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Assessing Five Citrus Rootstocks for NaCl Salinity Tolerance Using Mineral Concentrations, Proline and Relative Water Contents as Indicators

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ABSTRACT
Changes in concentrations of Na, Cl and K, proline and Relative Water Contet (RWC) of five citrus species (Cleopatra mandarin, Carrizo citrange, Tiwanica, Bacraii and Shaker) grown in four NaCl salinity levels (0, 25, 50 and 75 mM NaCl) for the purpose of assessing their magnitude of susceptibility to salinity and estimating the salt tolerance degree were studied. The Na and Cl concentrations increased whilst K concentration in leaves decreased with increasing NaCl concentration in the rootzone. Shaker and Cleopatra mandarin accumulated optimal concentration of the ions in their tissues. Increasing NaCl concentration reduced RWC and increased proline content in plant tissues. Shaker and Cleopatra mandarin rootstocks maintained relatively higher leaf RWC and proline in comparison to other rootstocks. Cleopatra mandarin and Shaker showed to be markedly less affected by salinity up to 50 mM NaCl and could bear the highest salinity levels (75 mM) imposed in this study.

Key words: NaCl salinity, citrus rootstocks, salt tolerance

INTRODUCTION

Citrus is one of the most commercially grown fruits in the world. The main areas of citrus production are located in tropical and subtropical regions having arid and semi-arid climates (Davies and Albrigo, 1999; Lindaya, 2008). In these areas, hot and dry weather together with limited water resources cause elevation of salinity concentrations which restrict or delay crop production (Yadav *et al*., 2011). Effects of salinity differ among genotypes and selecting those that are salinity-tolerant considered to be an effective method for salinity adaptation (Najafian *et al*., 2008). Further decrease of osmotic potential compared to total water potential have caused the turgor pressure to be maintained in the plants under increasing or long lasting NaCl salinity (Parida and Das, 2005). Tabatabaie and Nazari (2007) concluded that increased NaCl concentration induces an increase in Na in lemon Verbena. According to Navarro *et al*. (2010), the leaf water content in citrus plants decreased with increasing salinity. Under saline conditions, Na and Cl are the abundant and major ions which cause severe physiological damages in citrus plants and limit their growth and development (Balal *et al*., 2011).

Citrus plants show various adaptive strategies in response to salinity which should be studied individually in terms of their salinity tolerance (Ruiz *et al*., 1997; Abadi *et al*., 2010). There are significant differences among citrus species in terms of both structural and operational properties of roots which may cause differences in nutrients absorption, their transport and the assimilation of other elements (Fernandez-Ballester *et al*., 2003). However, the capability of citrus rootstocks for regulation of Na and Cl absorption and their transport from roots to shoots is very important (Fernandez-Ballester *et al*., 2003). Thus, the relative tolerance of citrus rootstocks is based on the accumulation of Cl in leaves (Levy and Syvertsen, 2004). Tozlu *et al*. (2000) concluded that with increasing NaCl, K content mostly decreased in root tissue and increased in leaves of some citrus rootstocks. Citrus rootstocks have an important control over accumulated Na and Cl content in their leaves and roots. Accumulation of Na and Cl around the roots has been correlated with reductions in citrus plants growth and fruit yield. Therefore, differences of rootstock for salinity tolerance are considered a major concern for citrus growers (Storey and Walker, 1998).

It seems that proline accumulation has an important adaptive role in plant tolerance to stress and it has been recommended that proline acts as a compatible osmolyte and its accumulation could be considered as a mechanism for carbon and nitrogen storage. Proline also improves salinity tolerance through balancing the osmotic potential and maintains the enzymatic activities in presence of toxic ions (Xue *et al*., 2009). In this regard, Abadi *et al*. (2010) reported that proline content of leaves and root of citromelo, rough lemon, sour orange and Volkameriana lemon increased with increasing NaCl salinity. Most of citrus growers in arid and semi-arid areas use the salt sensitive citrus rootstocks that lead to a decrease in growth of scions and fruit production.

This study evaluated the response of citrus rootstocks to saline conditions. In order to achieve this goal and to explain the possible adaptive mechanisms to salinity involved at physiological and biochemical levels, changes in Na, Cl and K, as well as in leaf proline and Relative Water Content (RWC) were recorded and analyzed in five citrus rootstocks.

