50 and 75 mg L^{-1}. The culture tubes were grown on the same condition as previously described, the replication were 10-fold, and number of adventitious shoot and their length were recorded after 8 weeks (Figure 1 C & D).

Induction of rooting. The newly formed shoots obtained in the previous step were separated with a small amount of callus and transferred to a rooting medium consisting of half strength MS medium supplemented with 0.1 mg L^{-1} NAA. A single shoot was cultured in each tube and the replication was 10-fold. The tubes were cultured on the same condition of temperature and light intensity as refereed to above. Rooted shoots were obtained within 8 weeks of culture on this medium (Figure 1 E).

Effect of sodium chloride on growth of regenerated plantlets. Uniform regenerated plantlets were selected and cultured on basal culture medium consisted of Murashige & Skoog (1962) salts supplemented with (per liter) sucrose 25 g, 100 mg myo-inositol, 2 mg glycine, 200 mg glutamine, 1 mg nicotinic acid, 1 mg biotin, and 5 g agar. Activated charcoal was added at 500 mg L^{-1}.

Then, NaCl was added at the following concentration: 0, 10, 20, 30, 40, and 50 mM. Single plantlets were cultured in each tube and the replication was 10-fold.

The culture tubes were incubated at the same growing conditions of light intensity and temperature. A after 4 weeks, the following growth measurement were taken: plantlet height, leaf number, fresh weight of the shoot and root system (Figure 2 A).

