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, which have already been investigated at morphological, physiological and biochemical level. Conversely, corresponding molecular mechanisms are still poorly investigated.

By using an integrated approach, we here described the changes in growth, oxidative damage and osmolytes accumulation in stressed plants, together with corresponding modification of physiological parameters and proteomic repertoire in *Chétoui* olive cultivar.
Result 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, integrity of photosystem II and leaves nitrogen content, and corresponding representation of proteins involved photosynthetic apparatus, Calvin-Benson cycle and nitrogen metabolism; the most significant changes occurred under salinity stress conditions. Oxidative stress was also observed, in particular lipid peroxidation which could be tentatively balanced by the 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 unveiled by olive plants under these stresses. All together, these findings suggest that *Chétoui* variety is quite sensitive to both drought and salt stress, although it seems more incline to tolerate water depletion.

Keywords: 2-DE, leaf, physiology, proteome, salinity, water depletion.

Abbreviations: 2-DE: two-dimensional electrophoresis; A: CO_2-assimilation rate; ATP syn: ATP synthase; BSP: bark storage protein; C: control samples; 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: malondiadehyde; 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.

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 peculiar 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 plant can decrease CO_2 availability caused by diffusion limitations through the stomata and the mesophyll (Flexas *et al*., 2007) or alterated 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 retained more devastating than drought for two reasons: first, the osmotic effect reduces the capacity of plant water uptake, by its effect on the ionic homeostasis within the plant cell, and causes a slow plant growth; second, ion toxic effect, due to the increased salts uptake, cause tissue damage (Munns *et al*., 2006). In fact, it is reported that Na^+, at concentration above 100 mM, inhibits enzymes that require K^+ as a cofactor, including photosynthetic ones.

A common overlap in the signaling pathways, which include cellular redox status, reactive oxygen species, hormones, protein kinase cascades and calcium gradients, have been reported to be commonly used by plants to counteract drought and salt stress (Zhu, 2002).

Olive tree (*Olea europaea* L.) is one of the 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 implications for olive production (Galan e*t al*., 2008). Specific morphological, physiological and biochemical mechanisms of adaptations of this plant to drought have been described, such as modification of leaves anatomy (Sofo *et al*., 2007), together with the regulation of gas exchange (Moriana *et al*., 2002), osmolytes content (Chartzoulakis *et al*., 1999) and antioxidant system (Bacelar *et al*., 2007). Olive plants are also moderately tolerant to NaCl excess (Rugini *et al*., 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).

Most of reported studies on olive response to salt and drought stresses have mainly focused on morphological, physiological and chemical responses, whereas corresponding molecular mechanisms 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, nucleic acids and lipids, which make extraction procedures very problematic and tissues recalcitrant to investigation; ii) the lack of information on *O. europaea* L. genome, which creates difficulties towards 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 response (Das *et al*., 2016). Among the most commonly used methods in proteomics, two-dimensional eletrophoresis (2-DE) easily allows resolution and visualization of thousands of protein species on a single gel. Recently, we developed an effective method for protein extraction from leaves and corresponding 2-DE analysis of a Tunisian olive variety, named *Chétoui*, which is is the second main variety cultivated in the north of Tunisia, characterized by an extremely high content of phenolic compounds. The oil obtained from this variety is valued for its high amounts of total phenols and tocopherols, and good resistance to oxidation (Ben Temime *et al*., 2006; 2008). Moreover, *Chétoui* olive leaves are a good source of flavonoids which have potent antioxidant activity (Abaza *et al*., 2011). All these features make *Chétoui* a very economically interesting olive variety. However, previous physiological, biochemical and agronomical analyses showed that this cultivar is quite 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.

Our current investigation aims to provide a complete picture of the response of *Chétoui* olive plant to drought and salinity stresses, describing the changes in plant growth, oxidative damage and osmolytes accumulation in leaves, in combination with corresponding changes in physiological parameters and proteomic repertoire.

Materials and Methods

Plant Material and growth conditions

Seven months-old self rooted plants (*Olea europaea* L.), cultivar '*Chétoui*', were transplanted in 10 L pots filled with inert sand and kept in a glasshouse under controlled environment 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 were irrigated every two days with 100% Hoagland solution, ii) drought stress (named D), plants were grown in completely water depletion for 21 d, and iii) salt stress (named S), plants were 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 2 days until the 200 mM concentration was reached. The nutrient solutions were renewed each 2 days.

