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Short communication

Responses of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) to continuously and gradually increasing NaCl concentration

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Abstract – The effect of continuous or gradual stress due to NaCl on *in vitro* growth, proline and sugar accumulation and nutrient acquisition of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) explants was studied. Apical shoot tips obtained from previous subculture were transferred to a Murashige and Skoog nutrient medium for proliferation and were exposed to continuous or gradual salinity stress for 42 days. The salt used to induce salinity was NaCl added in six concentrations: 0, 50, 100, 150, 200 and 300 mM. Gradual salinization was achieved by transferring the explants sequentially every week to the above mentioned NaCl concentrations. Most salt treatments had a negative effect on the growth parameters of explants. Sodium concentration of explants increased in all NaCl treatments compared to control and it was higher in the treatments with gradual exposure to salinity. Potassium concentration was reduced, mostly in the treatments with continuous exposure. Calcium and Mg concentrations increased in all saline treatments. In general, the high salinity level in the substrate enhanced the proline and sugar concentrations of the studied explants. In conclusion, salinity had significant impacts on the growth and chemical status of *P. trifoliata*.

Keywords: Carbohydrates, micropropagation, *Poncirus trifoliata*, proline, salinity stress

Introduction

Citrus is one of the most economically important crops worldwide. However, in arid and semi-arid regions drought and saline irrigation water are a common problem and a major consideration in citrus production (FERGUSON and GRATTAN 2005). The damage to plants exposed to salinity has been attributed to ion toxicity, nutrient imbalance, osmotic and

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oxidative stress (GRATTAN and GRIEVE 1998, ZHU 2001). Compared with other crops, *Citrus* and *Poncirus* are among the most sensitive to soil salinity, although the ability of citrus trees to tolerate salinity varies among species and depends on the rootstock. For orange, the values of electrical conductivity of the soil solution that correspond to a 100%, 50%, or zero yield potential are 1.7, 4.8 and 8.0 dS m^{-1}, respectively (MAAS 1984).

The genera *Citrus* and *Poncirus* are commonly used rootstocks and ornamental species worldwide. Field screening in saline sites to select salt tolerant genotypes of citrus rootstocks is complicated because of differential ontogenic reactions of the plant to salinity and a large genotype in combination with environment interaction. *In vitro* tissue culture is a simple technique that offers a suitable alternative for the study of physiological mechanisms of tolerance to salinity, since it provides relatively fast responses, needs a short testing time and a controlled environment, especially in tree species that have long reproductive cycles (PEREZ-TORNERO et al. 2009).

The aim of this experiment was to examine nutritional, physiological and growth responses of *in vitro* cultivated trifoliate orange (*Poncirus trifoliata* (L.) Raf.) to continuously and gradually increasing external NaCl concentration.

Materials and methods

Apical shoot tips (length approximately 20 mm) of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) obtained from previous subculture, were transferred to a Murashige and Skoog nutrient medium (MURASHIGE and SKOOG 1962) for proliferation and were exposed to continuous or gradual salinity stress *in vitro*. The salt used to induce salinity was NaCl added in six concentrations: 0 (control), 50, 100, 150, 200 and 300 mM (or 0, 3, 6, 9, 12 and 16 g L^{-1}, respectively). Gradual salinization was achieved by transferring the explants sequentially every week to the above mentioned NaCl concentrations. All media contained 2 mg L^{-1} BA (6-benzyladenine), 0.1 mg L^{-1} IBA (3-indolebutyric acid), 30 g L^{-1} sucrose and 6 g L^{-1} agar (Oxoid No3). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 minutes. Cultures were incubated at 25±2 °C under cool white fluorescent tube lamps (90 µmol m^{-2} s^{-1}), with a 16-hours photoperiod, for six weeks. Each treatment consisted of 15 replicates (tubes). For the explants grown under continuous salt stress, growth characteristics (shoot number and length, fresh and dry matter weight) were recorded at the end of the experiment, while for the explants exposed to gradual salinity stress, growth parameters were recorded at each NaCl treatment after each transfer. At the same time, samples of explants were analyzed for proline and carbohydrates according to KHAN et al. (2000). Furthermore, after growth assessment, explants were dried at 75 °C for 48 hours, ground to a fine powder and ashed at 500–550 °C. Subsequently, the shoot samples were analyzed for concentration of K, Ca, Mg, Mn, Zn and Na ions by atomic absorption spectroscopy (Perkin-Elmer 2380) and for concentration of Cl ions by titration with $AgNO_3$. The experimental layout was completely randomized and the experiment was repeated twice. The reported data are the means of the two experiments. Means were compared using the Duncan multiple range test for $p \leq 0.05$.

