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Alleviation of salt stress in citrus seedlings inoculated with arbuscular mycorrhizal fungi depends on the rootstock salt tolerance☆

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ABSTRACT

Seedlings of Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) and Alemow (*Citrus macrophylla* Wester) were inoculated with a mixture of AM fungi (*Rhizophagus irregularis* and *Funneliformis mosseae*) (+AM), or left non-inoculated (−AM). From forty-five days after fungal inoculation onwards, half of +AM or −AM plants were irrigated with nutrient solution containing 50 mM NaCl. Three months later, AM significantly increased plant growth in both Cleopatra mandarin and Alemow rootstocks. Plant growth was higher in salinized +AM plants than in non-salinized −AM plants, demonstrating that AM compensates the growth limitations imposed by salinity. Whereas AM-inoculated Cleopatra mandarin seedlings had a very good response under saline treatment, inoculation in Alemow did not alleviate the negative effect of salinity. The beneficial effect of mycorrhization is unrelated with protection against the uptake of Na or Cl and the effect of AM on these ions did not explain the different response of rootstocks. This response was related with the nutritional status since our findings confirm that AM fungi can alter host responses to salinity stress, improving more the P, K, Fe and Cu plant nutrition in Cleopatra mandarin than in Alemow plants. AM inoculation under saline treatments also increased root Mg concentration but it was higher in Cleopatra mandarin than in Alemow. This could explain why AM fungus did not completely recovered chlorophyll concentrations in Alemow and consequently it had lower photosynthesis rate than control plants. AM fungi play an essential role in citrus rootstock growth and biomass production although the intensity of this response depends on the rootstock salinity tolerance.

Introduction

Soil salinity is an important abiotic stress, which strongly affects crop productivity by way of accumulation of toxic Na and Cl ions and nutrient imbalance (Grattan and Grieve, 1992; Munns and Tester, 2008). Citrus are widely cultivated in arid regions where the scarcity of water resources forces growers to use low-quality waters from aquifers containing excessive concentrations of soluble salts mainly sodium chloride. Citrus plants are classified as a salt-sensitive crop because relatively low salinity levels lead to physiological disturbance and a reduction in growth and fruit yield (Maas, 1993; Storey and Walker, 1999). The ability of citrus trees to tolerate salinity varies among species and depends on the rootstock (Maas, 1993). Salt-tolerance growth has been related with the ability of citrus rootstocks to restrict the uptake and/or transport from the roots to the shoots, primarily of Cl (Romero-Aranda et al., 1998; García-Sánchez et al., 2002) and secondarily of Na (Storey and Walker, 1987). For example, the rootstocks Cleopatra mandarin and Alemow, one of the most-common rootstocks employed in Spain, have differing characteristics, resulting in different responses to salinity. Whereas Cleopatra mandarin is considered among the rootstocks most capable of limiting Cl but not Na uptake, the rootstock Alemow seems to be able to exclude Na (Zekri and Parsons, 1992; Conesa et al., 2011). High concentrations of Cl and Na in leaves reduce net assimilation of CO_2 by a direct biochemical inhibition of photosynthetic capacity rather by decreases in stomatal conductance (García-Sánchez and Syvertsen, 2006). Osmotic adjustment, a net increase in intracellular inorganic and/or organic solutes, can maintain turgor and reduce the deleterious effects of salt stress on plants (Munns and Tester, 2008). The osmotic adjustment in salinized citrus leaves is very effective because even when leaf water potential is reduced, the high leaf Cl and Na concentrations reduce the osmotic potential such that leaf turgor is maintained or even increased (García-Sánchez and Syvertsen, 2006). In citrus, salt can also damage plants by causing

Abbreviations: AM, arbuscular mycorrhizal; −AM, non-inoculated plants; +AM, inoculated plants; DW, dry weight; MDA, malondialdehyde; RWC, relative water content; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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nutritional imbalances that have been attributed to the depressed absorption of some nutrients. A decrease in the concentration of Ca, Mg and, sometimes, K was found in citrus when salt concentration in the irrigation water was increased (Romero-Aranda et al., 1998).

Arbuscular mycorrhizal (AM) fungi have been considered as important bio-amelioratorsfor saline soils (Rabie and Almadini, 2005). It is well documented that AM colonization can enhance plant growth and vigour, alleviating damage of host plants caused by soil salinization (Evelin et al., 2009). When plants enter into a symbiotic relationship with AM fungi, morphological, nutritional and physiological changes occur, increasing the plant resistance to abiotic stresses (Augé, 2004; Wu et al., 2010a,b). Citrus is very dependent of AM colonization (Levy et al., 1983). Most citrus species, such as sour orange, trifoliate orange, Cleopatra mandarin, swingle citrumelo, and Carrizo citrange, have short or even rare root hairs, and are thus fairly dependent on AM colonization (Wu and Xia, 2006). Root architectural alteration in AM-colonized citrus could increase root functioning to explore more water and nutrients under salt stressed conditions (Wu et al., 2010a,b). Mycorrhizal inoculation not only affects root morphology but also physiological status in host plants. It has been shown that the citrus plants colonized by AM fungi had larger leaf area and higher leaf P concentration, and the tree growth and photosynthesis were more vigorous than those of noncolonized ones (Sherstha et al., 1995).

