Journal of Horticultural Science & Ornamental Plants 10 (1): 17-27, 2018
ISSN 2079-2158

DOI: 10.5829/idosi.jhsop.2018.17.27

In vitro Evaluation and Selection for Salinity Tolerance in Some Citrus Rootstock Seedlings

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Abstract: Salinity is considered a critical problem especially in citrus trees since they are one of the most globally important horticultural salt sensitive crops. This study was conducted to evaluate the performance of some citrus rootstocks under different salinity concentrations. Seeds of seven citrus rootstocks [four accessions of sour orange *Citrus aurantium* L. (Balady, Brazilian, Spanish and Russian), Macrophylla (Alemow) *Citrus macrophyla*, besides, Trifoliate orange (*Poncirus trifoliate* (L.) Raf.); and Pomeroy trifoliate (*Poncirus trifoliate* (L.) Raf.)] were cultured *in vitro* on Murashige and Skoog salts (MS) supplemented with NaCl. The effects of NaCl concentrations at 0.0, 1000, 2000, 3000, 4000 and 5000 ppm were studied. Salinity tolerance of these rootstock seedlings were noticed *in vitro* and *in vivo*. Moreover, proline accumulation in leaves, protein and isozyme banding patterns through gel electrophoresis were analyzed. Results showed that, number of burned leaves increased during the first subculture more than the second subculture at 5000 ppm with Brazilian sour orange. Moreover, both seedling height and emerged shoots were enhanced by low levels of salts compared to control. Results also cleared that, the increment of sodium chloride levels (up to 5000 ppm) in culture medium lead to a significant accumulation of proline in leaves of citrus rootstocks. Moreover, Balady sour orange considered the most citrus rootstock tolerant to salinity stress since it exhibited the highest survival percentage and proline accumulation followed by Spanish sour orange while Brazilian, Russian sour orange, Alemow and Trifoliate orange found to be moderate to salt tolerance. On the other hand, Pomeroy trifoliate was the most sensitive rootstock, which gave the lowest values. Protein banding patterns revealed a total number of nineteen bands with molecular weight (MW) ranged from 22.2 to 287.45 KDa. Thirteen monomorphic bands were detected while the remaining six bands were polymorphic with 31.51 % polymorphism. On the other hand, peroxidase and polyphenyl oxidase, isozymes banding patterns represent differences with the tested rootstocks in some bands existence or its density with the NaCl treatment of 4000 ppm compared with control treatment.

Key words: *In vitro* · Evaluation · Selection · Citrus · Rootstocks · NaCl · Salt stress · Proline accumulation · Gel electrophoresis · Protein · Isozyme · Banding patterns

INTRODUCTION

Resistance to abiotic stresses such as drought, salinity and alkalinity is an important trait for selection of fruit trees rootstocks, which affect the nutritional status of the scion and appreciably influence scion tolerance to salinity [1]. Salinity is the major abiotic stress confronting citrus orchards, which inhibits plant growth and reduces productivity due to water deficit, nutritional imbalance and ionic toxicity resulting in massive loss in yield [2].

Interest in salinity tolerant citrus rootstocks has increased as land and water are becoming increasingly limited. The identification and evaluation of salt tolerant citrus rootstocks could help in solving salinity problems in the newly reclaimed lands. Therefore, developing rootstocks that tolerate different biotic and abiotic stresses is one of the major goals of breeding programs of citrus rootstocks to insure rootstocks suitable for different environmental conditions.

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Sodium chloride (NaCl) represent the major component of salt in irrigation water and soil solutions [3]. Many detailed studies on the usage of *in vitro* techniques for genotypes screening, adaptation and selection against many abiotic stresses have been done and proved to be better applicable system for testing salt tolerance in a number of fruit species such as pomegranate cultivars, citrus species, rootstocks of sweet cherry and pistachio [4-7].

Plants respond to salinity stress by accumulation nontoxic and compatible osmolytes such as proline. Proline can increase the protection of cellular structures against oxidative damage by scavenging free radicals [8]. Salt tolerance in citrus is associated with various mechanisms such as accumulation of Cl^- and/or Na^+ in cell vacuoles [9]. Proline is one of the organic compounds that might be involved in facilitating osmotic adjustment under salinity stress or act as an enzymatic regulator during stress conditions and sink of energy for growth after stress [10].

Isozyme markers provide a convenient method for cultivars and rootstocks identification. In citrus, isozymes have been used as molecular markers to differentiate between different species and to detect genetic changes [11, 12]. Moreover, used as a useful markers for the assessment of tolerance or susceptibility for diseases [13-14]. Besides, the anti-oxidant activities and patterns of some isoenzymes such as Catalase (CAT), Peroxidase (POD) and Super-oxidase dismutase (SOD) that are altered significantly in plants subjected to salinity stress, i.e. the induction of a new SOD isozymes were found in salt-tolerant embryonic callus cultures of lemon (*Citrus limon* L. Burn) [15]. Thus, the objective of this study was to evaluate the performance of the tested seven citrus rootstocks under different concentrations of salt added to nutrient medium to select the most tolerant plantlets, which are capable of surviving under salt stress conditions. Moreover, determine the survival rate of tolerant plantlets transferred into the greenhouse with continuous saline treatments as an attempt to introduce more salinity tolerant citrus rootstocks.

MATERIALS AND METHODS

This work was carried out through two successive seasons of 2014 and 2015 in tissue culture laboratory, Fruit Breeding Department, Horticulture Research Institute, Giza, Egypt.

