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Research Paper

Does salinity modify anatomy and biochemistry of *Olea europaea* L. fruit during ripening?

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ABSTRACT

The effect of salinity on physiology of olive (*Olea europaea* L.) fruits was studied using a salt-sensitive genotype (Leccino cv.) able to translocate sodium to the aerial part. Plants were treated with 0 and 60 mM NaCl for 49 days starting from the beginning of pit hardening to veraison. The effects of salt exposure were studied classifying olive in two Maturation Groups: MG_1 olives from green skin to < 50% purple skin; MG_2 olives with purple skin ≥50%.

Plants treated with NaCl present a significant reduction of shoot elongation after 35, 42 and 49 days of salt treatment (15%, 18% and 24%, respectively). Na accumulation occurs in fruit flesh depending on the MG: 559 mg kg^{-1} in MG_1 and 397 mg kg^{-1} in MG_2. In general, NaCl does not negatively affect fruit yield parameters while induce an increment of total phenols (58%) and DPPH scavenging activity (15%) in MG_1 in comparison with control. Cryo-SEM observations of freeze-fractured fruits revealed that salt treatment induce an increment of cells area and a thickening of cuticle, epidermis, hypodermis and outer mesocarp, that could be considered useful to protect fruits against other biotic and abiotic stress.

1. Introduction

Olive (*Olea europaea* L.) tree is adapted to climate of Mediterranean agroecosystems that is characterized by scarce and irregular rainfalls. Irrigation during the development of the fruits significantly increases tree performance (Gucci et al., 2011) improving olives and oil yield (Chartzoulakis et al., 2006; d'Andria et al., 2009). Since the availability of fresh-water in Mediterranean basin is limited, the use of saline water is becoming a practicable option (Ben-Gal et al., 2017; Chartzoulakis, 2005). However, saline water could represent a stress according to the level of salinity tolerance of the cultivars (Gucci and Tattini, 1997) and salinity level achieved (Weissbein et al., 2008). Examples of the use of moderate saline water (4.2 dS m^{-1} EC) for irrigation of twelve olive cultivars, coming from Mediterranean region, are reported by Weissbein et al. (2008). Despite cultivars showed significant differences in terms of growth and yield parameters, the saline irrigation treatment did not induce any effect on olive yield, suggesting a possible use of moderate salt irrigation. Long term responses under field salt treatment (EC = 5 and 10 dS m^{-1}) has been monitored in the salt tolerant Picual cultivar and data showed that annual yield were not affected by treatments (Melgar et al., 2009). Moreover, in field experiment with Barnea trees (considerate salt-tolerant) under EC 1.2, 4.2, and 7.5 dS m^{-1}, Wiesman et al. (2004) established that, while 7.5 dS m^{-1} EC retarded growth and fruit production, 4.2 dS m^{-1} EC retarded growth only in the first year of the study showing more fruit per tree than the 1.2 dS m^{-1} EC treatment used as control.

Studies on fruits physiology under salt stress are actually scarce: data available showed that saline water (EC = 6.5 dS m^{-1}) can increase fruits dry weight in the cultivars "Manzanillo" and "Uovo di Piccione" (Klein et al., 1994), while a decrease in drupes weight, volume and diameter, associated with olive oil content decrease, was observed in the cultivar "Chemlali" under EC = 7.5 dS m^{-1} (Ben Ahmed et al., 2009).

The physiological effect of salinity is also related to the ability of olive plants to translocate sodium ions (Na^+) from roots to aerial parts. This characteristic can explain salinity tolerance and sensitivity among olive genotypes (Chartzoulakis et al., 2002; Tattini et al., 1992). On this basis, the cultivar "Leccino" has been classified as a salt-sensitive genotype (Gucci and Tattini 1997) and recently Rossi et al. (2015) showed that "Leccino" has a very high Na^+-translocation factor (T_f) in the aerial part (T_f = 1.06 ± 0.121 at 120 mM NaCl) and more Na^+ in old- than in new-leaves (4260 ppm versus 2960 ppm, respectively) (Rossi et al., 2016).