MATERIALS AND METHODS

Plant materials and growth conditions: The plant materials used in this study were Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.), Carrizo citrange [(*Citrus sinensis* (L.) Osbeck×*Ponicrus trifoliate* (L.) Raf.], Tiwanica (*Citrus taiwanica* Tan. and Shimada), Bacraii (*Citrus limettioides*×*Citrus reticulate*) and Shaker [(*Citrus limettioides*×*Citrus reticulate*)×*Citrus reshni*]. All seeds were provided by the Safiabad Agricultural Research Centre, Iran. Seeds of the rootstocks were sown in nursery sand beds at University Agriculture Park nursery of Universiti Putra Malaysia. After six months, uniform sized seedlings were selected, uprooted carefully to ensure minimum damage to roots and transplanted into 25×30 cm black plastic bags filled with 5.5 kg sandy-loam soil with pH 6.5, EC 0.6 dS m^{-1} and 0.64% organic matter. The plants were grown in a greenhouse with an average photoperiod of 12 h at the 27 (±5)°C day $night^{-1}$ temperature and 65 (±10)% RH.

NaCl treatments: The seedlings were watered with tap water for 60 days under greenhouse conditions. After 60 days of growth in poly bags, four levels of NaCl salinity (0, 25, 50 and 75 mM) were applied to citrus rootstock plants for 90 days. The salt levels were increased gradually over 2 weeks to avoid osmotic shock. The control water was maintained with a complete nutrient solution containing (mg L^{-1}) 232 N, 67 P, 239 K, 120 Ca, 30 Mg, 3 Fe, 80 S, 0.62 Mn, 0.44 B, 0.02 Cu, 0.11 Zn and 0.048 Mo. The electrical conductivity of four solutions according to the NaCl treatments were 1.8, 4.22, 6.63 and 9.03 dS m^{-1} for 0, 25, 50 and 75 mM NaCl, respectively.

Mineral ion contents: The roots samples and expanded leaves were harvested and washed with deionized water and dried at 70°C for 72 h. The nutrient concentrations were measured using the method described by Awang *et al*. (2009).

Relative water content: The leaf Relative Water Content (RWC) was measured in fully expanded leaves. Five leaf discs of 10 mm diameter were cut using a cork borer and their Fresh Weight (FW) was recorded. They were floated in small dishes containing deionized water for 4 h to regain turgidity and then re-weighed (TW). The discs were then dried at 80°C for 24 h to determine the Dry Weight (DW). The leaf Relative Water Content (RWC) of the fresh leaf samples was estimated using the following formula and the values were expressed in percentage (Smart and Bingham, 1974; Ghoulam *et al*., 2002):

$$\text{RWC (\%)} = \frac{\text{FW-DW}}{\text{TW-DW}} \times 100$$

Proline assay: Free proline content was extracted from the leaf tissues according to the method described by Bates *et al*. (1973). Plant tissues were snapped-frozen in liquid nitrogen. The 0.5 g of frozen leaf sample was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and filtered through Whatman's No. 2 filter paper. Two milliliter of the filtrate was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The reaction mixture was extracted with 4 mL toluene and the chromophore containing toluene was aspirated and the absorbance was quantified spectrophotometrically at 520 nm.

Experimental design and data analysis: This experiment was conducted in a Randomized Complete Block Design (RCBD) with 4 salinity levels×5 citrus rootstocks arranged in a factorial combination with four replications. The data obtained was subjected to ANOVA using SAS (Version 9, SAS Institute Inc. Cary, North Carolina, USA) and differences between treatment means were compared using Duncan's Multiple Range Test (DMRT) at $\leq$0.05% level of probability.

RESULTS

Na, Cl and K concentrations: The interaction of salinity levels and rootstocks showed a marked effect on Na, Cl and K concentration in leaves and roots of citrus rootstocks (Table 1). The leaves of Shaker and Cleopatra mandarin contained lower Na compared to the other rootstocks at all tested salinity levels. At 75 mM NaCl, leaves of Shaker contained. 51.9% less Na compared to those of Carrizo citrange and Tiwanica (Fig. 1a). The corresponding value for Cleopatra mandarin was 43.0%. The reverse trend was observed for Na root contents, suggesting that roots of Shaker had a higher ability to reduce