Plant Materials: Seven rootstocks were used throughout this study [four accessions of sour orange *Citrus aurantium* L. (Balady (local genotype), Brazilian, Spanish and Russian), Macrophylla (Alemow) *Citrus macrophyla*, besides, Trifoliate orange (*Poncirus trifoliate* (L.) Raf.) and Pomeroy trifoliate (*Poncirus trifoliate* (L.) Raf.)]. Seeds were extracted from mature fruits of rootstocks, which are grown in the orchard of El Qanater El Khayreya Experimental Station, Horticulture Research Institute, Kaluobia Governorate.

Culture Conditions: Seeds for each rootstock were subjected to continuous flow of tab water for about 90 min., in order to remove viscous materials covering the seeds. After that, seeds were sterilized by using 50 % sodium hypochlorite (NaOCl) solution of commercial bleach (Clorox) for 30 min. and rinsed three times (10 min. for each rinse) in sterile distilled water. By using a scalpel (blade no. 11) and forceps to removed outer and inner off seeds on sterile filter paper. The cotyledons (seeds after removing the seed coat) were cultured on different saline media. The saline medium that used for *in vitro* evaluation experiments consisted of Murashige and Skoog (MS) salts and vitamins at full strength [16] plus different salt concentrations of sodium chloride (NaCl) at 0.0, 1000, 2000, 3000, 4000 and 5000 ppm. The medium solidification was achieved by 7 g/l Agar, the pH of different concentrations was adjusted to 5.7 using KOH and HCl before autoclaving. The media were autoclaved at 100 K.pa (15 P.S.I) and 121°c for 20 min. then left to cool and stored at 25°C ±2 for 2 days before being used. Cultures were incubated at temperature of 25°C ± 2°C and photoperiod of 16-hrs supplied by fluorescent lamp (2 lamps per shelf) to provide light intensity of 2200-2400 lux at explants level (30 cm from the light). Two subcultures were carried out every four weeks on a medium with the same concentration which used for the first culture in each group of seedlings.

Data Were Recorded as Follow:

- Number of burned leaves (50-100 % of the blade was brown).
- Seedlings height (cm).
- Number of lateral bud emerged to new shoots on citrus rootstock seedlings: (some rootstock seedlings which had dieback, emerged new shoots from basal lateral buds). The mean of emerged shoots according to the total number of each rootstock seedlings (replicates) were calculated.

Transplanting of Salt Tolerant Plantlets and Acclimatization Procedure: Seedlings of citrus rootstocks with different saline media**,** which tolerate the stress conditions, were washed carefully with water to remove the excess culture media from roots. Roots were rinsed in 0.1 % BENLATE (fungicide solution). Then, seedlings were individually transplanted into plastic bags filled with fine sand and transferred to greenhouse. The plantlets were irrigated with half strength MS medium plus different concentrations of salts (NaCl). Survival percentages were recorded after twelve weeks from transplanting.

Biochemical Analysis

Proline Determination: At the end of salinity treatments, the proline content was determined from the leaves of survived seedlings grown under greenhouse conditions according to the method described by Bates *et al*. [17].

Protein Electrophoresis: Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was used to study protein profiles of seven citrus rootstocks treated with only two concentrations of sodium chloride at 0.0 and 4000 ppm. Total soluble protein were extracted according to Laemmli [18]. Water-soluble protein fractionation performed on 1.5 mm-thick of 12% (W/V) acrylamide vertical slab gels using "BIO-RAD PROTEAN® II xi Cell" according to the method modified by Studier [19]. Molecular weight of protein bands were relatively estimated using a wide range of molecular weight related to protein marker (Fermentas Company).

Isozymes Electrophoresis: Isozymes were extracted according to Wendel and Weeden [20]. Native-polyacrylamide gel electrophoresis (Native-PAGE) was performed on 12% (W/V) slab gels then gels were stained for isozymes of peroxidase (Px) and polyphenyl oxidase (PPO) according to the procedures described by Wendel and Weeden [20]. The stained gels were incubated at 37 °C in dark conditions to complete staining after adding the appropriate substrates and staining solutions. Gel bands were scanned and analyzed using Gel Doc., Bio-Rad system.

Experimental Design and Statistical Analysis: The experiment were set up as a factorial experiment in a completely randomized design. Each treatment consisted of five replicates, three seeds for each replicate (jar). Data subjected to analysis of variance (ANOVA). Duncan's multiple range test at 5% level of significance (p=0.05), was used for means comparisons according to Snedecor and Cochran [21].

RESULTS AND DISCUSSION

During the first weeks of establishment rootstock seedlings, the germination percentage in all rootstocks at saline medium reached 100 % after four weeks. In addition, seedlings development exceeded 3 cm in length and the main root emerged. From this step, the subcultures were carried out every four weeks in the same concentration.

Effect of *in vitro* Salinity Treatments, Type of Citrus Rootstocks and Their Interaction on the Number of Burned Leaves, Seedling Height and Number of Emerged Shoots During Two Subcultures.

