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Research Paper

Unraveling physiological, biochemical and molecular mechanisms involved in olive (*Olea europaea* L. cv. *Chétoui*) tolerance to drought and salt stresses

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ABSTRACT

Olive (*Olea europaea* L.) is an economically important crop for the Mediterranean basin, where prolonged drought and soil salinization may occur. This plant has developed a series of mechanisms to tolerate and grow under these adverse conditions.

By using an integrated approach, we described in *Chétoui* olive cultivar the changes in plant growth, oxidative damage and osmolyte accumulation in leaves, in combination with corresponding changes in physiological parameters and proteome.

Our results showed, under both stress conditions, a greater growth reduction of the aboveground plant organs than of the underground counterparts. This was associated with a reduction of all photosynthetic parameters, the integrity of photosystem II and leaf nitrogen content, and corresponding representation of photosynthetic apparatus proteins, Calvin-Benson cycle and nitrogen metabolism. The most significant changes were observed under the salinity stress condition. Oxidative stress was also observed, in particular, lipid peroxidation, which could be tentatively balanced by a concomitant photoprotective/antioxidative increase of carotenoid levels. At the same time, various compensative mechanisms to cope with nitrogen source demands and to control plant cell osmolarity were also shown by olive plants under these stresses. Taken together, these findings suggest that the *Chétoui* variety is moderately sensitive to both drought and salt stress, although it has greater ability to tolerate water depletion.

1. Introduction

Global water resource scarcity and soil salinization are becoming two main threats affecting agricultural losses in the Mediterranean climate regions (Askari et al., 2006; IPCC, 2014). Mediterranean vegetation, dealing with these particular soil characteristics, has developed a series of mechanisms to tolerate and grow under these adverse conditions. The primary effect of drought on plant cell is to generate osmotic stress, whereas salinity induces osmotic stress more indirectly through its effect on the ionic homeostasis within the plant cell (Zhu, 2002). The effects of both stress conditions on plants can decrease CO_2 availability caused by diffusion limitations through the stomata and the mesophyll (Flexas et al., 2007) or altered photosynthetic metabolism (Lawlor and Cornic, 2002). They also disrupt cell membrane function and elicit lipid peroxidation, protein degradation, and disturb redox homeostasis by forming reactive oxygen species (ROS) (You and Chan, 2015). However, salinity is generally more devastating than drought for two reasons: first, the osmotic effect reduces the capacity of the plant for the water uptake, and consequently, it has an effect on the ionic homeostasis within the plant cell; and second, the ion toxic effect, due to the increased salt uptake, causing tissue damage (Munns et al., 2006). In fact, it has been reported that Na^+ at a concentration of above 100 mM inhibits enzymes that require K^+ as a cofactor, including photosynthetic ones.

Abbreviations: 2-DE, two-dimensional electrophoresis; A, CO_2-assimilation rate; ATP syn, ATP synthase; BSP, bark storage protein; C, control sample; Ci, intercellular CO_2 assimilation; CA, carbonic anhydrase; Car, carotenoids; Chl, chlorophyll; D, drought-stressed sample; E, transpiration rate; FNR, ferredoxin-NADP reductase; Fv/Fm, maximum quantum efficiency of PSII; GCc, glutamine synthetase cytosolic isoenzyme; GCn, glutamine synthetase nodule isoenzyme; g_s, stomatal conductance; MDA, malondialdehyde; PSII, photosystem II; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; S, salt-stressed sample; SABP, salicylic acid binding protein; OEE1, oxygen evolving enhancer proteins 1; OEE2, oxygen evolving enhancer proteins 2; WUE, water use efficiency; Y, quantum yield of PSII photochemistry

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A common overlap among the signaling pathways, which operate on cellular redox status, reactive oxygen species, hormones, protein kinase cascades and calcium gradients, is commonly used by plants to counteract drought and salt stress (Zhu, 2002).

The olive tree (*Olea europaea* L.) is one of the most widely diffused and economically important crops of the Mediterranean basin that is well-known for its tolerance to prolonged drought periods, although very long and severe droughts may have significant effects on olive production (Galán et al., 2008). Species-specific morphological, physiological and biochemical mechanisms of adaptation of this plant to drought have been described, such as modification of leaf anatomy (Sofo et al., 2007), together with the regulation of gas exchange (Moriana et al., 2002), osmolyte content (Chartzoulakis et al., 1999) and the antioxidant system (Bacelar et al., 2007). Olive plants are also moderately tolerant to NaCl excess (Rugini and Fedeli, 1990). Nevertheless, under both stress conditions, a considerable tolerance variation among the different cultivars has been reported (Chartzoulakis et al., 1999; Bacelar et al., 2007).

Chétoui olive is the second main variety cultivated in the north of Tunisia. The oil obtained from this variety is valued for its physicchemical characteristics and in particular for the high amounts of total phenols, tocopherols, and flavonoids (Ben Temime et al., 2006, 2008; Abaza et al., 2011) that make *Chétoui* an economically interesting olive variety.

Previous physiological, biochemical and agronomical analyses have shown that this cultivar is considerably sensitive to drought (Guerfel et al., 2009a,b; Ben Ahmed et al., 2009; Dbara et al., 2016), while its response to salinity is completely unknown. Molecular mechanisms that underpin the stress response of the *Chétoui* plant and other olive varieties are still poorly investigated for two main reasons: i) the occurrence of many interfering compounds, *i.e.* oxidative enzymes, phenolic compounds (simple phenols, flavonoids, condensed tannins, lignin), carbohydrates, and nucleic acids and lipids, which make extraction procedures very problematic and tissues recalcitrant to investigation; ii) the lack of information about the *O. europaea* L. genome, which creates difficulties for the characterization of molecular factors and signal transduction pathways.