Results and discussion

Some explants treated continuously with 300 mM NaCl showed signs of chlorosis and/or wilting (data not shown). These salt injuries could be due to accumulation of Na or Cl

(or both) resulting in excessive levels of transpiring leaves, building up rapidly in the cytoplasm and inhibiting enzyme activity, or in the cell walls, altering their functioning (FLOWERS and YEO 1986). It is well established that salinity stress is followed by chlorophyll degradation that leads to a chlorotic appearance in explants. High concentrations of NaCl applied under *in vitro* conditions reduced the chlorophyll content in *Citrus* (PEREZ-TORNERO et al. 2009) and other woody species (CHATZISSAVVIDIS et al. 2008). Similarly, GHALEB et al. (2010) experimenting with sour orange cultivated under *in vitro* conditions recorded complete leaf damage at 300 mM NaCl.

In all NaCl treatments, a decreased number of shoots per explants was observed (Tab. 1). Similarly, in explants of *Citrus macrophylla* a significant decrease in the shoot number was observed at 30 mM NaCl (PEREZ-TORNERO et al. 2009). Also, it was reported that continuous or gradual exposure to high NaCl in nutrient medium led to a reduced number of shoots in sour orange (SHIYAB et al. 2003). The length of shoots produced at and above 150 mM NaCl was found to be significantly decreased in continuous maintenance treatments and increased in the gradual transfer treatments, except for the treatment with 300 mM NaCl (Tab. 1). Likewise, SHIYAB et al. (2003) observed that shoot length of sour orange explants was negatively affected by continuous and gradual exposure at a salinity level of 150 mM NaCl or higher. Similarly, bitter almond (*Prunus dulcis* (Miller) D. Webb.) explants showed decreased shoot length when cultured on a substrate with high NaCl content (SHIBLI et al. 2003). The weight of fresh and dry matter of shoots was decreased by NaCl treatments (Tab. 1). The fact that the explants gradually exposed to 150–300 mM NaCl had higher dry matter weight than those exposed to 50–100 mM seems to be an unexpected result. A possible explanation is that the explants grown under high salinity benefit from gradual exposure (as compared to continuous one) and develop a mechanism that directs salt ions to build up in the apoplast of cells, or be isolated within the vacuole. In any case, further research on gradual and continuous exposure of plants to salinity would elucidate this point. In agreement to these results, SHIYAB et al. (2003), experimenting with sour orange cultured under NaCl stress, observed a negative effect on explant weight, mainly in continuous exposure treatments. The inclusion of NaCl in the nutrient medium also led to a decrease in dry

Tab. 1. Effect of continuously (C) and gradually (G) increased NaCl levels on certain growth parameters, total sugar and proline concentrations of trifoliate orange explants. Mean values followed by different letters in each column are statistically different (Duncan's multiple range test, $p \leq 0.05$).