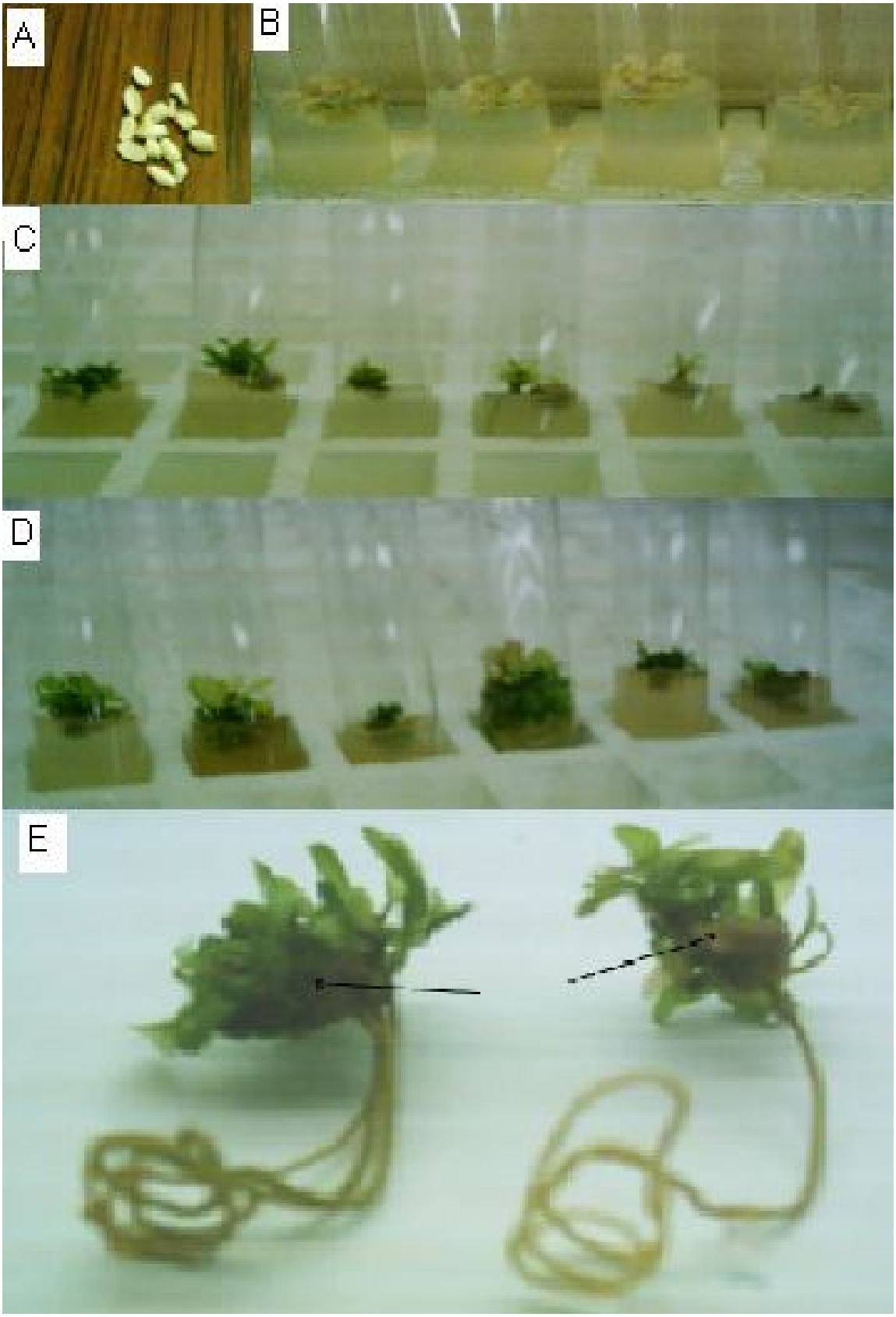

Figure 1. Organogenesis in C*itrus sinensis* cv. Local orange; A - Isolated nucleus tissue; B - Primary callus; C - Effect of NaCl on organogenesis; D - interaction between praline and NaCl on organogenesis; E - rooted adventitious shoots.