Physiological analysis

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 weight 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 successively dried at 80 °C for 48 h before being weighed for DW.

Gas exchange, pigment and fluorescence measurement

Gas exchange characteristic measurements of net CO_2 assimilation (A), transpiration rate (E) and 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: 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 Car/Chl was then calculated.

Chlorophyll fluorescence was measured with a portable fluoremeter (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 15.000mmol m^{-2} s^{-1} for 0.7 s. *F*0 and *F*m were used afterward 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 (*F*s) 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 ~~e~~ffective quantum yield of PSII photochemistry was calculated [Y= (*F*0m-*F*s)/*F*0m].

Chemical analysis

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's, 1988) with some modifications, as already reported in Trupiano *et al*. (2012). Concentration of lignin was calculated by measuring the absorbance at 280 nm, using a specific absorbance coefficient of 6.0 $l \cdot g^{-1} \times cm^{-1}$. Three biological replicates were used for statistical analysis ($p < 0.01$).

Proline and Sugar content

For proline extraction, we used dry leaf samples 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 were quantified with the anthrone reagent according to Yemm and Willis (1954) using L-glucose for the standard curve.

Lipid peroxidation assay

Lipid peroxidation was determined using the thiobarbituric acid (TBA) reaction followed by measurement of malondiadehyde (MDA) content (Heath and Packer, 1968).

Lyophilized tissue (100-200 mg) was extracted with 0.1% trichloroacetic acid (TCA). After centrifuging, 250 µl of supernatant was added to 1 mL of 0.5% TBA prepared in 20% TCA solution. The mixture was incubated at 95 °C for 30 min, cooled in an ice bath, and then centrifuged at 10000 x *g* for 15 min. Specific absorbance of the supernatant was measured at 532 nm, while non-specific absorbance was measured at 600 nm. After substracting the non-specific absorbance, the MDA concentration was calculated by using a specific molar extinction coefficient (155 mM^{-1} cm^{-1}); the results were expressed as nmol/ g. FW.

Hydrogen peroxide content

The H_2O_2 concentration was measured on lyophilized tissue crushed in 0.1% cold TCA and then centrifuged for 15 min at 12000 x *g* in a refrigerated centrifuge. The supernatant (0.5

mL) was added to 10 mM phosphate buffer (pH 7) and 1 M of iodate potassium (KI) solution; the corresponding absorbance was measured at 390 nm (Sergiev *et al*., 1997). The amount of hydrogen peroxide was calculating using a standard curve prepared with known concentration values of H_2O_2.

Electrolyte leakage determination

Fresh leaves and roots (200 mg) were excised into small discs and placed in 10 ml of bi-distilled water for 3 h, at 37 °C. Following incubation, the conductivity of the solution was measured with a conductivity meter (value A). The solution was then incubated at 95 °C for 30 min, and then the conductivity was measured again (value B). The ion leakage (%) was calculated as follows: (Value A/Value B)*100.

Nitrogen content

Leaves samples were over-dried at 70 °C for 48 h and then ground. Total N was determined in accordance with the Kjeldahl method, with a 25 mL aliquot of pure extract, followed by distillation and titration, as described by Bremmer and Mulvaney (1982).

Statistical analyses

The statistical differences among treatments were determined through one–way variance analysis (ANOVA); the Duncan test ($p < 0.05$) was used for separation of the means. Data were analyzed using SPSS software (Version 20.0).

Proteomic analysis

Protein extraction, separation and analysis

Total proteins from 2 g of leaf lyophylized tissue from control, drought stressed and salt stressed samples were extracted following the TCA/Acetone-Methanol-Phenol/SDS protocol, previously described by Ben Abdallah *et al*. (2017)**.** The Bradford assay (Bradford, 1976) was used to quantify protein concentration, using bovine serum albumin as standard.

For isoelectric focusing (IEF) analysis, immobilized pH gradient (IPG) strips (17 cm; pH 4–7 linear; Bio-Rad) were rehydrated overnight with 300 mL of rehydration buffer [6 M urea, 2% (w/v) CHAPS, 0.5% (v/v) Triton X-100, 20 mM dithiothreitol (DTT) and 1% (w/v) carrier ampholytes pH 3–10] and 700 mg of total proteins. IEF was performed in a PROTEAN IEF Cell (Bio-Rad) set up with the following program: (1) 250 V for 90min in linear mode; (2) 500 V for 90 min in linear mode; (3) 1000 V for 180 min in linear mode; and (4) 8000 V in

rapid mode until 56 kVh was reached. After IEF, the IPG strips were equilibrated in a equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS] supplemented with 1% (w/v) DTT for 20 min; then, they were treated with equilibration buffer containing 2.5% (w/v) iodoacetamide, for 20 min.

