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Salt stress modifies apoplastic barriers in olive (*Olea europaea* L.): a comparison between a salt-tolerant and a salt-sensitive cultivar

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ABSTRACT

Olea europaea L. (Olive tree) is a glycophytic species showing differences in salinity response depending on the genotype and the cultivar. Despite several studies have demonstrated which olive cultivars are tolerant and which are sensitive to salt stress, the role of anatomical adjustment in olive roots under salinity has not been completely clarified by scientific evidence. To address this problem one-year cuttings of olive cultivar Leccino (salt-sensitive) and Frantoio (salt-tolerant), were grown in an aeroponic system under controlled condition. Plants were sprayed (5 s of spray every 15 min-intervals) with the nutrient solution with and without 120 mM NaCl for 40 days. Free hand section of roots were taken and stained with 0.01% Fluorol yellow 088 to allow suberin lamellae visualization under a fluorescence microscope. Moreover, Cryo-SEM and energy dispersive X-ray microanalysis were used for ion localization in root cells. Both treated cultivars with Na^+ showed a significant reduction in shoot length, besides that, Na^+ concentration in leaves increased only in Leccino (salt-sensitive) as confirmed by translocation factor. Microscopic analyses showed that apoplastic barriers in the endodermis developed closer to the root apex in Leccino compared to Frantoio. Moreover, a significant Na^+ gradient concentration from exodermis to stele tissues between genotypes and cell types has been detected and it resulted stronger in Leccino than in Frantoio. In conclusion the different apoplastic adjustments in roots play a role in reducing ion fluxes to the shoots depending on genotype and Na^+ concentration.

1. Introduction

Olea europaea L. (olive tree) is the landmark tree of the Mediterranean Basin. Olive orchards are in fact visual fingerprints of the rural landscape of regions with a Mediterranean climate, as well as testimony of a long cultural tradition of usage of olive oil as food and medicinal supplement (Obied et al., 2012). Nowadays more than 95% of the world production of olive oil takes place in the Mediterranean countries with the EU member states being the largest producers accounting for about 75% of the world production (EUROSTAT, various years).

However, the yield of olives and olive oil could be seriously compromised because of climate changes in the areas of its major cultivation. Despite olive is a plant well adapted to withstand relatively high solar radiation, low temperatures, drought and salinity (d'Andria et al., 2009; Gucci and Tattini, 1997; Malik and Bradford, 2009; Sebastiani, 2011; Fernández, 2014), extended periods of drought and salt stress strongly affect the development of fruits and, therefore, endanger the annual crop yield. Salinity represents a hot topic, in fact, the extension of salt-affected areas varies according to published articles, but estimates are in the range of 6–7% of the world's total land. In addition to these, 77 Mha have been salinized due to human activities (Ghassemi et al., 1995). Approximately 20% of irrigated land are affected by salinity and since sporadic water supply with salty water is currently under evaluation, these values will very likely increase in the next future (Qadir et al., 2007).

According to the literature, olive is a glycophytic species of intermediate tolerance to salinity (Gucci and Tattini, 1997). Compared with other fruit trees that are generally salt sensitive, olive shows a good tolerance to salt stress, but presents wide differences in salinity response depending on the genotype (Bracci et al., 2008; Chartzoulakis, 2005). As observed in other non-halophytes (Møller et al., 2009) including olive, salt tolerance is associated with mechanisms of exclusion and retention of Na^+ and Cl^- in the root, which counteract ion translocation to the shoot (Chartzoulakis, 2005; Tattini, 1994) and seem to be a discriminant trait between tolerant and sensitive cultivars (Gucci and Tattini, 1997).

It is well known that roots are the main organ involved in the uptake of water and nutrients from soil, whereas xylem vessels allow their transport to aboveground tissues (Marschner, 1995).

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Besides this, roots function as the primary site for sensing salinity signal so that they can respond rapidly to maintain functionality, and meanwhile they transmit the signal to the shoot for appropriate changes in shoot function (Zhao et al., 2013). Moreover, roots are able to exclude and/or counteract potentially harmful substances by modifying their anatomy (Lux et al., 2011). In particular, the endodermis that separates the cortex from the central cylinder is characterized by the development of specific wall modifications, called 'apoplastic barriers' (Schreiber, 2010; Stoláriková et al., 2012) formed by a combination of Casparian strips and suberin lamellae (Schreiber et al., 1999). According to literature, also, exodermis (=hypodermis with Casparian bands) that separates the rhyzodermis from the cortex, plays an important role as barrier towards the passive apoplastic diffusion mechanism in roots (Clarkson, 1991).

Despite several studies have demonstrated which olive cultivars are tolerant and which are sensitive to salt stress, scientific evidence has not yet completely clarified the role of anatomical adjustment in olive roots under salinity. More scientific evidences on this topic could help to explain how Na^+ and Cl^- are excluded from the shoot in tolerant olive cultivar.

Our hypothesis is that the different responses to salinity in tolerant (Frantoio) and sensitive (Leccino) olive genotypes is related to changes in the development of apoplastic barrier. These modifications should affect radial transport of Na^+ across the root, and its accumulation in the shoot. To address this problem energy dispersive X-ray microanalysis combined with cryo-scanning electron microscopy (Cryo-SEM) analysis of frozen-hydrated samples has been used to determine cell-specific localization of elements in roots.