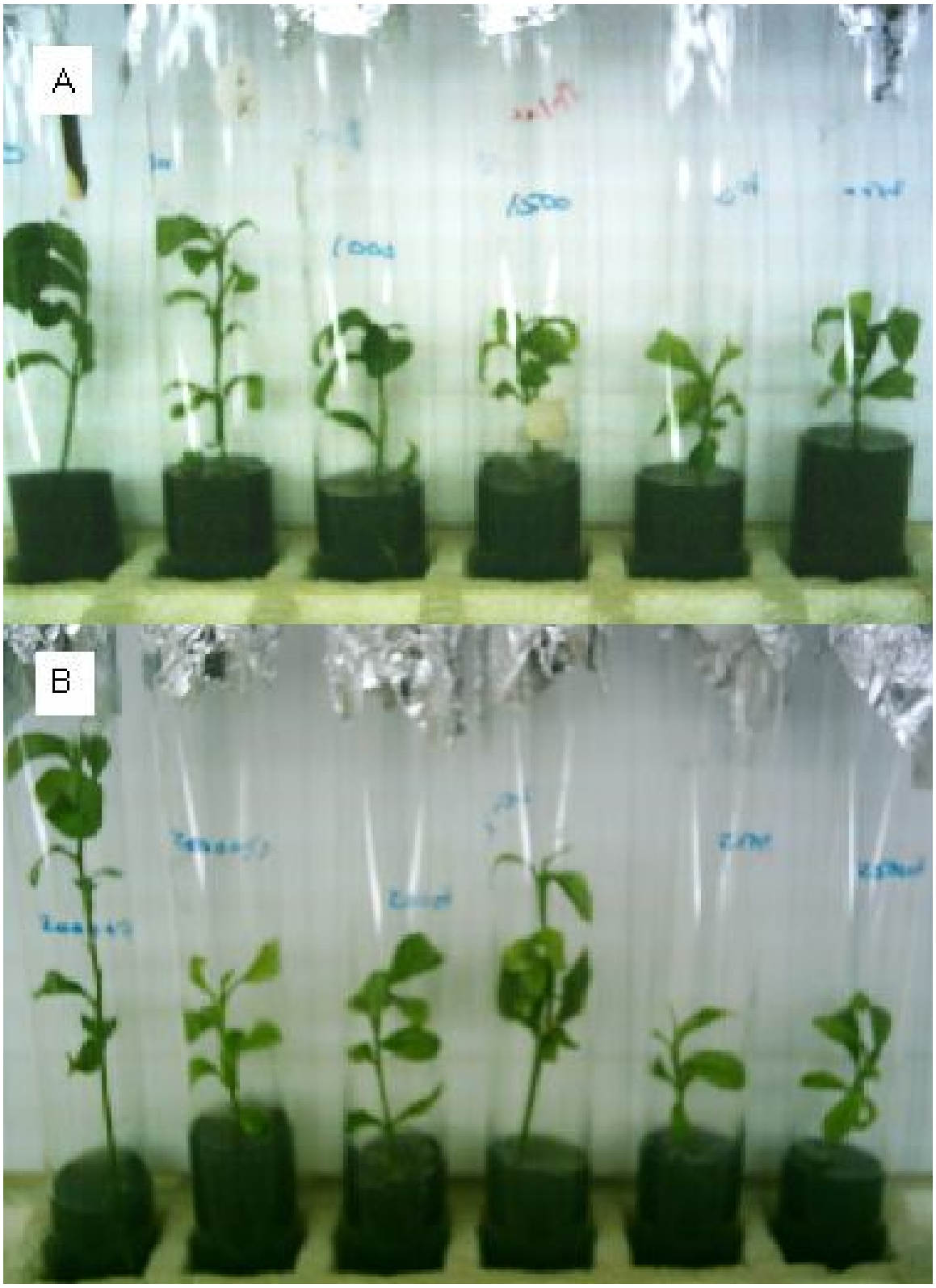

Figure 2. Effect of NaCl, and proline on growth of regenerated plantlets of *Citrus sinensis* cv. Local orange; A - from left to right: control, 10, 20, 30, 40 and 50 mM NaCl; B - from left to right: 40 mM NaCl+ 25mg L^{-1} proline, 40 mM NaCl+ 50 mg L^{-1} proline, 40 mM NaCl+ 75 mg L^{-1} proline, 50 mM NaCl+ 25 mg L^{-1} proline, 50 mM NaCl+ 50 mg L^{-1} proline, 50 mM NaCl+ 75 mg L^{-1} proline.

The effect of NaCl, proline and their interaction on growth of the plantlet. For interaction experiments, NaCl was used at 0, 40 and 50 mM, while the amino acid proline was used at 0, 25, 50 and 75 mg L^{-1}. The plantlets were cultured on the same growth medium, as well the same growing conditions of light intensity temperature for 4 weeks, at the end of which the same growth parameters of the plantlets were determined (Figure 2 B).

Effect of NaCl, proline and their interaction on total soluble carbohydrates and free proline of the plantlets. NaCl was used at the following concentrations: 0, 10, 20, 30, 40 and 50 mM. For the interaction experiment, NaCl was used at 0, 40, and 50 mM, whereas proline conditions were 0, 25, 50 and 75 mg L^{-1}. 500 mg over dry sample were taken after four weeks from culture of the plantlets, and used for the determination of soluble carbohydrates, using the method of Dubois et al (1965). For proline determination 200 mg oven dry samples were used using the method of Troll & Lindsley (1955).

Plantlet acclimation. The process of acclimation was carried out on plantlets having good shoot and root system. The plantlets were removed from the culture vessels and washed with sterilized distilled water to clean the root system from the remains of the growth medium. The plantlets were placed in glass tubes containing distilled water, ensuring the submergence of the root system, for two weeks, during which time the distilled water was changed every two days (Figure 3 A). Then, the plantlets were planted into an autoclaved soil mix containing sand and peat moss (2:1). The plantlets were then covered with a glass cover for 4 weeks (Figure 3 B). The glass covers were removed gradually during the 5^{th} weeks (Figure 3 C). The plantlets were then watered every 2-3 days with liquid fertilizer (NPK) at 100mg L^{-1}. The process of acclimation continued for 8 months, and rate of survival was 100 % (Figure 3 D).

Statistical design and analysis. Completely randomized design was used, with 10 replicates. As for the interaction between NaCl and proline, the experiment was factorial with 10 replicates for organogenesis and parameters of the regenerated plantlets. As for the carbohydrate and free proline, the replication was three-fold.

The data were subjected to the analysis of variance and mean values were compared using revised LSD (Snedecor & Cochran 1980).