In the recent years, differential proteomic analysis has become an essential tool in the study of plant abiotic stress responses (Das et al., 2016) and we have developed an effective method for protein extraction from *Chétoui* leaves and corresponding two-dimensional electrophoresis (2-DE) analysis (Ben Abdallah et al., 2017).

In the present work, we provide a complete picture of the response of the *Chétoui* olive plant to drought and salinity stresses, describing the changes in plant growth, oxidative damage and osmolyte accumulation in leaves, in combination with corresponding changes in physiological parameters and proteome.

2. Materials and methods

2.1. Plant material and growth conditions

Seven months old self-rooted plants (*Olea europaea* L.) of the cultivar '*Chétoui*' were transplanted in 10 L pots filled with inert sand and kept in a glasshouse under controlled environmental conditions (day/night temperature regime of 25 °C/17 °C, 16 h photoperiod, light intensity of approximately 400 mmol m^{-2} s^{-1} and 70%–75% relative humidity) for 21 days. Three treatments (15 pots each) were defined by different conditions of plant growth: (i) control (named C), plants irrigated every two days with 100% Hoagland solution, ii) drought stress (named D), plants grown in complete water depletion for 21 d, and iii) salt stress (named S), plants irrigated every two days with 200 mM NaCl in 100% Hoagland solution. To avoid osmotic shock, NaCl concentration values were increased gradually by 50 mM every two days until the final 200 mM concentration was reached. The nutrient solutions were renewed every second day.

2.2. Physiological analysis

2.2.1. Estimation of plant growth and relative water content

After washing with distilled water, plants were dried with filter paper and corresponding shoots and roots were carefully removed. Thereafter, samples were dried in an oven at 60 °C until total desiccation and dry weights (DW) were noted.

Leaf relative water content (RWC) was calculated based on fresh (FW), turgid (TW) and DW of five fully expanded leaves, using the following formula: [(FW – DW)/(TW – DW)]100. All leaf samples were harvested between 9:00 h and 10:00 h in the morning and, immediately after their excision, the FW was determined. TW was determined after soaking the leaves in deionized water in the dark for 24 h. Leaves were dried at 80 °C for 48 h before being weighed for DW.

2.2.2. Gas exchange, pigment and fluorescence measurement

Gas exchange characteristic measurements of net CO_2 assimilation (A), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 assimilation (Ci) were made from 10.00 to 12.00 h on a fully expanded 3rd leaf (from top) of each plant (6 plants per treatment), using a portable open-system infrared gas analyser LCi instrument (Analytical Development Company Ltd., Hoddesdon, UK). The following conditions were used for the measurement: 398 ± 1 μmol mol^{-1} CO_2 concentration; 30 ± 0.3 °C leaf temperature; 1012 m Bar atmospheric pressure. Water use efficiency (WUE) was measured as the ratio of CO_2 assimilation to stomatal conductance (A/g_s).

Total chlorophylls (Chla + b) and carotenoids (Car) were determined spectrophotometrically using 80% acetone as a solvent. The following equations were used: (total Chl = $17.90A_{647} + 8.08A_{664.5}$, where A = absorbance in 1.0 cm cuvettes and Chl = mg per g of FW). The ratio of Car/Chl was then calculated.

Chlorophyll fluorescence was measured with a portable fluorometer (FMS-2, Hansatech Instruments Ltd, Norfolk, UK). Leaves were dark-adapted for at least 20 min using leaf clips. Maximum fluorescence in the light (*F*m) was then measured after applying a saturating actinic light pulse of 15.000 mmol m^{-2} s^{-1} for 0.7 s *F*0 and *F*m were used afterwards to calculate variable fluorescence (*F*v = *F*m − *F*0) and maximum quantum efficiency of PSII (*F*v/*F*m). The same leaf sections were used to measure light-adapted parameters, after adapting plants to ambient light for 30 min. Steady-state fluorescence values (*Fs*) were recorded. The same saturating actinic light pulse was subsequently applied, which temporally inhibited the PSII photochemistry, and maximum (*F*0 m) was recorded. Finally, the effective quantum yield of PSII photochemistry was calculated [Y = (*F*0m − *Fs*)/*F*0m].

2.3. Chemical analysis

2.3.1. Lignin content

Lignin content was measured in the five fully expanded leaves of C, D and S plants by using the thioglycolic acid-based method (Doster and Bostock, 1988) with some modifications, as already reported in Trupiano et al. (2012). The concentration of lignin was calculated by measuring the absorbance at 280 nm, using a specific absorbance coefficient of 6.0 l g^{-1} × cm^1. Three biological replicates were used for statistical analysis ($p < 0.01$).

2.3.2. Proline and sugar content

Dry leaf samples were used for proline extraction, homogenized in 3% (w/v) sulfosalicylic acid. Free proline content was determined according to Bates et al. (1973) using L-proline for the standard curve. Total soluble sugars (TSS) were extracted in 80% ethanol from dry leaf and quantified with the anthrone reagent according to Yemm and Willis (1954) using L-glucose for the standard curve.

2.3.3. Lipid peroxidation assay

Lipid peroxidation was determined using the thiobarbituric acid