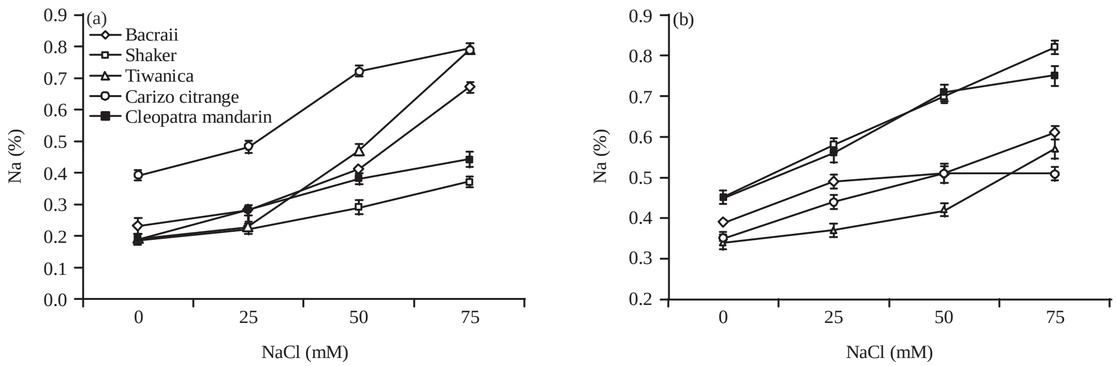

Fig. 1(a-b): Effects of NaCl salinity and rootstocks on (a) Leaf and (b) Root sodium contents. Values are expressed on dry weight basis (Mean±SE; n = 4)

Table 1: Effect of rootstock and NaCl salinity on concentration of Na, Cl, K, relative water content and proline content

Variables and factors	Na (%) Leaf	Na (%) Root	Cl (%) Leaf	Cl (%) Root	K (%) Leaf	K (%) Root	RWC (%)	Proline (μmol g^{-1} FW)
Rootstocks								
Bacraii	0.44^{c}	0.50^{c}	1.11^{b}	1.58^{b}	1.67^{c}	1.68^{b}	87.0^{b}	74.7^{c}
Shaker	0.27^{e}	0.43^{d}	0.80^{d}	1.90^{a}	1.94^{a}	1.55^{d}	88.7^{a}	95.7^{a}
Carrizo citrange	0.59^{a}	0.64^{a}	1.32^{a}	1.52^{c}	1.61^{d}	1.71^{a}	83.0^{d}	55.0^{e}
Cleopatra mandarin	0.32^{d}	0.49^{c}	0.99^{c}	1.89^{a}	1.79^{b}	1.64^{c}	87.7^{b}	87.5^{b}
Tiwanica	0.50^{b}	0.62^{b}	1.17^{b}	1.43^{d}	1.53^{e}	1.68^{b}	85.8^{c}	58.7^{d}
NaCl (mM)								
0	0.24^{d}	0.40^{d}	0.21^{d}	1.05^{d}	1.85^{a}	1.91^{a}	92.7^{a}	33.4^{d}
25	0.30^{c}	0.49^{c}	0.77^{c}	1.61^{c}	1.76^{b}	1.75^{b}	88.8^{b}	49.8^{c}
50	0.52^{b}	0.57^{b}	1.23^{b}	1.86^{b}	1.72^{c}	1.52^{c}	84.3^{c}	99.9^{b}
75	0.64^{a}	0.68^{a}	2.11^{a}	2.14^{a}	1.5^{d}	1.43^{d}	80.2^{d}	114.4^{a}
F-test (significant level)								
Rootstock	**	**	**	**	**	**	**	**
NaCl	**	**	**	**	**	**	**	**
Rootstock× NaCl	**	**	**	*	**	**	**	**

**Significant at 1% probability level, *Significant at 5% probability level. Concentration of Na, Cl and K are expressed on dry weight basis. Means in each column with different letters indicate significant differences at $p \leq 5\%$ level according to DMRT, RWC: Relative water contet, FW: Fresh weight

the upward movement of Na in plants. For example, at the highest level of salinity (75 mM NaCl), roots of Shaker contained 41.4% more Na compared to those in roots of Carrizo citrange. Root Na contents in the two rootstocks are 0.58 and 0.82%, respectively (Fig. 1b).

Similar to Na, the concentration of Cl in leaves and roots increased with NaCl salinity but the variations were depended on rootstock species as shown by significant interaction between rootstocks and salinity (p<0.01) (Table 1). Again, at the highest salinity, leaves of Carrizo citrange contained the highest concentration of Cl (2.63%) and this is followed by Bacraii (2.34%) (Fig. 2a). Results in Fig. 2a also revealed that leaves of Shaker contained the lowest concentration of Cl which was only 63.5% of Cl of that found in leaves of Carrizo citrange. Leaves of Cleopatra also contained relatively low Cl content. In contrast to K in leaves, Cleopatra mandarin and Shaker had higher Cl contents in their roots tissue compared to other rootstocks. At 75 mM NaCl, Cl contents in roots of Shaker was the highest (2.60%) (Fig. 2b).