In this study, we want to focus on "Leccino" fruits physiology in relation to salinity answering how Na: (i) accumulate in fruit tissues; (ii) modify the fruit mineral element status; (iii) changes fruit

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maturation index, polyphenols, *ortho*-diphenols and anthocyanins concentrations; (iv) modify fruit anatomy.

2. Materials and methods

2.1. Plant material and treatments

Four-years old olive trees (*Olea europaea* L.) of cultivar Leccino were grown in pots (33 cm Ø) filled with peat and pumice (60/40, v/v). Pots were accurately covered with a plastic foil in order to avoid rain water leaching effect. Plants were fertilized and irrigated at the beginning (day 1) of the experiment and one month after (day 30), with 2 L of 1/3 strength olive medium (Rugini 1984) at pH 5.8, containing a basal amount of 33 μM NaCl. The salt treatments started at fruit pit hardening stage (assessed by slicing fruits) and ended 49 days later. Two level of Na were applied (7 times – once a week): 0 mM NaCl (control plant, 2 L of 18.2 MΩ.cm Milli-Q water per plant) and 60 mM NaCl (2 L of 60 mM NaCl in 18.2 MΩ cm Milli-Q water per plant, corresponding to a measured EC of 6.44 dS m^{-1}).

2.2. Plant growth, PSII photochemistry and pigments analysis

The total number of fruits on each tree was counted weekly and on two randomly selected one-year-old fruiting shoots per plant, shoot length increment and fruit numbers were non-destructively measured. On the same shoots, chlorophyll *a* fluorescence was measured on the dark-adapted (30 min) leaves nearest to the infructescence, using a portable Chlorophyll fluorometer FMS2 (Hansatech, UK). Background fluorescence signal (Fo), maximum fluorescence (Fm) and potential quantum yield of PSII photochemistry [Fv/Fm = (Fm − Fo)/Fm] were determined. At the end of the experiment (49 days), two disks (Ø = 4 mm) were sampled (from 10:00 to 12:00 a.m.) from the same leaves used for the chlorophyll *a* fluorescence frozen in liquid nitrogen and stored at −80 °C until use. Disks were homogenized in 100% HPLC-grade methanol (1/10, w/v) overnight at 4 °C. A Zorbax ODS column was used for HPLC separation (5 μm particle size, 250 × 4.6 mm Ø; Agilent Technologies, Milan, Italy) and pigments were eluted under gradient of solvent A (acetonitrile/methanol, 75/25, v/v) and solvent B (methanol/ethyl acetate, 68/32, v/v) as reported in de las Rivas et al. (1989). Standard of lutein, zeaxanthin, Chl *b*, Chl *a* and β-carotene in a range of 0.0078–0.0625 mg mL^{-1} (Sigma-Aldrich Milan Italy) were used. Calibration equations, correlating peak area to pigment concentration (R^2 > 0.96), were used.

2.3. Fruit growth, biomass and morphological parameters

At the end of experiment olives were collected and divided into two Maturation Groups (MG), according to the classification used by Camposeo et al. (2013): MG_1 (olives with green skin and < 50% purple skin) and MG_2 (olives with ≥50% purple skin).

Fresh (FW) and Dry Weight (DW), flesh/pit ratio and fruit volume was determined on 15 olives per plant (n = 6) were analysed. Volume was calculated from the longitudinal and transverse diameters using the formula for a prolate spheroid ($V = 4/3\ \pi\ a^2\ b$, where *a* and *b* are the transverse and longitudinal radii, respectively).

2.4. Oil concentration

For the determination of the oil concentration, dried samples of olive flesh (2 g) were subjected to Soxhlet extraction using petroleum ether as solvent, for 6 h. After solvent evaporation, the flask containing the fat was dried at 105 °C and reweighted. Oil concentration has been expressed as percentage (flesh dry mass).