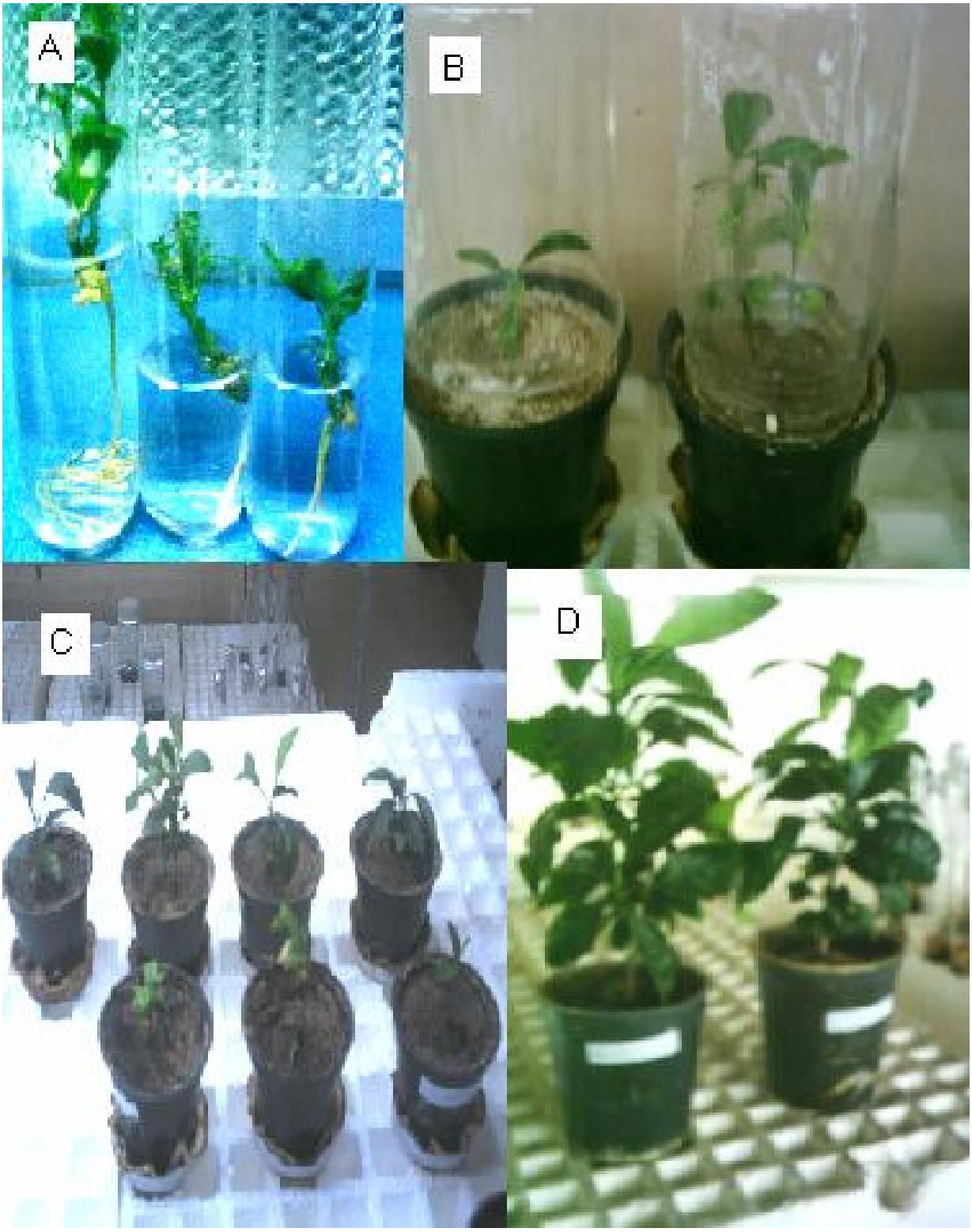

Figure 3. Stages of acclimatization of regenerated plantlets of in C*itrus sinensis* cv. Local orange: A - plantlets in dipping solution (distilled water); B - salt tolerant orange plantlet established in plastic pots and covered with glass covers during acclimatization; C - salt tolerant orange plants established in plastic pos after removal of the glass covers; D - 8-month old salt tolerant plants of local orange ready for planting in orchard.