Proteins were separated in the second dimension by 12% polyacrylamide gel (17 cm x 24 cm x 1mm) electrophoresis (SDS–PAGE); in detail, analysis was performed in a PROTEAN (Bio-Rad) vertical apparatus containing 25 mM Tris–HCl, pH 8.3, 1.92 M glycine, 1% (w/v) SDS as running buffer. A constant voltage of 70 V was applied for 16 h, until the dye front reached the bottom of the gel. For each sample, three replicates were run. Finally, separated proteins were fixed by treating gels with 40% (v/v) methanol, 7% (v/v) acetic acid, for 30 min, and then visualized by staining with Coomassie Brilliant Blue G-250 (Bio-Rad). Gels were scanned using a GS-800 calibrated densitometer (Bio-Rad); corresponding digital images were recorded and analysed using PDQuest software (Bio-Rad). Finally, statistical analysis was conducted applying a Student's t-test ($P < 0.01$). A 2-fold change (<0.5 and >2) of normalized spot densities was considered indicative of differential representation between samples.

In-gel digestion, mass spectrometry analysis and protein identification

Spots were manually excised from gels, triturated and washed with water. Proteins were *in-gel* reduced, digested and analyzed by nanoLC-ESI-LIT-MS/MS (liquid chromatography electrospray ionization-linear ion trap-tandem mass spectrometry), as previously reported (De Zio *et al*., 2016). MASCOT software package (Matrix Science, UK) was used to search mass spectrometric data against a homemade database made of available *Pentapetalae* EST sequence data plus non-redundant plant protein sequence data (NCBI nr 2016/03/22). In the first case, all possible sequence reading frameshifts were considered. Database searching was performed by selecting a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavage maximum value of 2, cysteine carbamidomethylation and methionine oxidation as fixed and variable modification, respectively. Candidates with at least two assigned unique peptides with an individual MASCOT score >25, corresponding to $P < 0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from two-dimensional electrophoresis.

Results

Plant growth and RWC

Olive plant growth was significantly influenced by both drought and salt stress conditions, as evidenced by a reduction in shoot dry weight biomass by -12 and -37%, respectively, compared to control (Fig. 1a). However, salt stress affected more significantly shoot growth, compared to drought. Roots biomass resulted increased in drought stressed plants by 8%, while it was decreased in salt stressed plants by -22%, when compared to control (Fig. 1b). Unstressed plants maintained RWC levels at 93%, while RWC declined in response to drought and salt stress (-43 and -8%, respectively; Fig. 1c). In drought, the RWC decline was significantly higher than that observed under salt stress.

Photosynthesis, pigments and chlorophyll fluorescence analysis

The analysis of photosynthesis parameters showed that the net photosynthesis rate (A) was more inhibited by salt stress than by water stress (-56.4 and -31.5%, respectively), when compared to control plants. Also, transpiration rate (E) and stomatic conductance (g_s) followed a similar decreased trend in both stressed conditions (Fig. 2a, b and c). However, while E equally decreased in salt and drought stresses, g_s where lower in salt than in drought stressed samples. Furthermore, intrinsic water-use efficiency (WUE) was significantly enhanced in both drought and salt stressed plants compared to control, reaching the maximum value in drought stressed plants (Fig. 2d). In addition, intercellular CO_2 assimilation (Ci) value was unchanged in salt stressed olive leaves and improved in drought stressed ones compared to control (Fig. 2e).

The analysis of pigments content revealed that drought and salinity did not have impact on chlorophyll amount, while carotenoids and, consequently, Car/Chl ratio increased in both stressed conditions, compared to control plants (Table 1).

The analysis of fluorescence parameters evidenced that drought stress had no significant effect on Fv/Fm, while salinity induced a decreasing by -17% compared to control (Table 1). Conversely, Y value decreased in both drought and salt stressed samples by -36 and -41%, respectively, when compared to counterpart recorded in control sample (Table 1).