Results showing the effects of NaCl salinity and type of rootstocks on K are shown in Table 1 and Fig. 3. Apparently K contents in leaf and roots were significantly affected by rootstocks and levels of salinity as well as by interactions between the two factors (p<0.01). Overall, K content in both leaves and roots decreased with increasing salinity from 1.85-1.50% in leaves and from 1.91-1.43% in roots, with their respective decrease of 18.9 and 25.1%. Figure 3a clearly shows that there was a clear difference in K decrease pattern among rootstocks in response to the increase in NaCl salinity. The K contents in leaves of Tiwanica, Carrizo citrange and Bacraii declined rapidly with increasing NaCl compared to those in leaves of Shaker and Cleopatra mandarin. In the case of Shaker, the decrease in leaf K was not affected by NaCl treatment and its K contents remained within the range of 1.8-1.95%.

Relative water content: As expected the leaf RWC was significantly affected by NaCl salinity (Fig. 4a) and the effects were clearly shown for various rootstocks at 50 and 75 mM NaCl with Cleopatra mandarin and Shaker having higher RWCs compared to the RWCs the other three rootstocks (p<0.01). At the highest level of salinity, the RWC of Cleopatra mandarin and Shaker were maintained above 80%, while the RWC for other rootstocks were reduced to less than

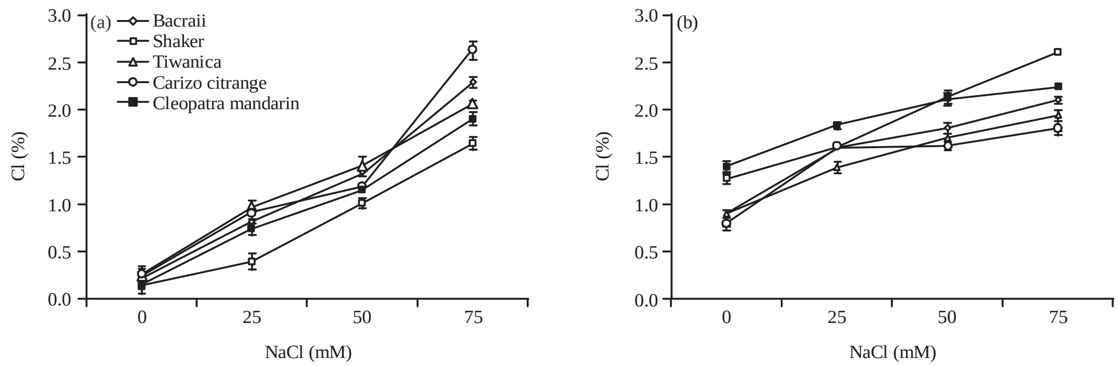

Fig. 2(a-b): Effects of salinity and rootstocks on (a) Leaf and (b) Root chloride contents. Values are expressed on dry weight basis (Mean±SE; n = 4)

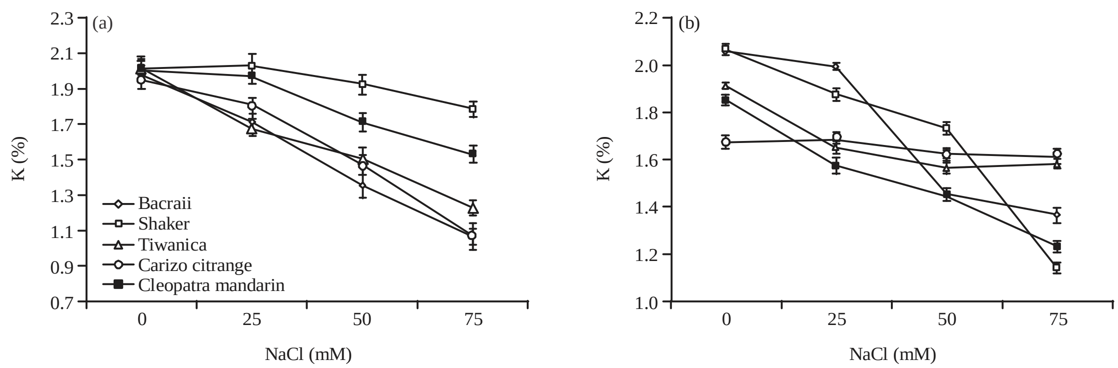

Fig. 3(a-b): Effects of salinity and rootstocks on (a) Leaf and (b) Root potassium contents. Values are expressed on dry weight basis (Mean±SE; n = 4)

72%. Among the rootstocks tested, in term of RWC, Carrizo citrange were the most sensitive rootstock to the increasing NaCl.

