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Effects of salinity on diploid (2x) and doubled diploid (4x) *Citrus macrophylla* genotypes

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ABSTRACT

Tetraploid (4x) citrus seedlings may be more tolerant to salt stress than diploid (2x) genotypes. Genome duplication in citrus changes both plant physiology and anatomy, leading plants to acquire differentiated capacities to uptake and transport mineral elements. To provide insight into this behaviour, 2x and 4x *Citrus macrophylla* (CM), seedlings were grown at moderate (40 mM NaCl) and high salinity (80 mM NaCl) for a period of 30 days. Moderate salinity reduced the biomass of 4x plants, but did not affect the 2x plants, even when diploids accumulated more chloride (Cl^-) and sodium (Na^+) ions in leaves than tetraploids. The leaf K^+ concentration descended in 2xCM leaves but not in the tetraploids. These differences were correlated to variations in uptake and transport rates between the genotypes. Leaf osmotic potential in salinized plants was lower in 2x than in 4x leaves, but water potential and turgor were similar between them. Tetraploid plants had a stronger decrease in their gas exchange parameters than diploids when subjected to moderate salt stress. Leaf damage was only observed in 2x plants subjected to high salinity media, and was correlated with higher Cl^- leaf concentration than 4x plants, while Na^+ did not differ between them. Taken together, our results suggest that genome duplication improves the tolerance to saline toxicity in CM, because the lower Cl^- accumulation in leaves delays the damage. This effect may be linked to the reduced transpiration rate of the 4x genotype.

1. Introduction

Citrus are frequently cultivated in semiarid areas where many soils are affected by salt, or present a high risk of salinization. The saline soil solution around roots can derive from either irrigation water, soil release from mother rock or sea intrusion in coastal lands. Irrigation water has a low quality when contains excessive concentrations of soluble salts (sodium chloride or NaCl, among others) and generates an electrical conductivity (EC) greater than 3 dS m^{-1} which is the critical level for citrus production (García-Sanchez et al., 2002a). Soil is considered saline when EC of soil saturated paste extract reaches 4 dS m^{-1} or more, which is approximately equivalent to 40 mM NaCl and generates an osmotic pressure of about 0.2 MPa (Munns and Tester, 2008).

Salinity reduces growth and causes physiological disorders in citrus trees (Gómez-Cadenas et al., 2003; Syvertsen and García-Sanchez, 2014). Major differences in salt stress tolerance have been found between citrus species, related genera and hybrids (Maas, 1992). The effects of salt stress are partly induced by adverse water relations, due to reduced soil solution osmotic potential and excessive concentrations of saline ions in leaves, causing specific toxicities and nutrient imbalance (García-Sanchez et al., 2002a; Forner-Giner et al., 2011) which alter leaf gas exchange parameters. Salt stress was shown to decrease the water potential (ψ_S), stomatal conductance (*gs*), transpiration (*E*) and net CO_2 assimilation rates (A_{CO2}) in leaves (Lloyd et al., 1990; Bañuls et al., 1990; García-Sanchez et al., 2002a; Perez-Perez et al., 2007).

Uptake and/or transport of saline ions to the scion is controlled by the rootstock, which chiefly determines chloride (Cl^-) and sodium (Na^+) accumulation in leaves (Bañuls and Primo-Millo, 1995; Bañuls et al., 1990; García-Sanchez et al., 2002a, 2006a; Forner-Giner et al., 2009). Since the main ion that causes damage is Cl^- (Bañuls and Primo-Millo, 1992; Hussain et al., 2012), the salt tolerance of some citrus rootstocks is usually established by their capacity to exclude Cl^- from leaves (Bañuls and Primo-Millo, 1992, 1995; Bañuls et al., 1990).