Chemical analysis

Results of lignin content measurements showed that this parameter increased by 1.3 and 1.4 fold in drought and salt stresses, respectively, compared to control (Fig. 3). Contrarily, proline content was reduced by -27 and -18% in drought and salt stressed plants, respectively, with

respect to control (Fig. 4a). Nonetheless, total soluble sugar showed an increase in both treatments (39 and 51% in D and S, respectively; Fig. 4b).

The analysis of leaf oxidative damage showed that lipid peroxidation (measured as MDA content with respect to control) was enhanced by 37 and 50% in drought and salt stressed olive leaves, respectively (Fig. 5a). Conversely, the H_2O_2 content was increased by 27% under drought stress, while it was found decreased by -32% under salt stress when compared to control (Fig. 5b). In comparison with control plants, the electrolyte leakage increased by 59% in drought stressed plants, while remaining unchanged in salt stress (Fig. 5c). Finally, nitrogen content decreased significantly by -52 and -53 % in drought and salt stressed olive leaves, respectively, compared to control (Fig. 6).

Proteomic analysis

Reproducible proteomic 2-D electrophoresis maps of *Chetoui* olive leaves were obtained for the first time; indeed, although highly abundant photosynthetic proteins (e. g., Rubisco) still dominated 2-DE gels, for this extremely recalcitrant tissue, our protocols (Ben Abdallah *et al.*, 2017) resulted effectively valid to obtain comparable 2-DE maps and to perform successive spot identification by mass spectrometry. In detail, 2-DE maps of control, drought and salt stressed olive plants were characterized by an average of about 300 well resolved spots, migrating in 11-100 kDa mass range. The comparison of these electrophoretic maps, through software-assisted analysis, revealed 26 protein spots as differentially expressed ($P \leq 0.01$) among all samples (Fig. 7), which were subjected to trypsinolysis and further nanoLC-ESI-LIT-MS/MS analysis for protein identification (Table 2). Based on Bevan et al. (1998) classification, the majority of identified proteins (16 in number) corresponded to proteins involved in photosynthesis (spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 19, 22, 23, 24, 25 and 26); additional differentially expressed components included factors involved in nitrogen metabolism (spots 14, 15 and 16), protein storage (spots 13 and 17) and energy (spot 18). The remaining four protein spots were grouped in the functional class named *Other*, which included salicylic acid-binding protein (spot 10), carbonic anhydrase (spot 11) and Fra 12.01 allergen (spots 20 and 21).

The profiles of all identified proteins in C (control), D (drought) and S (salt) samples are shown in Supplementary Data S1. A summary of the observed differences between control, drought stressed and salt stressed plants is expressed in Fig. 8. In detail, results showed that 12 components involved in photosynthetic processes were down-expressed in both stress conditions compared to control, namely ribulose-1,5-bisphosphate carboxylase/oxygenase

(RuBisCO) large subunit isoforms (spots 1, 2, 3, 6 and 19), oxygen-evolving enhancer protein 1 (OEE1) isoforms (spot 8, 23, 24 and 25), oxygen-evolving enhancer protein 2 (OEE2) isoforms (spots 12 and 26) and ferredoxin-NADP reductase (FNR) (spot 9). A similar condition was observed for a bark storage protein (BSP; spot 17), glutamine synthetase cytosolic isozymes (GSc; spots 14 and 16), salicylic acid-binding protein (SABP; spot 10) and a carbonic anhydrase isozyme (CA; spot 11). Conversely, ATP synthase subunit β (spot 18), GS nodule isoenzyme (GSn; spot 15) and Fra e 12.10 allergen isoforms (spots 20 and 21) were over-expressed in leaves from drought and salt stressed olive plants, when compared to control (Fig. 8).

Specific proteins were found differentially accumulated in drought and salt stressed samples. In detail, olive leaves from drought stressed plants were characterized by down-representation of four other RuBisCO large subunit components (spots 4, 5, 7 and 22) and an additional BSP isoform (BSP; spot 13). In olive leaves from salt stressed plants, these components resulted unchanged, *e.g.* RuBisCO large subunit component (spots 4, 5 and 7) or over-expressed, *e.g.* RuBisCO large subunit components (spot 22) and BSP (spot 13), when compared to control (Fig. 8).

