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Tetraploidy enhances the ability to exclude chloride from leaves in carrizo citrange seedlings

M. Ruiz[a,*], A. Quiñones[b], M.R. Martínez-Cuenca[b], P. Aleza[a], R. Morillon[c], L. Navarro[a], E. Primo-Millo[b], B. Martínez-Alcántara[b]

[a] Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA) Moncada, Spain
[b] Centro de Citricultura y Producción Vegetal, Instituto Valenciano de Investigaciones Agrarias (IVIA) Moncada, Spain
[c] UMR AGAP, Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), Montpellier, France

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ABSTRACT

Tetraploid citrus seedlings are more tolerant to salt stress than diploid genotypes. To provide insight into the causes of differences in salt tolerance due to ploidy and thus to better understand Cl^- exclusion mechanisms in citrus, diploid and tetraploid seedlings of Carrizo citrange (CC) were grown at 0 (control) and 40 mM NaCl (salt-treated) medium for 20 days. Chloride uptake and root-to-shoot translocation rates were on average 1.4-fold higher in diploid than in tetraploid salt-treated plants, which resulted in a greater (1.6-fold) Cl^- build up in the leaves of the former. Root hydraulic conductance and leaf transpiration rate were 58% and 17% lower, respectively, in tetraploid than in diploid control plants. Differences remained after salt treatment which reduced these parameters by 30–40% in both genotypes. Morphology of the root system was significantly influenced by ploidy. Tetraploid roots were less branched and with lower number of root tips than those of diploid plants. The cross-section diameter and area were lower in the diploid, and consequently specific root length was higher (1.7-fold) than in tetraploid plants. The exodermis in sections close to the root apex was broader and with higher deposition of suberin in cell walls in the tetraploid than in the diploid genotype. Net CO_2 assimilation rate in tetraploid salt-treated seedlings was 1.5-fold higher than in diploid salt-treated plants, likely due to the loss of photosynthetic capacity of diploid plants induced by Cl^- toxicity. Leaf damage was much higher, in terms of burnt area and defoliation, in diploid than in tetraploid salt-treated plants (8- and 6-fold, respectively). Salt treatment significantly reduced (37%) the dry weight of the diploid plants, but did not affect the tetraploids. In conclusion, tetraploid CC plants appear more tolerant to salinization and this effect seems mainly due to differences in morphological and histological traits of roots affecting hydraulic conductance and transpiration rate. These results may suggest that tetraploid CC used as rootstock could improve salt tolerance in citrus trees.

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1. Introduction

Most citrus are cultivated in warm climates located around the Mediterranean basin, the United States and Brazil (Syvertsen and García-Sánchez, 2014), where irrigation is required because rainfall is scarce and its seasonal distribution is very irregular. Many soils from these areas are salt-affected and others present a high risk of salinization (Grattan et al., 2015). Soils are considered saline when ECe (electrical conductivity of the saturated paste extract) is 4 dS/m or more, which is approximately the equivalent to 40 mM NaCl and generates osmotic pressure of approximately 0.2 MPa (Munns and Tester, 2008). Citrus is a salinity-sensitive crop (Maas, 1986) and therefore, high NaCl contents in the root medium stunt growth and cause major physiological disorders (Gómez-Cadenas et al., 2003 Syvertsen and García-Sánchez, 2014), although there are striking differences in saline ion uptake and tolerance to salt stress among citrus species and relatives (Maas, 1992). Responses of citrus to salinity are apparently induced by two separate components of salt solutions (Munns and Tester, 2008). Initial responses are attributed to the water-stress effects triggered by reduced soil water potential (due to the osmotic component of saline solution), while ion-specific toxicity occurs later when excessive concentrations of saline ions accumulate in old leaves and cause damage. These effects also reduce the plant's photosynthetic capacity, which

* Corresponding author.
E-mail address: mvruiz@outlook.es (M. Ruiz).