Average Number of Burned Leaves: Data in Table (1) indicated that the mean number of burned leaves increased by increasing salt concentration in the first and second subcultures. All salt concentrations significantly caused burning leaves when compared with control. The highest significant value was obtained with 5000 ppm during the first subculture. The mean number of burned leaves decreased in the second subculture. The highest significant mean was recorded with 4000 and 5000 ppm, without significant differences between them in the second subculture. Regarding the rootstock, the lowest affected seedlings were "Spanish sour orange" followed by "Pomeroy trifoliate", "Trifoliate orange" and "Balady sour orange" during the first subculture. They achieved the lowest significant mean in comparing with other rootstocks. Moreover, in the second subculture "Spanish sour orange" had the same trend and scored the lowest significant mean. The interactions between the variables showed the lowest significant number of burned leaves with "Spanish sour orange" on medium contained 1000, 2000 or 3000 ppm NaCl in the first and second subcultures.

Average Seedlings Height: Data in Table (2) showed the height of different seedlings cultured on saline medium *via* two subcultures. In the first subculture, the seedlings height gradually increased and significantly with salt treatments up to 4000 ppm, while decreased at 5000 ppm. Concerning rootstocks, "Brazilian sour orange" scored the highest average followed by "Mycrophylla", "Pomeroy" and "Russian sour orange". Regarding the interaction between the two variables, the highest average

Table 1: Effect of *in vitro* salinity treatments, type of citrus rootstocks and their interaction on the number of burned leaves (during two subcultures)

NaCl Treatments	Brazilian sour orange	Russian Sour orange	Spanish sour orange	Balady sour orange	Alemow	Trifoliate orange	Pomeroy trifoliate	Mean
				1st subculture				
0.0 ppm	0.00 p	0.00 p	0.00 p	0.00 p	0.00 p	0.00 p	0.00 p	0.00 F
1000 ppm	1.20 no	1.40 k-o	1.33 l-o	1.13 o	1.20 no	1.13 o	1.20 no	1.23 E
2000 ppm	1.46 j-n	1.59 h-l	1.26 m-o	1.20 no	1.66 g-k	1.19 no	1.53 i-m	1.41 E
3000 ppm	1.46 j-n	1.66 g-k	1.26 m-o	1.59 h-l	1.80 f-i	1.40 k-o	1.59 h-l	1.54 C
4000 ppm	2.26 cd	2.06 d-f	1.59 h-l	1.86 e-h	2.13 de	1.73 g-j	1.66 g-k	1.90 B
5000 ppm	2.86 a	2.60 b	1.66 g-k	1.93 e-g	2.39 bc	1.86 e-h	1.93 e-g	2.18 A
Mean	1.54 A*	1.55 A*	1.18 C*	1.29 BC*	1.53 A*	1.22 BC*	1.32 B*	
				2nd subculture				
0.0 ppm	0.00 k	0.00 k	0.00 k	0.00 k	0.00 k	0.00 k	0.00 k	0.00 D
1000 ppm	1.26 h-j	1.33 g-j	1.20 ij	1.19 ij	1.26 h-j	1.33 g-j	1.13 j	1.24 C
2000 ppm	1.33 g-j	1.33 g-j	1.13 j	1.13 j	1.46 f-j	1.12 j	1.26 h-j	1.25 C
3000 ppm	1.40 f-j	1.53 d-i	1.13 j	1.52 d-i	1.40 f-j	1.46 f-j	1.46 f-j	1.41 B
4000 ppm	2.00 ab	1.93 a-c	1.46 f-j	1.73 b-f	1.79 b-e	1.80 b-e	1.66 c-g	1.77 A
5000 ppm	2.13 a	1.93 a-c	1.60 c-h	1.79 b-e	1.73 b-f	1.66 c-g	1.86 a-d	1.81 A
Mean	1.35 A*	1.34 A*	1.09 B*	1.23 A*	1.27 A*	1.23 A*	1.24	A*

Means followed by the same letters in each column, row or interaction are not significantly different at 5% level.

Table 2: Effect of *in vitro* salinity treatments, type of citrus rootstocks and their interaction on seedlings height (during two subcultures)

NaCl Treatments	Brazilian sour orange	Russian Sour orange	Spanish sour orange	Balady sour orange	Alemow	Trifoliate orange	Pomeroy trifoliate	Mean
				1st subculture				
0.0 ppm	3.06 l-p	2.93 n-p	2.80 pq	2.86 o-q	3.20 m-p	2.80 pq	3.19 j-p	2.98 D
1000 ppm	3.33 i-p	3.39 h-o	3.52 f-m	2.39 q	3.66 d-k	2.99 m-p	3.13 k-p	3.20 C
2000 ppm	3.59 e-l	3.66 d-k	3.86 b-i	3.93 a-h	4.19 a-d	3.66 d-k	3.86 b-i	3.82 B
3000 ppm	4.26 a-c	4.13 a-e	4.00 a-g	4.13 a-e	4.13 a-e	4.19 a-d	4.00 a-g	4.12 A
4000 ppm	4.46 a	4.33 ab	3.93 a-h	4.19 a-d	3.6 e-l	4.13 a-e	4.06 a-f	4.10 A
5000 ppm	3.99 a-g	3.46 g-n	3.53 f-m	3.70 c-j	3.2 j-p	3.59 e-l	3.7 c-j	3.60 B
Mean	3.78 A*	3.65 A*	3.61 AB*	3.53 B*	3.66 A*	3.56 B*	3.66 A*	
				2nd subculture				
0.0 ppm	3.66 b-f	3.19 e-j	3.59 b-g	3.73 b-f	3.53 b-h	3.53 b-h	3.73 b-f	3.57 C
1000 ppm	3.99 a-d	3.93 a-e	4.06 a-c	3.66 b-f	3.79 b-f	3.59 b-g	3.7 b-f	3.82 B
2000 ppm	4.13 a-c	3.66 b-f	4.60 a	4.26 ab	3.99 a-d	4.26 ab	4.06 a-c	4.14 A
3000 ppm	3.92 a-e	4.13 a-c	3.13 f-j	3.26 b-j	3.60 b-g	4.06 a-c	2.79 i-k	3.56 C
4000 ppm	4.06 a-c	2.86 h-k	2.79 i-k	3.06 f-j	2.93 g-k	2.86 h-k	2.73 i-k	3.04 D
5000 ppm	3.39 c-i	2.59 jk	2.60 jk	2.26 k	2.86 h-k	2.60 jk	2.70 i-k	2.72 E
Mean	3.86 A*	3.39 B*	3.46 B*	3.37 B*	3.45 B*	3.48 B*	3.29 B*	