Proline: Consistent with other parameters measured, Cleopatra mandarin and Shaker showed to have a higher resistance to increasing salinity stress by accumulating higher concentration of proline in their leaves (Table 1 and Fig. 4b). At the highest salinity, proline content in leaves of Cleopatra mandarin was 141.4 μmol g^{-1} FW and this was followed by Shaker (at 75 mM NaCl (Fig. 4b).

DISCUSSION

Different methods maybe used in selection of citrus rootstocks for production under saline condition. In the present study the effect of NaCl salinity on mineral contents, relative water content and proline content in five citrus rootstocks were investigated. Results showed that Na concentration of all five citrus rootstocks increased with increasing salinity levels. Increase in Na concentration in shoots and roots of all five citrus species following NaCl treatments are in agreement with other reports in citrus trees (Ruiz *et al*., 1997; Anjum *et al*., 2001; Levy and Syvertsen, 2004).

As shown in Fig. 1a, higher accumulation of leaf Na was observed in Bacraii, Citrus Tiwanica and Carrizo citrange rootstocks. The result shows the susceptibility of these rootstocks to NaCl salinity and further proves the salt sensitivity of Carrizo citrange which have also been reported earlier (Al-Yassin, 2005; Garcia-Sanchez and Syvertsen, 2006). The evidence on Na concentration in plant tissues showed that Shaker and Cleopatra mandarin have the ability to restrict and store Na in their roots, thus prevent its excessive transportation to the shoots which would reduce damages caused by increasing salinity in shoots, making these species become more tolerance to salinity damages.

Roots of other rootstocks especially Carrizo citrange contained lower concentrations of Na that indicates a higher transportation of this element to shoots which could lead to serious injury. Results recorded here are in agreement with those reported by Garcia-Sanchez and Syvertsen (2006) and Anjum *et al*. (2001) and serve as an evidence for salinity tolerance of Shaker and Cleopatra mandarin rootstocks. Damages caused by high Na are seen as necrosis of leaves especially the older ones that begins first at tips and margins of the leaves and extends towards the center of leaf. Serious leaf injuries due to salinity is considered to be an obvious characteristic of salinity sensitive species and cultivars of fruit

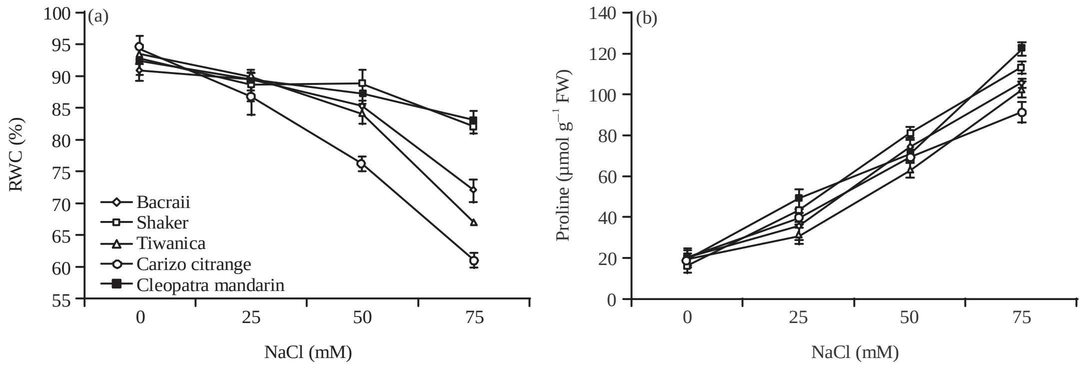

Fig. 4(a-b): Effects of salinity and rootstocks on (a) Relative water content (RWC) and (b) Proline contents (Mean±SE; n = 4)

trees and the injury can be used as index in salt-plant sensitivity assessment (Kozlowski, 1997; Murkute *et al*., 2005).