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cannot fulfill the carbohydrate requirements of organs, which further stunts growth.

Citrus species and relatives differ in their capacity to accumulate Cl^- and Na^+ in leaves. Walker and Douglas (1983) observed major differences in Cl^- accumulation in leaves from seedlings of three citrus species, but minor differences for Na^+. At moderate salt levels, Walker (1986) reported that Cleopatra mandarin (*C. reshni* Hort. ex Tan.) (CM) was more efficient excluding Cl^- from leaves, while trifoliate orange (*Poncirus trifoliata* (L.) Raf.) (TO) was more efficient excluding Na^+, but accumulated Cl^-. Trifoliate orange restricts Na^+ transport to leaves by accumulating high Na^+ concentrations in the basal stem and roots. Recent research has indicated that a higher root Na^+ concentration in the TO genotype, and thus lesser allocation of this ion in the shoot, is the result of enhanced Na^+ retrieval from the xylem stream and an impaired translocation to shoot tissues, probably as a result of the overexpression of the *SOS1* and *HKT1* genes (Martínez-Alcántara et al., 2015). Higher transcriptional levels of *NHX1*, together with the enhanced activity of the tonoplast proton pumps (V-ATPase and V-PPiase), probably led to an increase of the relative vacuolar Na^+ concentration in the roots of TO compared to that of CM. This effect might reveal preferential Na^+ sequestration in the vacuole of TO root cells, thus reducing the xylem loading of this ion (Martínez-Alcántara et al., 2015).

However in citrus, Cl^- is considered the more toxic ion, as evidenced by the clear relationship between Cl^- accumulation in leaves and toxic damage (Bañuls and Primo-Millo, 1992, 1995; Bañuls et al., 1997; Romero-Aranda et al., 1998). In the later phase, leaf Cl^- content correlates with the abscission rate of leaves, which depends on their ethylene production. This suggests that in salt-stressed citrus, leaf abscission is induced by Cl^- build-up through a mechanism that stimulates leaf ACC (1-aminocyclopropane-1-carboxylic acid) synthesis and further conversion to ethylene (Gómez-Cadenas et al., 1998).

In cultivated citrus trees, the rootstock largely controls the uptake and/or transport of saline ions to the scion, and salt tolerance of scion/rootstock combinations is mainly determined by the capacity of rootstock to exclude Cl^- and to limit its translocation to the scion (Ream and Furr, 1976; Walker et al., 1983; Lloyd et al., 1990; Bañuls and Primo-Millo, 1995; Bañuls et al., 1997; Forner-Giner et al., 2009; Hussain et al., 2012). It is noteworthy that the wide variability in leaf accumulation of Cl^- or Na^+ between seedlings from different citrus species is also maintained in leaves of scions when these species are used as rootstocks. For example, the differences in the relative transport of Cl^- and Na^+ between CM and TO seedlings (Walker, 1986) also occurred when these species were used as rootstocks budded with Valencia late orange (*C. sinensis* (L.) Osb.) scions (Walker et al., 1983; Lloyd et al., 1990; Bañuls et al., 1997).

Autotetraploid plants can be considered clones of their diploid ancestors as they differ from them in a doubled number of somatic chromosomes. In the apomictic diploid genotypes of citrus, doubled diploids usually arise spontaneously by chromosome set doubling in maternal nucellar cells, which then form somatic embryos (Aleza et al., 2011). Some phenotypic differences between ploidies have been described (Cameron and Frost, 1968; Romero-Aranda et al., 1997; Syvertsen et al., 2000; Allario et al., 2011). Compared with diploids, these citrus tetraploid plants present thicker greener leaves, and shorter thicker roots. Large changes in anatomy and physiology between diploid and their autotetraploid genotypes (Syvertsen et al., 2000; Allario et al., 2011) could affect mineral leaf content. Moreover, different behaviour of tetraploid genotypes in comparison with their corresponding diploids under saline conditions has been reported (Saleh et al., 2008; Mouhaya et al., 2010; Syvertsen and García-Sánchez, 2014). This suggests the possible use of tetraploid seedlings as salt stress-tolerant rootstocks, thus providing a suitable system to manage toxicity problems caused by excess saline ions, while maintaining most of the genotype's favourable characteristics.