Means followed by the same letters in each column, row or interaction are not significantly different at 5% level.

Table 3: Effect of *in vitro* salinity treatments, type of citrus rootstocks and their interaction on lateral buds emerged to shoots (during two subcultures)

NaCl Treatments	Brazilian sour orange	Russian Sour orange	Spanish sour orange	Balady sour orange	Alemow	Trifoliate orange	Pomeroy trifoliate	Mean
	1st subculture							
0.0 ppm	0.07 ab	0.07 ab	0.07 ab	0.13 ab	0.13 ab	0.13 ab	0.13 ab	0.10 BC
1000 ppm	0.13 ab	0.20 ab	0.07 ab	0.33 a	0.20 ab	0.26 ab	0.33 a	0.22 A
2000 ppm	0.13 ab	0.2 ab	0.066 ab	0.33 a	0.19 ab	0.13 ab	0.19 ab	0.18 AB
3000 ppm	0.07 ab	0.13 ab	0.13 ab	0.26 ab	0.13 ab	0.19 ab	0.19 ab	0.16A-C
4000 ppm	0.07 ab	0.13 ab	0.00 b	0.13 ab	0.07 ab	0.2 ab	0.13 ab	0.10 BC
5000 ppm	0.07 ab	0.2 ab	0.07 ab	0.07 ab	0.00 b	0.13 ab	0.00 b	0.08 C
Mean	0.09 B*	0.16 AB*	0.07 B*	0.21 A*	0.12 AB*	0.17 AB*	0.16 AB*	
	2nd subculture							
0.0 ppm	0.07 ab	0.13 ab	0.07 ab	0.19 ab	0.33 ab	0.19 ab	0.13 ab	0.16 BC
1000 ppm	0.13 ab	0.33 ab	0.13 ab	0.39 a	0.26 ab	0.39 a	0.33 ab	0.28 A
2000 ppm	0.19 ab	0.26 ab	0.13 ab	0.40 a	0.26 ab	0.26 ab	0.26 ab	0.25 AB
3000 ppm	0.13 ab	0.26 ab	0.13 ab	0.33 ab	0.26 ab	0.33 ab	0.19 ab	0.23 AB
4000 ppm	0.07 ab	0.26 ab	0.00 b	0.33 ab	0.33 ab	0.26 ab	0.19 ab	0.21 AB
5000 ppm	0.07 ab	0.19 ab	0.07 ab	0.20 ab	0.00 b	0.13 ab	0.00 b	0.09 C
Mean	0.11 B*	0.24 A*	0.09 B*	0.31 A*	0.24 A*	0.26 A*	0.18 AB*	

Means followed by the same letters in each column, row or interaction are not significantly different at 5% level.

was scored by "Brazilian sour orange", "Russian sour orange", "Balady sour orange" and "trifoliate orange" at 3000 and 4000 ppm. At the end of the first subculture, all cultures were visually noticed to fall the injured leaves (which had symptoms of necrosis and browning) besides the death of apical meristem. During the second subculture, the concentration of 2000 ppm achieved the highest significant values of seedling height. Furthermore, "Brazilian sour orange" recorded the highest significant average comparing with other rootstocks. Regarding the interaction, "Spanish sour orange" followed by "Balady sour orange", "Trifoliate orange" and "Brazilian sour orange" recorded the highest average of seedling height with 2000 ppm.

Average Number of Lateral Buds Emerged to Shoots: Data in Table (3) reveal the effect of salinity levels on rootstocks ability to renew their growth under stress conditions. The treatment of 1000 ppm demonstrated the highest significant average of emerged shoots 0.21 and 0.28 during the first and second subcultures, respectively. On the other hand, the lowest average number of emerged shoots was scored with 5000 ppm. Moreover, Balady sour orange recorded the highest mean of emerged shoots. Concerning the interaction, Balady sour orange with 1000 and 2000 scored the highest number of emerged shoots.

The obtained results are in agreement with the findings of Kaushal *et al.* [2] who reported that in all salt treatments, leaves of rough lemon seedlings showed severe injury symptoms of chlorosis as well as necrosis, which progressed fairly and rapidly in the tips and along with leaf margins, then advanced basepetaly. However, the control seedlings did not show any such symptoms. Moreover, Khoshbakht *et al.* [22] found that the lowest leaf damage were observed with cleopatra mandarin. In addition, Sykes [23] stated that small juvenile plants offer the advantage of high uniformity besides the age of citrus plants is a critical factor in measuring citrus responses to salinity because of the fact that older plants (i.e. two years old) may respond differently to young juvenile plants (i.e. several months old).