Treatments NaCl (mM)	Number of shoots		Length of shoots (cm)		Shoot weight (mg) fresh matter		Shoot weight (mg) dry matter		Total sugars (μmol g^{-1} f.w.)		Proline (μmol g^{-1} f.w.)	
	C	G	C	G	C	G	C	G	C	G	C	G
0	8.1^{a}	8.1^{a}	0.86^{ab}	0.86^{d}	580^{a}	580^{a}	93^{a}	93^{a}	27.03^{b}	27.03^{c}	37.13^{b}	37.13^{b}
50	4.3^{b}	3.9^{cd}	0.99^{a}	0.94^{c}	432^{b}	262^{e}	75^{b}	59^{d}	35.58^{ab}	36.47^{c}	46.80^{ab}	38.45^{b}
100	2.1^{cd}	2.7^{cd}	0.59^{bc}	0.34^{f}	237^{c}	187^{f}	62^{bc}	51^{e}	51.86^{a}	41.87^{bc}	54.51^{a}	38.79^{ab}
150	2.9^{bc}	5.1^{bc}	0.26^{cd}	1.09^{a}	206^{c}	409^{b}	55^{c}	81^{b}	40.60^{ab}	41.24^{bc}	49.95^{ab}	38.60^{ab}
200	1.7^{cd}	5.7^{b}	0.28^{cd}	1.03^{b}	154^{c}	400^{c}	48^{c}	75^{bc}	46.38^{a}	65.48^{a}	55.85^{a}	49.30^{a}
300	0.7^{d}	3.9^{cd}	0.11^{d}	0.68^{e}	141^{c}	313^{d}	52^{c}	69^{c}	40.63^{ab}	50.73^{ab}	56.40^{a}	42.74^{a}

weight of bitter almond explants (SHIBLI et al. 2003). The reduced vegetative growth of the NaCl treated explants may partly be attributed to impairment of their photosynthetic efficiency. Photosynthesis, together with cell growth, is among the primary processes to be affected by salinity (MUNNS et al. 2006). Moreover, since under *in vitro* conditions relative humidity is very high, the toxic effects of salinity are more likely to be due to ion toxicity rather than to water stress (MILLS et al. 2001).

Sodium concentration in the trifoliate orange explants increased significantly in all NaCl treatments compared to control and, specifically, it was higher in the treatments with gradual exposure to salinity. In the treatments above 150 mM NaCl (continuous exposure) and those above 50 mM NaCl (gradual exposure), Na concentration reached excessive or toxic levels (> 0.25%) for citrus leaves. This pattern, the result of increasing NaCl in the medium, is a common and expected response and has been reported for many citrus species (DIONISIO and ANTONII 1997, SHIYAB et al. 2003, PEREZ-TORNERO et al. 2009, GHALEB et al. 2010). Moreover, it is well documented that tissue concentrations of Cl in *Citrus* increase in response to NaCl treatments (DIONISIO and ANTONII 1997, PEREZ-TORNERO et al. 2009, GHALEB et al. 2010). The concentration of chloride in explants was several times higher than that of Na, and a similar response was also observed for jojoba (*Simmondsia chinensis* (Link) Schneid) explants under high salinity conditions (ROUSSOS et al. 2007). Interestingly, Cl increased more than Na; Cl was 578% higher at 300 mM NaCl with respect to 0 mM NaCl, whereas Na was only 24.5% higher (Tab. 2). According to PEREZ-TORNERO et al. (2009), these results suggest that the important deleterious effects in the *Poncirus trifoliata* explants grown *in vitro* at increasing NaCl concentration could be due to toxic intracellular levels of saline ions, mainly Cl. Since, in general, Cl concentration in orange plants above 1% may be toxic causing leaf burn and defoliation (KOO et al. 1984), Cl concentrations found in the explants treated with NaCl in the present experiment are considered to be high.

As regards K concentration, it was reduced in all saline treatments compared to control (Tab. 2). Specifically, in high NaCl treatments, the decline of K concentration was higher in the treatments with continuous exposure. A decline of K^+ concentration in *Citrus* explants caused by the inclusion of NaCl into the nutrient medium has also been reported by other researchers (DIONISIO and ANTONII 1997, SHIYAB et al. 2003, PEREZ-TORNERO et al. 2009, GHALEB et al. 2010). What is more, this reduction was less in gradually shocked cultures, as was also found by SHIYAB et al. (2003) for sour orange. The decrease of K concentrations of

Tab. 2. Effect of continuously (C) and gradually (G) increased NaCl levels on Na, Cl, K, Ca and Mg concentrations (% d.w.) of trifoliate orange explants. Mean values followed by different letters in each column are statistically different (Duncan's multiple range test, $p \leq 0.05$).