Carrizo citrange is the most widespread rootstock in Spain and is also widely used in other world citrus areas, nevertheless its use is sometimes limited because its sensitivity to salt (Maas, 1992). The purposes of this work were to check the behaviour of diploid and tetraploid seedlings of Carrizo citrange (*C. sinensis* x *P. trifoliata*) (CC) grown at elevated NaCl level in the external medium, to provide insight into the causes of the different behaviour of these plants under salt-toxicity conditions and to better understand Cl^- exclusion mechanisms in citrus, by comparing the factors affecting this process in two types of seedlings with high genetic similitude.

2. Materials and methods

2.1. Plant material

The plant material used for this study were seedlings originating from seeds of diploid (2x) and tetraploid (4x) Carrizo citrange (CC) trees that belong to the Citrus Germplasm Bank of pathogen-free plants at the Instituto Valenciano de Investigaciones Agrarias (IVIA; Valencia-Spain) (Navarro et al., 2002; Navarro, 2015).

2.2. Ploidy level determination

Carrizo citrange seedlings were obtained from the seeds of the aforementioned diploid and tetraploid CC trees and the ploidy level of all the nucellar seedlings used in the experiments (which are genetically identical to the mother plant) was determined by flow cytometry according to Aleza et al. (2009). Briefly, samples consisting in a small piece (approx. 0.5 mm^2) of leaf taken from each plant that was used in the experiments, with a similar piece from a diploid or tetraploid control plant, were chopped by a razor blade in the presence of nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec). Nuclei were filtered through a 30-μm nylon filter and stained with DAPI (4′,6-diamine-2-phenylindol) solution (High Resolution DNA Kit Type P, solution B; Partec). After 5 min of incubation, stained samples were run in a Ploidy Analyzer (PA; Partec) flow cytometer, equipped with a HBO 100-W high-pressure mercury bulb and both KG1 and BG38 filter sets. Histograms were analysed with the dpac v2.0 software (Partec), which determines peak position, coefficient of variation (CV) and the relative ploidy index of samples.

2.3. Experimental conditions

Carrizo citrange seeds (diploid and tetraploid) were germinated in a glasshouse using sterile substrate comprising peat, coconut fibre, sand and perlite (50:25:20:5), supplemented with 1.38 g kg^{-1} calcium superphosphate, and irrigated twice weekly with the following basal nutrient solution at half strength: 5 mM $Ca(NO_3)_2$, 1.4 mM KNO_3, 2 mM $MgSO_4$, 0.6 mM H_3PO_4, 20 μM Fe-EDDHA, 7.6 μM $ZnSO_4 \cdot 7H_2O$, 0.50 μM $CuSO_4 \cdot 5H_2O$, 50 μM H_3BO_3, 0.50 μM MoO_3, 54 μM $MnSO_4 \cdot H_2O$. The nutrient solution pH was adjusted to 6.0 with 1 M KOH or 1 M H_2SO_4. Water contained 3.8 mM Cl^- and 2.4 mM Na^+. After 8 weeks, seedlings were selected based on uniformity of size and were transplanted individually to opaque plastic 500 mL pots filled with substrate composed of peat, coconut fibre, sand and perlite (40:25:25:10). After transplanting, seedlings were habituated to the new medium with the same nutritive solution for 2 weeks. The seedlings of each ploidy were then separated into two groups and subjected to the different treatments. Six seedlings of each group were used for the ion analysis, six for the determinations of leaf greenness, leaf damage, defoliation and histological observations, and the remaining six seedlings were employed to measure the transpiration rate and root hydraulic conductance.

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