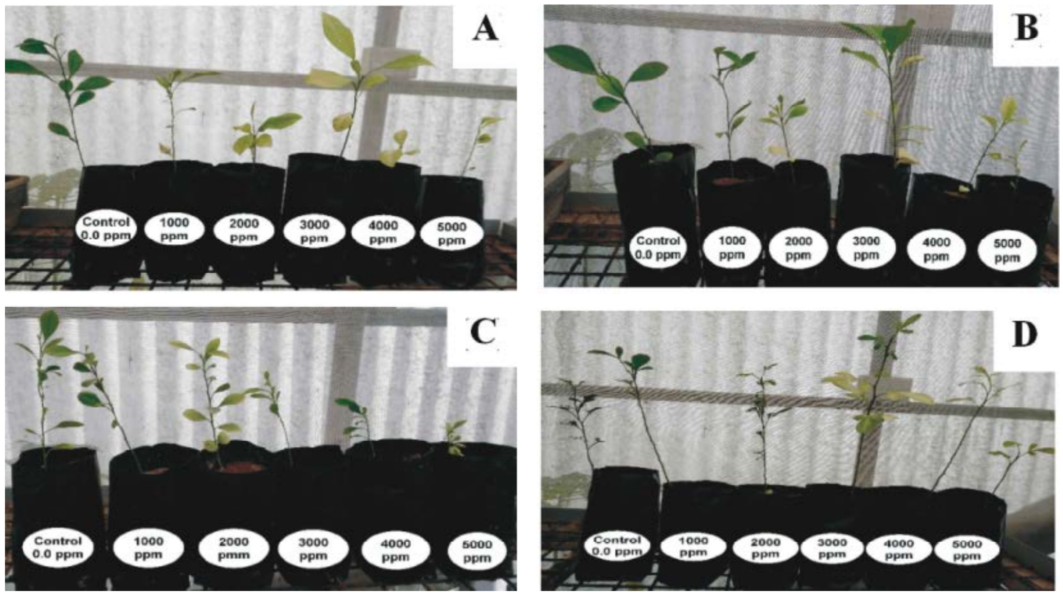

Fig. 1: Effect of NaCl concentrations on growth of some rootstock seedlings:
A) Balady sour orange B) Spanish sour orange C) Alemow D) Trifoliate orange

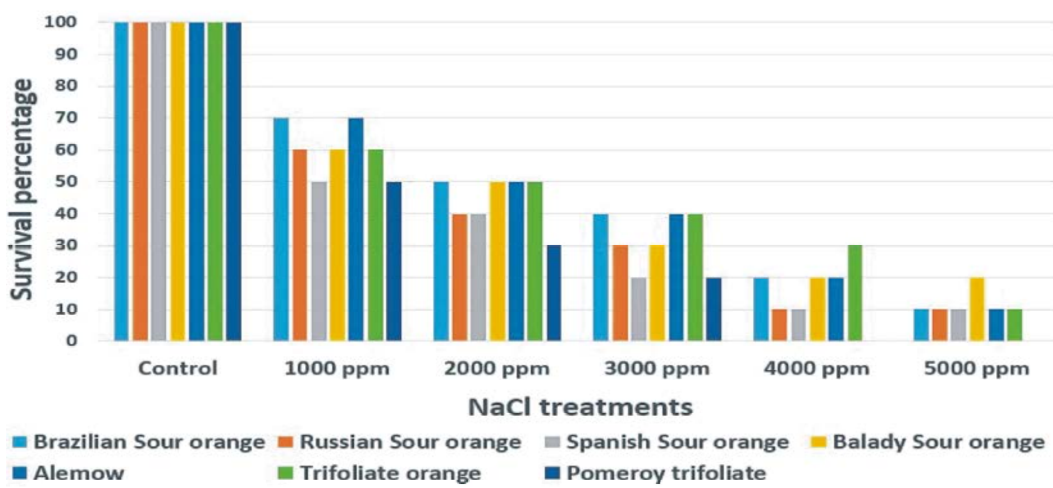

Fig. 2: Effect of NaCl concentrations on survival percentage of seven citrus rootstock seedlings during acclimatization stage

Survival Percentage During Acclimatization Stage: As shown in Figures (1 & 2), all seedlings treated with 0.0 ppm NaCl (control) recorded the highest survival percentage (100 %) for all rootstocks. Increasing salinity level reduces survival rate which ranged from 70 to 50 % with treatment of 1000 ppm (Brazilian sour orange and Alemow rootstocks scored 70 % while Spanish sour orange and Pomeroy trifoliate rootstocks scored 50 %) and ranged from 20 to 0.0 % with treatment of 5000 ppm that achieved by Balady sour orange and Pomeroy trifoliate, respectively. Besides, visual injury symptoms of salinity which were observed in high concentration such as growth reduction, chlorosis, necrosis and leaf burn. Balady sour orange rootstock achieved the highest survival percentage (20%) meanwhile; Pomeroy trifoliate gave the lowest value (0%).