Discussion

The present study comprehensively describes the influence of drought and salinity stresses on a widely cultivated Tunisian olive variety, *Chétoui*, by analyzing plant growth and corresponding physiological, chemical and proteomic responses. The results showed that after 21 days of growth under drought (water depletion) and salt (200 mM of NaCl solution) stress conditions, olive plants significantly reduced growth, as indicated by the decrease of shoot dry weight; this reduction was more marked in salt stressed olive plants. Furthermore, as reported in other studies (Parida and Das, 2005), the RWC decreased in both stress conditions, although the maximum effect was observed in drought stressed plants. For instance, Guerfel *et al.* (2009a) found that in the variety *Chétoui* the RWC value dropped to approximately 55% after 20 days of water stress.

A well-known mechanism to avoid plant dehydratation under drought and salt stress conditions is represented by osmolytes accumulation (Sofo *et al*., 2004; Munns and Tester, 2008), although it is considered species-specific and dose- and/or time-dependent (Ben Ahmed *et al*., 2009). Our results showed that, under both stresses, total sugars amount

significantly increased, reaching the maximum value in salt stressed plants, whereas proline content decreased in both stressed conditions. Soluble sugars play an important role in osmotic adjustment and cell protection by inducing direct ROS detoxification processes or stimulating indirectly antioxidative defense systems (Van den Ende and Valluru, 2009). Proline is another key component of cell osmoregulation generally accumulated in drought and salinity conditions (Ben Hassine *et al*., 2008). However, it was suggested that under stress conditions, where N limitation can occurs, proline can also act as alternative metabolic substrate by helping maintain cellular energy and $NADP^+$/NADPH balance and contributing to other pathways such as the tricarboxylic acid cycle and glutathione biosynthesis (Liang *et al*., 2013; Goel and Singh, 2015; Singh *et al*,. 2016). Thus, besides its direct role in plant stress tolerance, proline may represent a dynamic organic reserve that can remobilized to supply plant growth and stress response (Szabados and Savouré 2010). Considering that drought and salt stresses adversely affected N uptake/assimilation and related plant growth, in olive plants, compensatory mechanisms based on consumption of proline could be activated.

Our physiological data also revealed that both stress conditions negatively influenced olive leave photosynthetic parameters by A, g_s and E), with major effects induced by salinity. However, WUE was remarkably enhanced under both stress conditions, with a greater increase in drought stressed olive leaves, while Ci resulted unchanged in salt stress plants and increased in drought stressed plants compared to control.

It is widely known that drought and salinity, by inducing plant stomatal closure, reduce E and increase WUE to maintain vegetative growth and productivity (Khan *et al*., 2010). In parallel, stomatal closure decrease also CO_2 diffusion in mesophyll and reduce A, leading to an over-excitation and subsequent photoinhibitory damage of PSII reaction centre (Loreto *et al*., 2003; Bota *et al.,* 2004; Bacelar *et al*., 2007). Furthermore, at initial phase of drought stress, stomatal conductance reduction induced Ci declines (stomatal limitation of photosynthesis); successively, at a certain stage/intensity of water stress, Ci frequently increases indicating a predominance of non-stomatal limitations to photosynthesis (Flexas and Medrano, 2002). According to these evidences, we hypothesized that in both olive drought and salt stressed plants a predominance of non-stomatal limitations to photosynthesis seems to occur. However, our experiments showed that only under salinity olive leaves decreased Fv/Fm ratio and Y; under drought stress, Y showed a significant decrease, whilst Fv/Fm was unchanged relative to control. The Chl content was unchanged in both stresses condition.

Guerfel *et al.* (2009b) reported that in *Chetoui* olive leaves the slightly decreasing of FV/Fm and Y under drought stress allowing plant to maintain electron transport, avoiding

photodamage of photosynthetic apparatus. Furthermore, in another work Guerfel *et al.* (2009b), in contrast with our results, showed that Chl decreased in the variety *Chetoui* and *Chemlali* subjected to drought stress. Koyro *et al*. (2013) reported that in salinity condition the decline in photosynthesis might be caused by a reduction of the carboxylation activity of photosynthesis rather than any effect on CO_2 diffusion. Additionally, in the case of salt stress, the drastically photosynthesis reduction could be promoted also by direct toxicity effect of Na^+ and Cl^- in the leaf tissue and in particular on PSII (Munns *et al.*, 2006). -*….these observations are in contrast to other studies, where loss of Chl was observed during drought (Guerfel et al. 2009a);*In this condition, protection mechanisms against excess reducing power are important strategy to reduce photodamage (Takahashi and Badger, 2011). One of this strategy involve carotenoids, by direct quenching of Chl fluorescence by singlet-singlet energy transfer from Chl to Car or by trans-thylakoid membrane mediated, ΔpH-dependent indirect quenching of Chl fluorescence (Demmig-Adams and Adams, 1992). According to these findings, also in olive drought and salt stressed leaves, the significant increasing of Car contents, together with Car/Chl ratio may be considered as a mechanism that olive plants developed to limit, at least in part, photooxidative damage to phothosyntesis.