Results and Discussion. Table 1 show that NaCl at 10 mM caused a significant increase in the number and length of adventitious shoots formed on the surface of callus, as

compared with other treatment. Such increase in the number of adventitious shoots and their length, suggest that such concentration is probably optimal for growth with the resultant increased in the number and length of adventitious shoots. However, as concentration of NaCl increased, there is a significant decrease in the number and length of adventitious shoot produced, in particular at 50 mM NaCl such effects of salinity are probably due to the known effect salinity on growth, including osmotic effect, and ion cytotixicity. Other researchers, have found, that NaCl salinity significantly decreased the number of adventitious shoots in the tomato (El-Enany 1995, 1997).

Table 1

Effect of NaCl concentration (mM) on the number and length (cm) of adventitious shoots formed on the surface of the organogenic callus of *Citrus sinensis* (L.) Osbeck cv. Local orange

NaCl (mM)	*No. of adventitious shoots*	*Length of adventitious shoots*
0	9.333	0.433
10	11.000	0.933
20	5.000	0.500
30	3.333	0.466
40	1.666	0.166
50	0.666	0.066
Revised LSD (p = 0.05)	1.105	0.159

It is obvious from table 2 that NaCl significantly decreased the number and length of adventitious shoots produced on the surface of the organogenic callus of citrus, which is probably due to the inhibitory effects of salinity on growth and physiological processes. However, the main effect of proline was to increase significantly shoot number, in particular at 25mg L^{-1}. Proline is an important compatible solute that plays important roles in osmotic adjustment, stabilizes sub cellular structures, and clears free radicals, nitrogen source, as well as generation of ATP (Ashraf & Foolad 2007). In addition, proline in also known to induce the expression of salt responsive gene, with the resultant formation of new proteins, which increased salt tolerance (Khedr et al 2003).

Table 2

Effect of NaCl (mM), proline and their interaction on the number (A) and length (B) of adventitious shoots (cm)

A

NaCl concentration (mM)	*Proline concentration (mg L^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	9.33	14.00	12.00	11.0	11.58
40	1.50	11.33	5.33	4.0	5.23
50	0.50	6.00	3.33	2.66	3.36
Mean	3.88	10.44	6.88	5.88	-

RLSD p = 0.05 for NaC l= 0.55, Proline = 0.63, for the interaction = 1.21.

B

NaCl concentration (mM)	*Proline concentration (mg L^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	0.433	1.500	1.000	1.000	0.983
40	0.500	0.866	0.666	0.300	0.469
50	0.050	0.733	0.233	0.233	0.336
mean	0.222	1.033	0.633	0.511	-

RLSD p = 0.05 for NaCl = 0.130, Proline = 0.130, for the interaction = 0.384.

As for the interaction, it was also significant, in that the inhibitory effect of salinity on adventitious shoot formation was reduced by the addition of proline. Such effects are probably due to known stimulatory effect of proline on plant growth. Other research, also found that, the addition of proline alleviated the inhibitory effect of salinity and improve shoot formation in the tomato (El-Enany 1995).

Table 3 shows, that NaCl at 10 mM significantly increased plantlet height, leaf number, and fresh weight of shoot and root system in comparison with the control treatment. It is possible, that such low level of salinity is optimal for growth, and therefore stimulating the growth processes such as cell division and cell elongation. As the concentration of NaCl increased, these growth characteristics were significantly decreased, due to the known inhibitory effect of salinity. Similar results were obtained by El-Hammudy et al (1999) for grape plantlets and date palm plantlets cv. Barhi by Al-Kabi (2004), as well as in intact plants, such as tomato by Tawagen et al (2003a).

Table 3

Effect of NaCl concentration (mM) on certain vegetative characteristics of regenerated plantlets of *Citrus sinensis* (L.) Osbeck cv. Local orange

NaCl (mM)	*Plantlet height (cm)*	*Leaf number*	*Fresh weight of shoot system (g)*	*Fresh weight of root system (g)*
0	6.410	7.830	0.231	0.062
10	7.000	9.330	0.242	0.066
20	4.330	7.660	0.174	0.050
30	3.330	7.500	0.156	0.047
40	3.160	7.160	0.148	0.040
50	3.000	6.500	0.144	0.030
Revised LSD (p = 0.05)	0.999	1.230	0.049	0.010