2. Materials and methods

2.1. Plant materials and Na treatments

One-year cuttings of olive (*O. europaea* L.) cultivar Leccino (salt-sensitive) and Frantoio (salt-tolerant), having just root primordia, were grown in an aeroponic system inside a growth chamber (temperature 26–20 °C; relative humidity 55–75% day–night; photoperiod 16–8 h light–dark; maximum light intensity 400–500 $\mu mol\ m^{-2}\ s^{-1}$). Olive plants were supplied with 1/3 strength olive medium (OM), pH 5.8 (Rugini 1984). After twenty days from the beginning of the experiment (when the average length of the roots reached 5–6 cm) four cuttings per genotype ($n = 4$) were assigned to the treatment with 120 mM NaCl supplemented along with full strength OM for additional forty days, and fully renewed every week. Plants grown in OM without the addition of NaCl were used as a control.

Root were regularly sprayed during the whole 40 days period of experiment with an automatic pump that provide 5 s of spray at 15 min intervals.

2.2. Growth analyses and biomass partitioning

At the end of the experiment all plants were carefully rinsed with deionised water, divided into root, stem, and leaves, and separately weighted (FW). Plant material to be used for Cryo-SEM and X-ray microanalyses was quickly frozen in liquid nitrogen and stored at −80 °C, material to be used for microscopic observations was quickly conserved under pure methanol, or otherwise dried for 10 days in a forced-circulation oven at 70 °C for the final determination of biomass (DW) and atomic absorption. Mass ratio of root (RMR), stem (SMR) and leaves (LMR) were calculated as the ratio between the biomasses of each organ and whole plant.

2.3. Ion contents analyses

Dry root, stem and leaves (0.2 g) grounded with a laboratory mill (IKA-Werke GmbH & Co.KG, Staufen, Germany) were digested in HNO_3 and used for the Na and Cl quantification in an atomic absorption spectrometer (model 373; PerkinElmer, Norwalk, CT, USA) equipped with specific lamps. Analytical standards for sodium were used as a control (Sigma–Aldrich, Saint Louis, MO, USA). The response of olive plant to salt stress was evaluated in terms of Translocation factor (Tf), an unit-less index indicating the ability of the plant to transfer Na from root to leaves (modified by Zacchini et al., 2009). The Tf index is based on the ration of Na concentration in leaves and roots (Eq. (1)).

$$Tf = \frac{[Na]\ leaves}{[Na]\ roots} \quad (1)$$

2.4. Anatomical observations

Series of hand sections of new primary roots from each plant were prepared at 1 mm intervals from the root apex to the base up to 10 cm from the root tip, where the suberin lamellae were fully developed. The sections floating in drops of clearing solution (lactic acid 85% saturated with chloral hydrate) on microscope slides were heated (70 °C for 1 h) over a water bath in covered Petri dishes. For suberin visualization in fluorescence microscopy, the free hand sections were stained by 0.01% Fluorol yellow 088 dissolved in lactic acid for 30 min and washed in distilled water, as described by Lux et al. (2005). The samples were placed into a drop of 0.1% $FeCl_3$ dissolved in 50% glycerine prior to observation. The sections were observed under a Leitz Orthoplan epi-fluorescence microscope (Wetzlar, Germany) and documented by a Leica EC3 digital camera (Leica Microsystems, Germany).

2.5. Ions localization in root cells by Cryo-SEM and energy dispersive X-ray microanalysis

In order to estimate the ion distribution within the primary roots, 10 cm long segments of fresh roots treated with 120 mM NaCl were collected from three plants, immediately plunged in liquid N_2, and then stored until observation.

Frozen-hydrated (FH) samples were moved to a cryo-preparation chamber (SCU 020, Bal-Tech, Liechtenstein), freeze-fractured at 6 cm from the root tip, surface etched for 2 min at −80 °C, sputter coated with 10 nm of platinum and transferred to a cryo-stage (−180 °C) inside the scanning electron microscope (SEM 515, Philips, The Netherlands).

Energy dispersive X-ray microanalysis (EDXMA) was performed on the transversal fracture faces in the Cryo-SEM using an acceleration voltage of 17 kV, a take-off angle of 16.5°, and a working distance (sample to final lens) of 12 mm. Spectra from 0 to 20 keV were collected at increments of 10 eV per channel with the electron beam focused on a spot area in the center of selected root cells. The EDX-ray spectra with Na *K* (1.041 keV) Cl *K* (2.622 keV) and K *K* (3.313 keV) were recorded during an analysis period of 100 LSEC. Background and element-specific peak spectra were analysed using the EDAX DX-4 software (EDAX, San Francisco, USA), which fully deconvolutes the spectra and corrects for interference between elements. X-ray net counts from at least four spectra from the various cell layers (exodermis, cortical cell, pericycle and stele) of three primary root samples for plants were analysed. Values were expressed as the ratio between the specific emission intensity of the ions above background and the nonspecific emission intensity of the background (peak/background means ± standard deviation of three measurements per cell compartment). All acquired Na^+, Cl^- and K^+ p/b ratios were consistently above detection limits. The