The general reduced abundance of several proteins related to the photosynthetic Calvin-Benson cycle and electron transport system was in line with the detrimental effect of drought and salinity stress on the photosynthetic apparatus, and the concomitant reduced photosynthetic activity. This was the case of five RuBisCO large subunit isoforms that resulted down-represented by both drought and salt stress conditions; additional four RuBisCO large subunit isoforms also decreased their relative concentration in drought samples, while they resulted unchanged or over-represented in salt stressed leaves, respectively.

De-activation of the RuBisCO enzymes by low intercellular CO_2 has already been observed (Meyer and Genty, 1998) and represent one of the non-stomatal reasons for the lowering of the plant photosynthesis rate (Flexas *et al*., 2006). On the other hand, a previous study on tobacco reported that a salt stress condition can determine a RuBisCO abundance increase (Razavizadeh *et al*., 2009); in *Lolium perenne* salt stressed plants, this phenomenon was positively and linearly related to the corresponding increase of the leaf RWC value (Hu *et al*., 2013). These observations lead us to hypothesize the occurrence of the latter phenomenon also in olive leaves, justifying the concomitant relative higher value of leaf RWC and higher number of over-represented or constant RuBisCO large subunit isoforms observed in salt stressed compared to drought stressed plants.

A reduced representation of crucial proteins regulating electron transport activity, *e.g.* OEE1, OEE2 and FNR, may limit the PSII assembly/functionality and photosynthetic transport chain in both drought and salt stressed leaves (de Vitry *et al*., 1989; Mizobuchi and Yamamoto, 1989; Seeber *et al*., 2005). Nevertheless, the higher energetic need of both stressed plants was presumably coped with the over-expression of enzymes, such as ATP synthase subunit β, involved in ATP production.

In the present study, we showed a significant increase in H_2O_2, MDA and electrolyte leakage in olive drought stressed leaves. Conversely, MDA content increased in salt stressed olive leaves, while corresponding electrolyte leakage and H_2O_2 content unchanged and decreased, respectively. It is widely reported that electrochemical energy produced by electron transport chain inhibition generates ROS accumulation, as well as in membrane damage (Chawla *et al*., 2013). In stressed plants, ROS generation is a reaction accompanying lipid peroxidation (Bajji *et al*., 2002; Hernandez and Almansa, 2002) and electrolyte leakage (K^+, Cl^-, HPO_4^{2-}, NO_3^-, citrate^{3-} and malate^{2-}). However, salt stress plants have been reported to present a K^+ efflux that is rapidly induced, lasts up to 1 h, and leads to a significant decrease of cytosolic K^+ (Shabala *et al*., 2006). Based on these observations, we may hypothesize that in salt stressed olive plants, contrarily to drought stressed ones, ROS and electrolytes were accumulate only in the initial stress phase, producing a high oxidative damage.

Two important enzymes indirectly related to plant growth and stress tolerance, namely CA and SABP, were also down-represented in both stress conditions, resulting under the level of detection in salt stressed leaves. CA is a Zn^{2+}-containing metalloenzyme that contributes maintaining the activity of the RuBisCO machinery facilitating its interaction with CO_2 (Badger and Price, 1994). Its quantitative reduction may reflect the limited entrance and thus availability of CO_2 under drought and salinity conditions. CA, together with H_2O_2-scavenging enzymes, have also been identified as putative SABP, thus modulating salicylic acid (SA) action and plant systemic acquired resistance (Park *et al*., 2007).

SA is a phytohormone involved in a wide range of biotic and abiotic stress responses and tolerance in plants (reviewed in Rivas-San Vicente and Plasencia, 2011; Khan *et al*., 2015)..