Chlorine concentration in both leaves and roots of rootstocks was also increased by increasing NaCl salinity in this study. Such increase has also been observed in other studies (Ruiz *et al*., 1997; Abbaspour, 2008; Karimi *et al*., 2009). Zekri (1993) stated that Cl concentration in shoots of citrus plants was a better parameter for evaluating the damages caused by salinity compared to Cl concentration in roots and could therefore be a good parameter for categorization of citrus rootstocks. Similar to Na, Carrizo citrange, Tiwanica and Bacraii rootstocks accumulated higher Cl concentrations in their leaves compared to Shaker and Cleopatra mandarin rootstocks (Fig. 2a). However, rootstocks used in this study were different for their ability to accumulate Cl in their roots (Fig. 2b). Shaker and Cleopatra mandarin stored the highest concentrations of Cl in their roots and prevented its transportation to the shoots. Carrizo citrange rootstock stored the lowest concentration of Cl in its roots and transported most of ions to the shoots which is in agreement with what has been reported by Anjum (2008) and Al-Yassin (2004). Capability of some citrus rootstocks for Cl transportation control to shoots or having a restrictive system for Cl absorption in roots has been reported earlier by several researchers (Storey and Walker, 1998; Al-Yassin, 2004; Murkute *et al*., 2005). There is also the possibility that some citrus rootstocks prevent the accumulation of Cl in their shoots by producing new roots and removing or abscission of dead roots due to Cl toxicity as it is the case in some mango rootstocks (Zuazo *et al*., 2003).

Investigating the concentration of K under salinity stress is of utmost importance because K as an osmotic regulator plays an important role in maintaining the plant's osmotic potential especially in roots and its presence is necessary for water transportation through xylem and preservation of water in plant (Grattan and Grieve, 1998). There are also reports that indicate higher K concentration in tissues of citrus plants under salinity stress was one of the characteristics of salinity tolerant cultivars (Al-Yassin, 2004; Murkute *et al*., 2005). In this study, K concentration decreased in both leaves and roots with increasing NaCl salinity.

Roots had relatively lower concentrations of K compared to shoots. Decreased K concentration in tissues indicates a reduction in plant's ability to accumulate sufficient K in order to neutralize the effects of Na and as a result, a reduction in its ability to regulate plant physiological activities (Grattan and Grieve, 1998). Similar effect of salinity on K in fruits has been reported by many researchers such as those in citrus (Ruiz *et al*., 1997; Murkute *et al*., 2005), strawberry (Awang and Atherton, 1994), pistachio (Karimi *et al*., 2009) and mango (Zuazo *et al*., 2003). Shaker and Cleopatra mandarin rootstocks accumulated higher K concentrations in their shoots compared to other rootstocks, thus alleviating harmful effects of Na accumulation (Fig. 3a-b).

Increasing salinity also reduced RWC of all rootstocks. Under saline conditions, cell elongation rate and turgidity decreases and cell wall becomes thicker and rigid. This situation occurs as a result of decrease in water potential and higher absorption of Na and Cl and their impact on particular processes such as synthesis of cell wall would lower the plant's salt resistant or tolerant (Fricke and Peters, 2002). Cleopatra mandarin and Shaker rootstocks had relatively higher RWC at the highest level of salinity compared to other rootstocks (Fig. 4a), which may be associated with their ability to accumulate larger amount of soluble sugars and proline that are effective for osmotic adjustment.

Shaker rootstock showed the highest leaf proline content at the start of salinity treatment, followed by Cleopatra mandarin, Bacraii, Tiwanica and Carrizo citrange rootstocks. Consistent with the results obtained in this study, Anjum *et al*. (2001) also found that proline concentration was higher in Cleopatra mandarin and Troyer citrange when grown under salinity stress conditions. Similar results were also reported by Ghaleb *et al*. (2010) in citrus.

CONCLUSION

This study investigated some physiological and biochemical characteristics of citrus rootstocks in a saline environment. Results showed that Na and Cl concentration increased with increasing salinity. We also observed that K concentration decreased in plant tissues under the same conditions. It is clear that an increase in Na and Cl together with a decrease in K concentration may cause damage to the plant under saline conditions but considering genetic differences in salinity tolerance among citrus rootstocks, it was found that Shaker and Cleopatra mandarin accumulated optimal concentrations of these ions in their tissues. Generally, increasing salinity had reduced RWC but Shaker and Cleopatra mandarin have been shown to have the ability to maintain a higher water status. Shaker and Cleopatra mandarin had also been shown to contain higher proline at high salinity compared to other rootstocks.

Overall, Shaker and Cleopatra mandarin can be regarded as salinity tolerance rootstocks due to their capability in restricting Na and Cl absorption or limiting transportation of the ions to shoots, keeping high K and proline concentrations in their tissues while maintaining high plant's water status.

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