Effect of Salinity Stress on Proline Accumulation: Proline has increased with the increasing of salinity stress in all tested rootstocks. This indicate a possible role of proline in osmotic adjustment of citrus leaves. Maximum proline accumulation was recorded at higher salinity level, Balady sour orange showed the highest proline accumulation (94 µmol/g) with 5000 ppm of NaCl, whereas

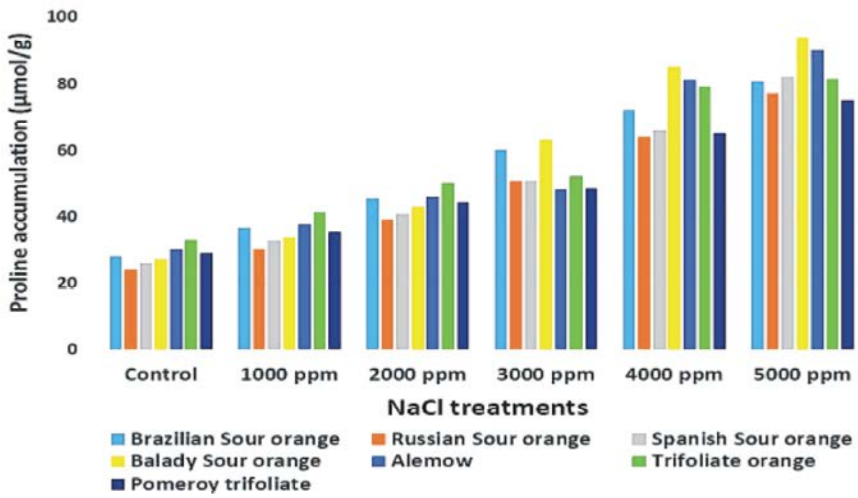

Fig. 3: Effect of NaCl concentrations on proline accumulation of seven citrus rootstocks.

the lowest proline accumulation was obtained with Pomeroy trifoliate at 5000 ppm (75 µmol/g) as shown in Fig. (3).

Results cleared that the increment of sodium chloride levels up to 5000 ppm in culture medium lead to a significant accumulation of proline in leaves of citrus rootstocks. Moreover, Balady sour orange rootstock considered to be the most citrus rootstock tolerant to salinity stress since it exhibited the highest survival percentage and proline accumulation followed by Spanish sour orange while Brazilian, Russian sour orange, Alemow and Trifoliate orange found to be moderate to salt tolerance. On the other hand, Pomeroy trifoliate was the most sensitive rootstock, which gave the lowest values. These results agreed with those mentioned by Ashraf and Foolad [8] who stated that, accumulation of proline under stress in many plant species has been correlated with stress tolerance and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants. Moreover, Rayan and Awad [24] stated that the pear plantlets content of Na, Cl and proline were gradually increased by raising sodium chloride concentrations *in vitro*. Nevertheless, Khoshbakht *et al.* [22] reported that increasing salt levels lead to significant increase in proline content of leaves in citrus rootstocks. In addition, they found that sour orange and Cleopatra mandarin were the most salt tolerant rootstocks followed by Rangpur lime and Bakraii which is considered to be moderate to high salt tolerant in comparing with trifoliate orange, Citrange and Swingle citrumelo were the most sensitive rootstocks while rough lemon and Macrophylla proved to be low to moderate salt tolerant rootstocks. Similarly, salt tolerant calli of *"citrus limon"* had significantly higher proline concentration compared with salt sensitive calli [15]. In addition, *in vitro* high accumulation of proline under salinity was reported for the salt tolerant citrus cell lines [25].

SDS-Protein Banding Patterns: SDS-PAGE of seven citrus rootstocks treated with two concentrations of sodium chloride (0.0 and 4000 ppm) revealed a total number of nineteen protein bands with molecular weight (MW) ranged from 22.2 to 287.45 KDa. Thirteen monomorphic bands were detected while the remaining six bands were polymorphic with 31.51 % polymorphism. As shown in Table (4) and Figure (4). Data represent a band with molecular weight of 287.45 KDa only present in control plants of rootstock no. 1 and 2 and the treated plant of rootstock no. 7. The band with 110.15 KDa was present in the control and treated plants of rootstock no. 1 and present with the control plants only of rootstocks no. 1, 3, 4 and 6, while absent in all plants of rootstocks no. 5 and 7. Band with 104.65 KDa was present in all plants of rootstock no. 3 and 4 and present only in control plants of rootstock no. 2 and 6, while absent with all plants of rootstock no. 1, 5 and 7. Band with 98.20 KDa was present in all control and treated rootstock plants except the treated plants of rootstock no. 6 and 7. Band with 80.90 KDa was present in all plants of rootstocks no. 3, 6 and 7 and present only in treated plants of rootstock no. 2 and 4, while absent with all plants of rootstock no. 1. Band with 63.50 KDa was present in all control and treated plants of rootstocks no. 1, 2, 5, 6 and 7 except rootstock no. 4 and the control plants of rootstock no. 3.

Table 4: Densitometric analysis for leaf protein of seven Citrus rootstocks treated with two concentrations of sodium chloride (0.0 and 4000 ppm).