Table 4 underline the main effect of proline NaCl and their interaction on vegetative characteristics of regenerated plantlets. It is clear, that proline at all concentrations significantly increased plantlet height, leaf number, fresh weight of shoot and root system, in particular at 25 mg L^{-1}. These effect are due to the fact that proline is a nitrogen source, respiratory substrate generate ATP, and also increase the process of cell division, as well as its role as osmoticum (Heuer 2010) NaCl at both concentrations, significantly decreased all growth parameters (Table 4 A - D) of the regenerated plantlets. These are due to the known inhibitory effect of salinity on growth and physiological processors. Furthermore it has been suggested that the reduction of growth in response to salinity is the result of the great portion of the respiratory energy diverted toward processes, resulting in salt tolerance rather than growth (Munns & Tester 2008). Similar results were reported by other workers on grape plantlets (El-Hammady et al 1999) and date palm plantlets (Al-kabi 2004), as well as intact plants, such as tomato and grape (Tawagen et al 2003a; El-Morshedy 1995).

Table 4

The effect of NaCl (mM), proline and their interaction on the height (A) and leaf number (B), fresh weight of the shoot system(C) and the root system (D)

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.

C

NaCl concentration (mM)	Proline concentration (mg L^{-1})				
	0	25	50	75	Mean
0	0.231	0.360	0.320	0.304	0.303
40	0.148	0.291	0.225	0.215	0.219
50	0.144	0.253	0.173	0.162	0.183
Mean	0.174	0.340	0.239	0.227	-

RLSD p=0.05 for NaCl=0.001, Proline=0.001, for the interaction = 0.003.

D

NaCl concentration (mM)	Proline concentration (mg L^{-1})				
	0	25	50	75	Mean
0	0.062	0.145	0.120	0.106	0.108
40	0.040	0.086	0.066	0.057	0.062
50	0.030	0.070	0.053	0.052	0.051
Mean	0.044	0.100	0.079	0.071	-

RLSD p = 0.05 for NaCl = 0.001, Proline = 0.001, for the interaction = 0.003.

As for the interaction, it was also significant, in that, the addition of proline significantly alleviated the inhibitory effects of salinity on the growth parameters of the regenerated plantlets. Other workers have also found that the addition of proline significantly alleviated the inhibitory effect of salinity on the growth of the plantlets, (El-Hammady et al 1999; Al-Kabi 2004) as well as, intact plants (El-Morshedy 1995; Tawagen et al 2003a).

It is clear from figure 4, that NaCl at 10 mM significantly increased total soluble carbohydrate in plantlet leaves for the reasons referred to that low levels of salinity increase carbohydrate content of plantlets was reported by Al-Kabi (2004) for date palm cv. Barhe and Tawagen et al (2003a) for intact tomato plants. As the concentration of NaCl increased, the concentration of soluble carbohydrates was significantly decreased. Such effect are due to the effect of Na^+ on cell metabolism, by increasing the respiration rate, due to the effect of Na^+ on the respiratory chain, as well as due to the use of ATP for salt tolerance, rather than growth. Furthermore, salinity also induces oxidative stress, which intern influence enzyme activity causing a reduction in carbohydrate metabolism (Mass 1986; Huang & Liu 2002; Wang et al 1999).