Enhancing salt tolerance of citrus plants is probably one of the most important objectives in the citrus management. In sustainable agriculture, solutions to salinity problems should include both plant breeding for salt tolerance and the application of biological factors such as AM fungi. The objective of this work was to evaluate the effects of fungal inoculation, on the growth, mineral nutrition and physiological response of citrus plants. In an attempt to better understand the behaviour of rootstocks with different response to salinity, the most commonly rootstocks Cleopatra mandarin (*Citrus reshni* Hort ex Tan) and Alemow (*Citrus macrophylla* Wester) were used, which are often irrigated with saline waters and which show different tolerance levels to salinity.

Materials and methods

Plant culture and experimental design

Seeds of Cleopatra mandarin and Alemow were surface sterilized for 10 min in 20% $NaClO_4$, rinsed four times with sterile distilled water and germinated into plastic trays containing moistened vermiculite. Forty-day-old seedlings were transplanted to plastic pots (1.1 L) containing substrate (soil:sand 3:1, v/v), previously sterilized in an autoclave for 1 h at 100 °C, three times on alternate days.

Twenty-five gram of *Rhizophagus irregularis* and *Funneliformis mosseae* in equal shares (a mix of spores, mycorrhized roots and substrate) mycorrhizal inoculum, propagated with the hybrid of *Sorghum bicolor* (L.) Moench and *Sorghum sudanense* (Piper) Hitch (*S. bicolor* × *sudanense*) as trap plant, was inoculated into the pots before transplanting. The inoculum was supplied by the Mycology-Mycorrhizas Laboratory, Department of Plant Biology, University of Murcia (Spain). The combination of these fungi species was selected as best inoculum according to previous results (data not shown). The non-inoculated plants (−AM) were watered regularly to maintain the relative humidity and 250 mL of modified Hoagland's solution (Hoagland and Arnon, 1950), with 2 mM $H_2PO_4^-$. Plants inoculated (+AM) were also irrigated regularly with the same solution but without P.

The seedlings were acclimated for 50 days and then were subjected to salinity treatments. Half of +AM and −AM seedlings was randomly used as the non-salt treatment (S1), and the other half was used as salt-stressed treatment (S2). The salt-stressed treatment was applied by 50 mM NaCl solution step wisely using 25 mM NaCl per day to avoid osmotic shock, whilst the non-salt treatment received 0 mM NaCl solution as the control. The plants were randomly placed in the same growth chamber.

The experiment was laid out in a completely randomized block design. Experimental treatments consisted of factorial combinations of two factors: mycorrhization (−AM and +AM) and salinity (S1 and S2). Each of four treatments had eight replicates and was repeated for each rootstock leading to a total of 32 pots per rootstock. The pots position was changed every week for eliminating the environmental error. The seedlings were harvested 45 days after NaCl treatments.

Growth and mineral analysis

Plant roots were separated carefully from the substrate and washed with distilled water. The fresh root systems were divided into two parts: one for the determination of mycorrhization and another was oven-dried for the dry weights (DW) and nutrients analysis. After roots and stems were oven-dried at 60 °C for 48 h (until constant weight) and leaves were freeze-dried, their DW were determined. Relative water content (RWC) of the leaves, stems and roots was measured. Freeze-dried leaves were ground and stored for chemical analysis. Dried and ground plant tissues were digested; the ashes were dissolved in HNO_3 and then P, Na, K, Mg, Ca, Fe. Cu, Mn and B were analyzed by an Inductively Coupled Plasma Optical Emission Spectrometer (Varian MPX Vista). Chloride was extracted with bidistillated water using the method of Guilliam (1971) and determined by ionic chromatography with a Dionex ICS-3000 ion chromatograph equipped with a conductivity detector and an AS11-HC anion exchange column.

Determination of mycorrhizal colonization

Root pieces of root fragments from every seedling were taken from the middle part of the root systems for estimation of mycorrhizal colonization. Samples were cleaned and stained with trypan blue according to the method of Phillips and Hayman (1970), but using lactic acid instead of lactophenol. One hundred root segments per plant were mounted on slides, squashed by pressing on the cover-slips and quantified for AM colonization according to McGonigle et al. (1990).

Physiological parameters determination

Chlorophyll contents in leaves were estimated after extracting 20 mg of the ground material, following the procedure described by Inskeep and Bloom (1985). Samples were kept at 4 °C and dark during 48 h with *N,N*-dimethylformamide. The absorbance of extracts was recorded at 664.5 and 647 nm and total chlorophyll concentrations (mg kg^{-1} DW) were calculated by the equation 250 ($17.9A_{647} - 8.08A_{664.5}$).

Gas interchange parameters were measured by an infrared gas analyzer (Li-6400, Li-Cor, Lincoln, NE, USA) on three replications per treatment from 9:30 to 11:30 am before harvest. Measurements were recorded when the total coefficient of variation was less than 0.5%.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) using the thiobarbituric acid (TBA) method (Heath and Packer, 1968). Dry tissue (25 mg) was homogenized in 1.5 ml of a 20% TBA and 0.5% trichloroacetic acid (TCA) mixture. The mixture was incubated at 95 °C for 45 min and then centrifuged at 4000 × *g* for 35 min at 4 °C. The supernatant was diluted in a 1:1 (v:v) ratio with water. Absorbance was read at 532 nm and correction for unspecific turbidity was done by subtracting the absorbance