2.5. Extraction and assay of L-phenylalanine ammonia-lyase (PAL) activity

Fresh olive flesh from a group of 5 olive fruits per plants (n = 6), were frozen in liquid nitrogen for determine PAL activity (EC 4.3.1.5). Flesh powder (1 g) and 25 mL of potassium phosphate buffer 0.05 M (pH 6.6) were homogenized in vortex at 4 °C and then 0.2 g of Triton X-100 and 25 mg of insoluble polyvinylpyrrolidone was added. The suspension was centrifuged at 4 °C during 15 min at 10,000*g*. Olive flesh homogenate (0.4 mL) and 4.1 mL of sodium phosphate buffer were mixed in test tubes and the reaction started when 1 mL of L-phenylalanine (10 mg mL^{-1}) was added to the solutions. The tubes were then heated for a period of 1 h at 37 °C. The reaction ended after adding 0.5 mL of 35% trifluoroacetic acid (w/w). The quantification was done by measuring the absorbance at 290 nm and the enzymatic activity was expressed in μmol cinnamic acid h^{-1} on a flesh DW basis.

2.6. Total polyphenolic compounds, ortho-diphenols, anthocyanins and scavenging activity from flesh

For the total polyphenolic compounds and *ortho*-diphenols extraction, fresh olive flesh (5 g) from a group of 5 olive fruits per plants (n = 6), were macerated with 50 mL of a mixture of methanol/water (50/50, v/v) and incubated at room temperature for 30 min. After, the supernatant was decanted and extractions were repeated three times. The liquid phase was centrifuged at 10,000*g* for 10 min. To remove the fat phase, the mixture was washed twice with hexane (50 mL) using a separators funnel and the organic phase was discarded. Each extract was introduced into a 200 mL round bottom flask, which was filled up to the mark with methanol/water (50/50, v/v) (Machado et al., 2013).

The content of total phenolic compounds was determined using a Folin–Ciocalteu reagent with gallic acid as standard. One mL of properly diluted methanolic extracts was mixed with 500 μL of the Folin–Ciocalteu reagent, 2 mL of 7.5% sodium carbonate solution and 6.5 mL of water. The mixture was shaken and the absorbance of the standard and samples was measured at λ = 750 nm after 30 min reaction at 70 °C. All measurements were performed in triplicate. The results were expressed as milligrams of gallic acid equivalents per gram of olive flesh (DW).

The concentration of *ortho*-diphenolic compounds in the same methanolic extract was determined. One milliliter of 5% (w/v) sodium molybdate in 50% ethanol was added to the aliquots of the methanolic extract (5 mL). The contents were mixed for 1 min and the absorbance was measured after 15 min at 370 nm against a blank reagent prepared by adding 1 mL of 50% ethanol to the aliquot of the extract instead of sodium molybdate solution. A calibration curve with equation: $y = 0.0009x – 0.0004$ ($R^2 = 0.975$) was constructed using gallic acid solutions in the range of 200–0 mg L^{-1}.

For the anthocyanins extraction, frozen fruit tissue (100 mg) was ground in chilled mortar and extracted into 5 mL methanolic HCl (1%) and incubated overnight at 4 °C in darkness. After centrifugation at 5000*g* for 5 min, the supernatant was filtered and used for spectroscopic analysis.

Concentration of anthocyanins was expressed as cyanidin-3-glucoside equivalents per g DW determined with a spectrophotometer at 535 nm. Kuromanin chloride (cyanidin-3-glucoside chloride) was used as standard to a calibration curve $y = e^{-0.5x} + 0.0004$ ($R^2 = 0.9991$) in a range of 0–600 ug L^{-1}.

With the same extract used for total polyphenols and *o*-diphenols determination, the DPPH method proposed by Kontogiorgis and Hadjipavlou-Litina (2005) was used to determine antioxidant activity of olive fruit's flesh for each maturation group.

The optical density (OD) of the solution was measured at 517 nm, after 60 min. The percent reduction values were determined and compared to gallic acid as standards. Inhibition of the free radical DPPH, in percent (*I%*) was calculated using the following Eq. (1):

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