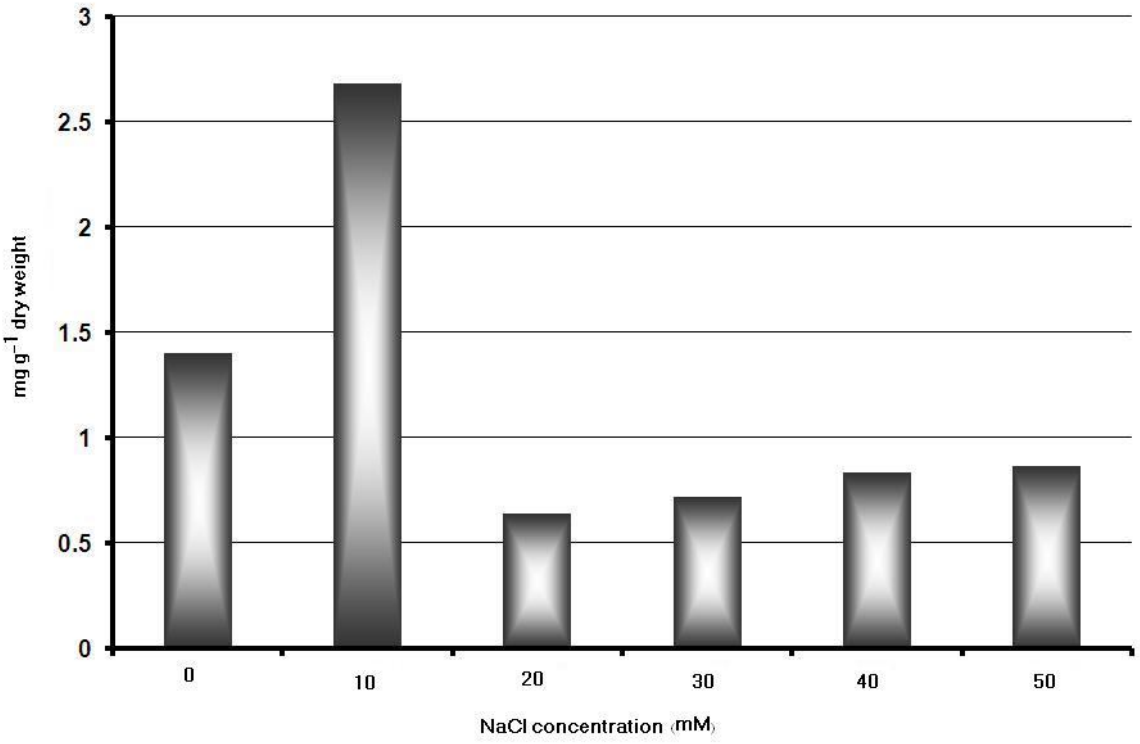

Figure 4. Effect of NaCl concentration on carbohydrate content of leaves of *Citrus sinensis* cv. Local orange plantlets.

Table 5 highlights that proline at all concentration significantly increased level of soluble carbohydrates, in particular at 25 mg L^{-1}. Such effect of proline on carbohydrate levels in plantlet leaves are due to the stimulation of plantlet growth by proline, as well as the reasons mentioned earlier, with the resultant accumulation of carbohydrates.

Table 5

The main effect of NaCl, proline and their interaction on soluble carbohydrate levels (mg g^{-1} dry weight), in leave of the plantlets

NaCl concentration (mM)	*Proline concentration (mg L^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	1.405	2.880	1.920	1.810	2.003
40	0.834	1.015	0.930	0.888	0.916
50	0.867	0.457	0.858	0.832	0.878
Mean	1.035	1.617	1.236	1.176	-

RLSD p = 0.05 for NaCl = 0.001, Proline =0.001, for the interaction = 0.003.

The main effect of NaCl on carbohydrates was to reduce significantly their accumulation in the leaves. Such effect is due to the inhibitory effects of salinity referred to earlier. As for the interaction, it was also significant, in that proline increased the levels of carbohydrates in all treatments, to which it was added, as compared to treatments of NaCl alone. Such effects are due to the known effects of proline in alleviating the adverse effects of salinity.

Figure 5 show that NaCl at all concentrations significantly increased proline concentration in leaves as compared to controls. Such increase is probably due to the inhibition of proline oxidizing enzymes, as well as an increase in its biosynthesis due to feedback inhibition. All these contribute to the accumulation of proline under salt stress conditions (Stewart & Larhar 1980; Berteli et al 1995; Heuer 2010). Other workers have also reported an increase in proline levels in leave of grape and date palm plantlet under

NaCl stress, as well as intact plants growing under salt stress (El-Morshedy 1995; El-Hammady et al 1999; Al-Kabi 2004; Tawagen et al 2003b).