Treatments	Na		Cl		K		Ca		Mg	
NaCl (mM)	C	G	C	G	C	G	C	G	C	G
0	0.220^{f}	0.220^{e}	0.84^{f}	0.84^{e}	1.69^{a}	1.69^{a}	0.91^{e}	0.91^{d}	0.15^{e}	0.15^{e}
50	0.235^{c}	0.247^{d}	2.56^{e}	2.50^{d}	1.53^{b}	1.27^{c}	1.04^{d}	1.76^{a}	0.18^{d}	0.27^{a}
100	0.228^{e}	0.252^{c}	2.64^{d}	2.55^{d}	1.44^{c}	1.43^{b}	1.13^{c}	1.49^{b}	0.19^{cd}	0.25^{b}
150	0.232^{d}	0.253^{c}	3.27^{c}	3.16^{c}	0.88^{d}	1.29^{bc}	1.36^{b}	1.21^{c}	0.20^{c}	0.21^{c}
200	0.261^{b}	0.265^{b}	4.55^{b}	4.74^{b}	0.84^{d}	1.31^{bc}	1.61^{a}	1.14^{c}	0.23^{a}	0.18^{d}
300	0.270^{a}	0.278^{a}	5.66^{a}	5.73^{a}	0.77^{e}	1.31^{bc}	1.64^{a}	1.21^{c}	0.21^{b}	0.18^{d}

plantlets in the presence of Na may be attributed to Na-K competition. Growth and K concentration in gradual exposure treatments declined less than in cases of continuous exposure, or even increased slightly, which may be an indication of the high adaptability of trifoliate orange to high salinity. In addition, it shows that, for salinity tolerance studies, gradual transfer is preferable to continuous exposure.

Calcium and Mg concentration increased in all saline treatments (Tab. 2). These results are in agreement with those of other researchers experimenting with Carrizo (*Poncirus trifoliata* × *Citrus sinensis*) hybrids and *Citrus macrophylla* explants (GARCIA-SANCHEZ et al. 2003, PEREZ-TORNERO et al. 2009). Calcium concentration in explants presented a lower increase in the treatments with gradual transfer than in those with a continuous exposure to high NaCl concentration in the nutrient medium. It has been reported that Mg concentration in plants is diminished, enhanced or not affected by salinity (DIONISIO and ANTONII 1997, ROUSSOS et al. 2007). This discrepancy found in proliferated explants, lacking a root system, could be related to differences in the diffusion rates of mineral nutrients in the culture medium with high NaCl concentrations (PEREZ-TORNERO et al. 2009).

In all NaCl treatments, Mn and Zn concentrations of explants were significantly reduced by salinity (data not shown). It is possible that the diffusion rate of the above ions decreases with increasing osmotic potential of the medium due to salinity, resulting in lower ion availability.

Finally, to counter increasing external salinity, plant cells must adjust osmotically, either by accumulating ions or by increasing their synthesis of organic solutes such as proline and sugars (FERGUSON and GRATTAN 2005). In the present experiment, proline concentration presented a significant increase in the explants cultured with 200 or 300 mM NaCl (and with 100 mM NaCl under continuous exposure) (Tab. 1). Similar results were observed in *Citrus macrophylla* explants where even at 60 mM NaCl the proline levels increased significantly, rising in line with the external salt concentration (PEREZ-TORNERO et al. 2009). Accordingly, there was a positive effect of salinity on sugar concentration (except the treatment 300 mM NaCl in continuous exposure) (Tab. 1). Increased proline and sugar levels were also reported by ROCHDI et al. (2003) working with sour orange explants treated with 137 mM NaCl.

In conclusion, the inclusion of NaCl in the nutrient medium leads to significant alterations in the growth, chemical status and proline and sugar concentrations of trifoliate orange explants.

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