It is generally accepted that a low concentration of SA enhances plant growth and tolerance, while high levels of this phytohormone induce ROS production in photosynthetic tissues high levels of oxidative stress, leading to decreased abiotic stress tolerance (Borsani *et al*., 2001; Kang *et al*., 2012),The basic physiological and molecular mechanisms that potentially underpins SA- induced plant-tolerance to drought and salt stresses remains to be fully elucidated. However, previous studies have shown that SA, at low concentration, promotes

plant tolerance to salinity and water deficit, enhancing photosynthetic activity and stomatal conductance (Hussein *et al.,* 2007; Yazdanpanah *et al.,* 2011; Khan *et al.,* 2014; Miura and Tada, 2014). Furthermore, Cao *et al*. (2009) showed that in *Arabidopsis* SA-deficiency lines salinity damages was increased by diminishing antioxidant enzymes activity.

Based on these findings, we can hypothesize that the down-regulation of CA and SABP in drought stressed plants may be associated with a low SA concentration therein, guaranteeing olive plant growth and tolerance. On the other hand, the total absence of both enzymes in salt stressed plants may be an indication that SA signaling was there arrested, thus reducing olive plant growth and stress tolerance. However, extensive efforts are still required to gain a comprehensive understanding the role of this hormone, and interaction with and between other phytohormones, such as abscisic acid and ethylene, in drought and salt stress response (Gururani *et al*., 2015; Tao *et al.,* 2015).

The GS, candidate gene for N-utilization efficiency, could have another important role in olive plant growth regulation under drought and salinity condition. In particular, the cytosolic (GSc) and nodule (GSn) isoforms had an opposite behavior, being down- and over-represented under both salinity and drought stress conditions, respectively. GS is widely distributed in all plant organs and occurs in two major forms, one in the plastid and one in the cytosol. Furthermore, a specific nodule isoform, originally observed by Morey *et al*. (2002) as expressed in all plant tissues, showed the highest levels in N_2-fixing root nodules. All isoforms have essential role in the assimilation of inorganic N through the reassimilation of NH_4^+ from photorespiration and proteolysis processes (Thomsen *et al*., 2014). Reduction of GSc expression under drought and salt stress conditions has been already reported (Plomion *et al*., 2006). Whereas Das *et al*. (2016) hypothesized that GSc reduction in soybean stressed leaves acts a protective mechanism toward reactive $NO^{\cdot}$, Singh *et al*. (2016) suggested that this quantitative trend in salt stressed plants is related to an impairment of the N assimilation process. Based on these studies and our concomitant observations of proteomic and N content profiles in both drought and salt stressed olive leaves, we agree with the second hypothesis.

GS plays also a decisive role in BSP accumulation by modulating its induction or degradation (Zhu and Coleman, 2001). The major role of BSP is N storage, although it resulted differently accumulated in response to environmental stresses (De Zio *et al*., 2016; Trupiano *et al*., 2014; Plomion *et al*., 2006). Our proteomic data revealed a down-presentation of two BSPs under drought stress condition, while a BSP isoform was induced and the other was repressed in salt stress one. Together with a general GSn over-representation, this different BSP accumulation

trend in drought and salt stressed plants may be related to the corresponding need of N balance under nitrogen limitation conditions (Kosová *et al*., 2013).

Both drought and salt stress conditions also induce in olive leaves the over-expression of two Fra e 12.10 protein isoforms having a high sequence identity with the main *Fraxinus excelsior* (European ash) allergens (Niederberger *et al*., 2002), thus confirming the strict structural relation between the Ole (olive) and Fra (ash) allergens already described (Mas *et al*., 2014). Jimenez-Lopez *et al*. (2013) reported that this olive allergen corresponds to a putative isoflavone reductase-like protein, a key enzyme in flavonoid and lignin/lignan metabolism (Jimenez-Lopez *et al*., 2013; Castro *et al*., 2015). It is tempting to speculate that its over-representation in drought and salt stressed plants may be related with corresponding lignin accumulation therein. An increase in lignification, as observed in this case, may correspond to a tolerance strategy adopted by olive plants to enhance mechanical strength of cell wall to minimize water loss and cell dehydration (Sofo *et al*., 2004; Cabane *et al*., 2012) displacing space occupied by mesophyll water which, unlike lignified tissue, exchanges readily with the transpiration stream.