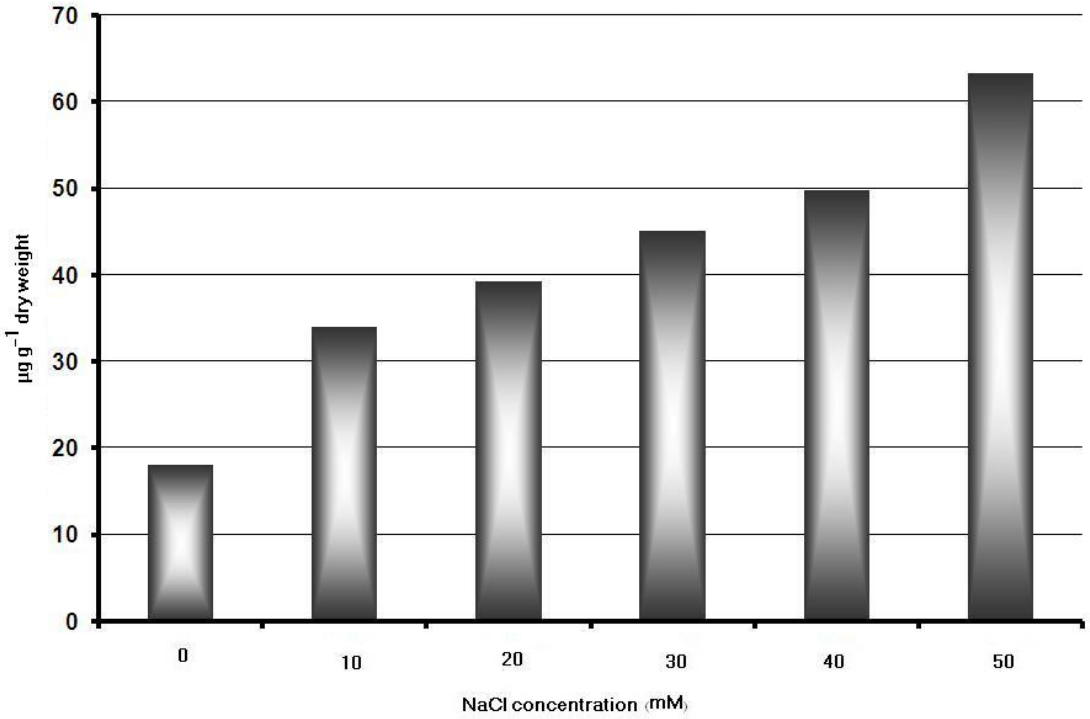

Figure 5. Effect of NaCl concentration on free proline content of leaves of *Citrus sinensis* cv. Local orange plantlets.

Table 6 emphasize that exogenous proline treatment caused a significant increase in its levels in leaves of orange plantlets, which is due to the absorption of proline from the culture medium, with a resultant increase in its concentration. Similar increase in proline concentration of the plantlets due to the exogenous application of proline has also been reported for other plantlets, such as grape and date palm. As for the interaction, it was also significant, in that proline accumulation due to NaCl stress, increased by the addition of proline, which is due to the synergism between proline and NaCl. The best treatment was NaCl 50 mM and proline 75 mg L^{-1} which gave a concentration of 95.69 µg g^{-1} dry weight of free proline as compared to 18.0 µg g^{-1} of the control treatment.

Table 6

The main effect of NaCl, proline and their interaction on the concentration (µg g^{-1} dry weight) of free proline in leaves of the plantlets

NaCl concentration (mM)	*Proline concentration (mg L^{-1})*				
	0	*25*	*50*	*75*	*Mean*
0	18.020	36.000	25.100	20.100	24.805
40	49.820	60.400	78.490	87.090	68.950
50	63.260	71.500	88.170	95.690	79.650
Mean	67.920	63.920	55.966	43.790	-

RLSD p = 0.05 for NaCl = 0.209, Proline = 0.242, for the interaction = 0.420.

Conclusions. The result obtained in the present work, clearly demonstrated the possibility of producing NaCl tolerant orange plants. The process began from the nucleus derived callus, which was grown under various concentration of NaCl. This resulted in the production of salt-tolerant primary callus. This primary callus was used for the production of NaCl tolerant organogenic callus. From this adventitious shoots were obtained, and

from which whole plantlets tolerant of NaCl stress were regenerated. Those NaCl-tolerant plantlets were successfully acclimatized into a soil mix and the process of acclimatization continued for 8 months. It is clear, therefore, that the trait of salinity tolerance was achieved using the technique of plant tissue culture and this salinity tolerance was further improved by the exogenous application of the amino acid proline.

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