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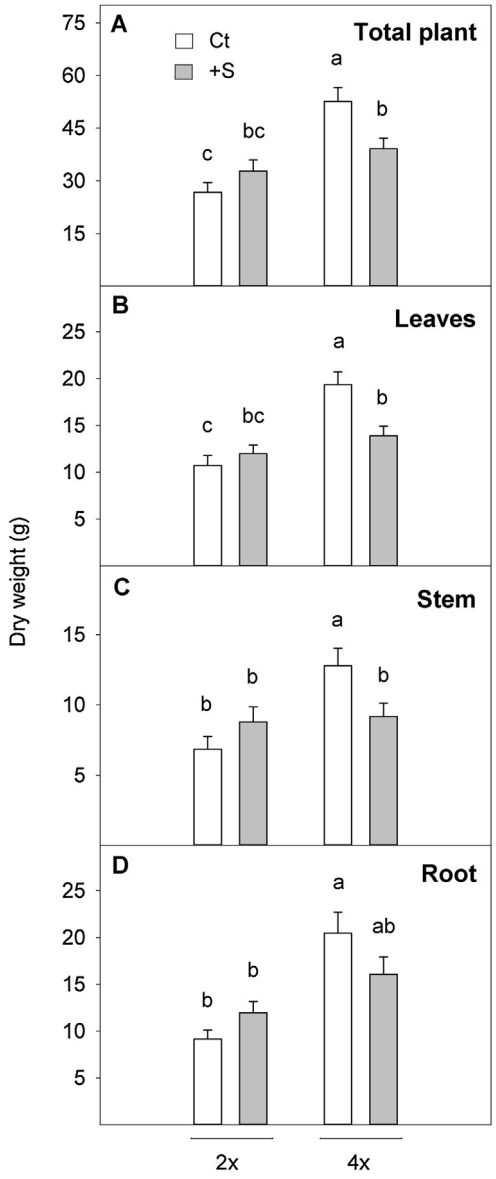

Fig. 1. Effect of 40 mM NaCl treatment for 30 days on the dry weight (g) of (A) total plant, (B) leaves, (C) stem and (D) root of diploid (2x) and tetraploid (4x) *Citrus macrophylla* seedlings. +S: salinized plants; Ct: control plants. The values are means of six independent plants (n = 6). Different letters indicate significant differences at $P < 0.05$ using Duncanís multiple range test.

Doubled diploid (4x) plants can be considered clones of their diploid (2x) ancestors and differ from them in a doubled number of somatic chromosomes. In apomictic 2x citrus genotypes, 4x plants usually arise spontaneously by chromosome set doubling in maternal nucellar cells, which then form somatic embryos (Aleza et al., 2011). Some phenotypic differences between ploidy levels have been described in citrus. When compared with 2x, 4x seedlings have depressed growth, thicker and greener leaves with larger volume in mesophyll cells, as well as shorter and thicker roots and larger fruits (Cameron and Frost, 1968; Romero-Aranda et al., 1997; Syvertsen et al., 2000; Allario et al., 2011). These differences may underlie a different performance of 4x plants with respect to 2x plants when subjected to salt stress, especially regarding the ability of 4x roots to exclude toxic saline ions from leaves (García-Sanchez et al., 2002b; Saleh et al., 2008; Grosser et al., 2012). Thus, it was proposed to use 4x genotypes as salt-stress tolerant rootstocks to provide a suitable system to manage the toxicity problems caused by saline ions excess, but maintaining most of the favourable characteristics from the 2x genotype.

The objective of this work was to study the behaviour of 2x and 4x seedlings of *Citrus macrophylla* Wester (CM), grown under salt stress conditions to elucidate if tetraploidy could further improve NaCl stress response in citrus providing insight into the causes of the differences in salt tolerance between both genotypes. To achieve this objective, two experiments were performed: In the first one, 2x and 4x seedlings were treated with a moderate salt concentration (+S) in the nutrient solution to check the effects on growth, ion concentration in organs, water relations and gas exchange parameters. In the second one, 2x and 4x seedlings were irrigated with a solution containing a high salt concentration (+HS) in order to induce leaf damage caused by ion toxicity and evaluate its impact. This specie (CM) was chosen because it is used as a rootstock to provide salt tolerance to citrus trees (Fernández-Ballester et al., 2003).

2. Materials and methods

2.1. Plant material

The plant material used for this study was originated from diploid (2x) and doubled diploid (4x) *C. macrophylla* (CM) seeds of trees from the Citrus Germplasm Bank of pathogen-free plants at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Spain.

2.2. Methods

2.2.1. Ploidy level determination

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

2.2.2. Experimental conditions

Diploid and 4x *C. macrophylla* seeds were germinated in a glasshouse using a sterile substrate composed by peat moss, 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. The Cl^- and Na^+ concentrations in irrigation water were 3.8 mM and 2.4 mM, respectively. After eight weeks, seedlings were selected according to uniformity of size, checked for ploidy level and transplanted individually to opaque plastic 0.5 L pots filled with a substrate composed by peat, coconut fibre, sand and perlite (40:25:25:10). Seedlings were acclimated for two weeks before the beginning of the experiments.