Band No.	M.W. KDa	Citrus rootstocks													
		R.1		R.2		R.3		R.4		R.5		R.6		R.7	
		C	T	C	T	C	T	C	T	C	T	C	T	C	T
1	287.45	1	0	1	0	0	0	0	0	0	0	0	0	0	1
2	170.40	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	110.15	1	1	1	0	1	0	1	0	0	0	1	0	0	0
4	104.65	0	0	1	0	1	1	1	1	0	0	1	0	0	0
5	98.20	1	1	1	1	1	1	1	1	1	1	1	0	1	0
6	80.90	0	0	0	1	1	1	0	1	1	0	1	1	1	1
7	68.40	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	63.50	1	1	1	1	0	1	0	0	1	1	1	1	1	1
9	58.20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	47.70	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	45.33	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	39.20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	36.40	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	31.00	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	29.45	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	27.50	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	25.80	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	23.75	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	22.20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Total		17	16	18	16	17	17	16	16	16	15	18	15	16	16

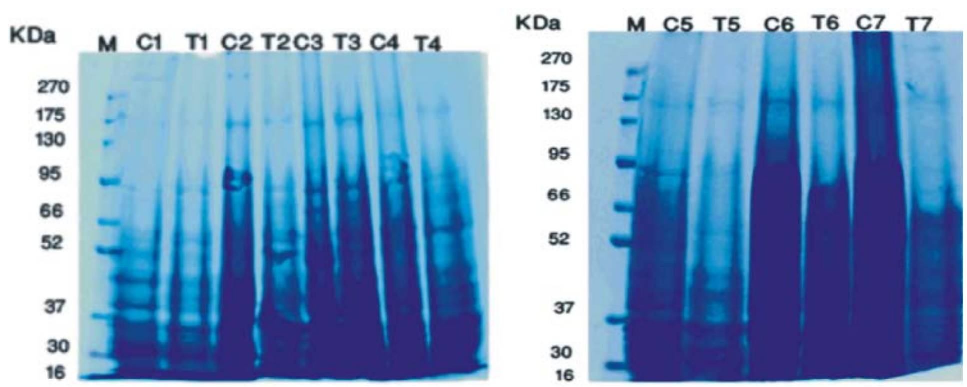

Fig. 4: SDS-leaf protein banding patterns for seven citrus rootstocks treated with two concentrations of sodium chloride (0.0 and 4000 ppm). (M) Protein marker (KDa) (C) control plants treated with 0.0 ppm NaCl (T) treatment of 4000 ppm NaCl

1) Brazilian sour orange 2) Russian sour orange 3) Spanish sour orange 4) Balady sour orange 5) Alemow 6) Trifoliate orange 7) Pomeroy trifoliate

Peroxidase Banding Patterns: The electrophoretic patterns of peroxidase isozymes exhibited differences in destiny among the seven citrus rootstocks treated with two concentrations of sodium chloride (0.0 and 4000 ppm). As illustrated in Table (5) and Figures (5 & 6), the seven citrus rootstocks were characterized by the presence of five groups of isozymes with relative mobilities of (0.2, 0.6, 0.7, 0.8 and 0.9) as Px1, Px2, Px3, Px4 and Px5, respectively. There were no differences in the density of bands between the two treatments with citrus rootstocks no. 2 and 7 (Russian sour orange and Pomeroy trifoliate, respectively). While, in the rootstocks no. 3, 4 and 5 the density of bands were raised with the treatment of 4000 ppm of NaCl in comparing with the control treatment. On the other hand, a reduction in banding density appeared in the second treatment in rootstocks no.1 and 6 (Brazilian sour orange and Trifoliate orange, respectively) as compared with the treatment of control. Moreover, the isozyme group of Px5 has disappeared in second treatment with rootstock no. 6 (Trifoliate orange).

Polyphenyl Oxidase Banding Patterns: The electrophoretic patterns of polyphenyl oxidase isozymes exhibited differences in destiny among seven citrus rootstocks treated with two concentrations of sodium chloride (0.0 and 4000 ppm). As illustrated in Table (6) and Figures (7 & 8), the seven citrus rootstocks characterized by the presence of six groups of isozymes with relative mobilities of (0.2, 0.4, 0.6, 0.7, 0.8 and 0.9) as PPO1, PPO2, PPO3, PPO4, PPO5 and PPO6, respectively. There were no differences in the density of bands between the two treatments with citrus rootstocks no. 3 (Spanish sour

Table 5: Densitometric analysis for leaf peroxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride

Perosidase groups	Relative Mobility	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Px1	0.2	1^{+}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{+}	1^{-}	1^{+}	1^{+}
Px2	0.6	1^{++}	1^{+}	1^{++}	1^{++}	1^{-}	1^{++}	1^{+}	1^{++}	1^{+}	1^{++}	1^{+}	1^{+}	1^{++}	1^{++}
Px3	0.7	1^{+}	1^{-}	1^{+}	1^{+}	1^{-}	1^{+}	1^{+}	1^{++}	1^{+}	1^{+}	1^{++}	1^{+}	1^{++}	1^{++}
Px4	0.8	1^{+}	1^{-}	1^{+}	1^{+}	1^{-}	1^{+}	1^{+}	1^{++}	1^{+}	1^{++}	1^{++}	1^{++}	1^{++}	1^{++}
Px5	0.9	1^{+}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	-	1^{+}	1^{-}	-	-	-	1^{-}	1^{-}

Table 6: Densitometric analysis for leaf polyphenyl oxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride.