Conclusion

In the present study, we demonstrated that drought and, more significantly, salinity stress highly reduce *Chétoui* plant growth and photosynthetic performances, determining a reduced representation of enzymes involved in the Calvin-Benson cycle or in electron transport, and concomitant augmented levels of compounds associated with oxidative cell damage. Furthermore, our results clearly showed the occurrence of non stomatal limitation of photosynthesis in both drought and salt stressed olive leaves. In detail, in drought stress condition, where g_s was low but Ci value was increased, RuBisCO down-regulation could be the main metabolic limitation of photosynthesis. Conversely, in salt stress condition, where g_s reaching the minimum value but Ci, together some RuBisCO isoforms, resulted unchanged, the direct salt toxicity could represent the main photosynthesis limitation.

A parallel reduction in GSc representation levels and N content also suggest that a reduced nitrogen assimilation occured in olive stressed plants. Nonetheless, they may use alternative nitrogen and energy sources to cope with a higher stress related to amino acid and energy demand. Increased soluble sugar content and lignin accumulation in drought and salt stressed plants, as also supported by isoflavone reductase-like protein levels, seem to enhance mechanical strength of cell and to minimize water loss and cell dehydration therein. Conversely, the reduced expression in drought stressed plants of factors involved in SA

modulation allowed us to hypothesize that the low SA levels occurring therein may confer olive a major resistance. The almost completely absence of these factors in salt stressed plants suggests that SA signaling was arrested there, reducing plant tolerance. However, further analysis are required to verify the role of this phytohormone and others, such as ethylene and abscisic acid, in olive plant response to drought and salt stresses; this knowledge would contribute to the clarification of the specificity of plant responses to abiotic stresses.

In conclusion, the under expression of factors strongly related to plant growth, together with physiological and oxidative damage measurements, suggested that *Chétoui* variety is quite sensitive to both drought and salt stress conditions. In particular, these data indicate that it tolerates better water depletion than salinity. They also provide important information for developing novel amelioration strategies to improve cultivar tolerance to these adverse conditions.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures caption

Figure 1: Changes in the shoot biomass (panel a), root biomass (panel b) and relative water content (RWC; panel c) of olive seedlings cv. "*Chétoui*" grown under control (C), drought stress (D; water depletion for 21 d) and salinity stress (S; 200 mM NaCl for 21 d) conditions. Values are means (n=6) ± standard deviation; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters.

Figure 2: CO_2-assimilation rate (A; panel a), stomatal conductance (g_s; panel b), transpiration rate (E; panel c) and water use efficiency (WUE; panel d) of olive plants grown in different conditions. Values are means (n=6) ± standard deviation; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters. C=control plants; D= drought stressed plants; S= salt stressed plants.

Figure 3: Lignin content in leaves from control, drought stressed and salt stressed olive plants. Lignin content is referred to the value in S plants (100%). Data represent the mean of three independent extractions and measurements ± standard deviation. Values marked with the same letter are not statistically significant (T Test, $p < 0.01$). C=control plants; D= drought stressed plants; S= salt stressed plants.

Figure 4: Proline (panel a) and soluble sugar (panel b) content in leaves from control, drought stressed and salt stressed olive plants. Values are means (n=6) ± standard deviation; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters. C=control plants; D= drought stressed plants; S= salt stressed plants.

Figure 5: H_2O_2 content (panel a), malondiadehyde content (MDA; panel b) and electrolyte leakage (panel c) percent (c) amount in leaves from control, drought stressed and salt stressed olive plants. Values are means (n=6) ± standard deviation; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters. C=control plants; D= drought stressed plants; S= salt stressed plants.

Figure 6: Nitrogen content in leaves from control (C), drought stressed (D) and salt stressed (S) olive plants. Values are means (n=6) ± standard deviation; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters.

Figure 7: Reproducible 2-DE maps of leaves from control (panel A), drought stressed (panel B) and salt stressed (panel C) olive plants showing 26 differentially represented proteins. Arrows indicate the position of each protein spot; spot identification information are reported in Table 2.

Figure 8: Representation levels of 26 variably represented proteins in leaves from drought stressed or salt stressed plants compared to control. The main color code represents the functional classification according to Bevan et al. (1998). Over-represented proteins are indicated by dark chart color, while down-represented components are represented by light chart color. In the bracket is reported the spot number listed in Table 2. ATP syn: ATP synthase; BSP: bark storage protein; CA: carbonic anhydrase; FNR: ferredoxin-NADP reductase; GCc: glutamine synthetase cytosolic isoenzyme; GCn: glutamine synthetase nodule isoenzyme; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; SABP: salicylic acid binding protein; OEE1: oxygen evolving enhancer proteins 1; OEE2: oxygen evolving enhancer proteins 2.