Polyphenyl Oxidase groups	Relative Mobility	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PPO1	0.2	1^{+}	1^{-}	1^{+}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{+}	1^{-}	1^{-}	1^{-}
PPO2	0.4	1^{+}	1^{-}	1^{+}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{++}	1^{-}	1^{-}
PPO3	0.6	1^{++}	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}	1^{+}	1^{-}	1^{-}	1^{-}	1^{-}	1^{-}	1^{++}	1^{-}
PPO4	0.7	1^{++}	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}	1^{++}	1^{++}	1^{++}	1^{+}	1^{+}
PPO5	0.8	1^{+}	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}	1^{+}	1^{++}	1^{-}	1^{+}	1^{++}	1^{+}	1^{++}	1^{++}
PPO6	0.9	1^{+}	-	-	1^{+}	1^{-}	-	-	1^{-}	1^{+}	-	-	-	1^{-}	-

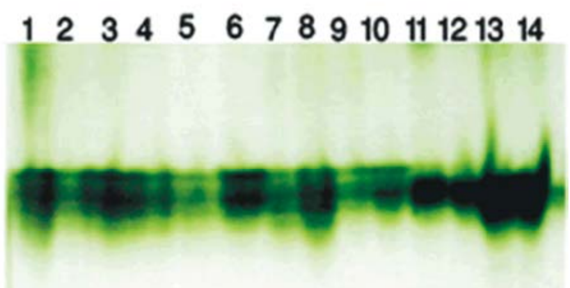

Fig. 5: Leaf peroxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride.

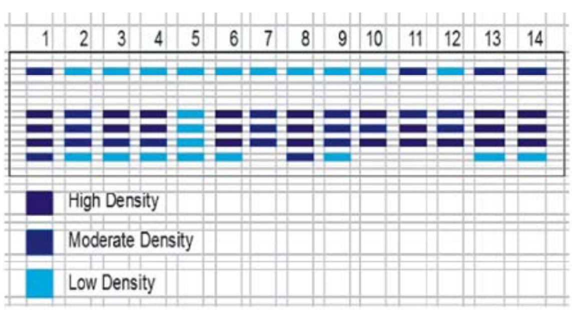

Fig. 6: Ideogram analysis for leaf peroxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride.

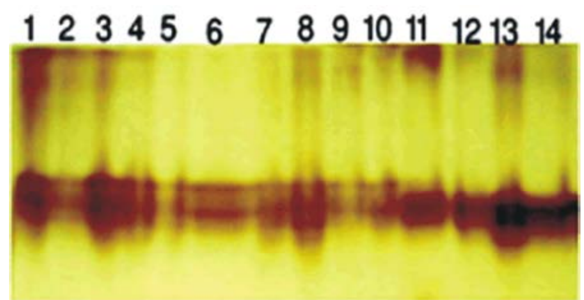

Fig. 7: Leaf polyphenyl oxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride.

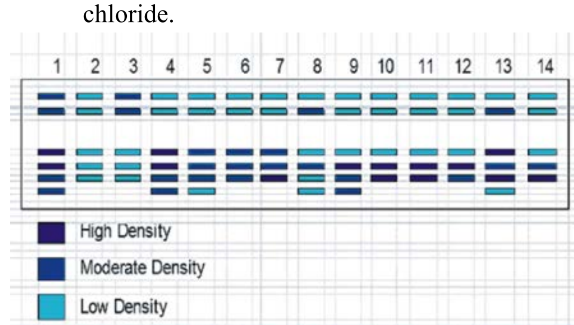

Fig. 8: Ideogram analysis for leaf polyphenyl oxidase isozyme banding patterns of seven citrus rootstocks treated with 0.0 and 4000 ppm concentrations of sodium chloride.

orange) except the absent of PPO 6 with the treatment of 4000 ppm as compared to control. On the other hand, there were decrements in banding density with the rootstock no. 1 (Brazilian sour orange) in all PPO groups except the PPO6 which was absent in comparing with control. While, there were obvious differences in banding pattern density with the remaining rootstocks, which appeared by density increasing at PPO2 with rootstock no. 6 (Trifoliate orange), PPO3 with rootstock no. 2 (Russian sour orange) and PPO4 with rootstocks no. 2 and 5 (Russian sour orange and Alemow, respectively) in comparing with control. Besides, density reductions at PPO1 and PPO2 with rootstock no. 2 (Russian sour orange) and at PPO3 with rootstock no. 4 and 7 (Balady sour orang and Pomeroy trifoliate, respectively) in comparing with control. Moreover, the absence PPO6 (band with relative mobility 0.9) in rootstocks no. 1, 3, 5 and 7 (Brazilian sour orange, Spanish sour orange, Alemow and Pomeroy trifoliate, respectively).

These results agreed with Rayan *et al.* [26] and Rayan *et al.* [27], who clarified that peroxidase and polyphenyl oxidase banding patterns represent differences in bands existence or absence and its density with different treatments in comparison to control for inducing somaclonal variations in plum cultivars and apple rootstocks. In addition, several isozymes such as peroxidase and polyphenyl oxidase have been routinely used in citrus to differentiate between different species [11]. Moreover, Gill *et al.* [28] reported that, in citrus the nucellars could be distinguished from the zygotic seedlings based on banding pattern of different isoenzymes such as peroxidase and amylase.

In conclusion, increasing NaCl levels in growth media lead to reduce plant growth of all tested rootstocks. Moreover, Balady and Spanish sour oranges proved to be more tolerant to NaCl levels followed by trifoliate orange and Alemow than other remained tested rootstocks. However, high concentration of NaCl caused a negative influence on all studied parameters.

Overall, all salinity tolerant rootstock seedlings were acclimatized into greenhouse to be transplanted into field conditions for further studies under both *in vitro* and *in vivo* conditions through incorporation breeding and selection procedures with molecular genomics in trying to release more stress tolerant citrus genotypes and rootstocks.

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