Antioxidant status, biochemical and hormonal responses involved in the response of *Olea europaea* L. to water deficit induced by PRD irrigation

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Abstract

Changes in antioxidant status, biochemical and hormonal responses, were investigated in three olive cultivars (Arbequina, Arbosana and Chetoui) grown in a super-high-density orchard under partial root-zone drying (PRD) irrigation. Four irrigation treatments were applied during two growing seasons (2015 and 2016); control treatment (100% ETC) irrigated at 100% of crop evapotranspiration on both parts of the root-zone and three PRD irrigation treatments (100% PRD, 75% PRD and 50% PRD) irrigated at 100%, 75%, and 50% of crop evapotranspiration only on one alternated part of the root-zone. The results indicated that the three studied olive cultivars showed a clear difference in their response to PRD irrigation.

In fact, PRD irrigation has led to a strong activation of secondary metabolites with antioxidant properties such as pigments, phenols and flavonoids. Other metabolic changes, including the accumulation of soluble sugars and proline have been also triggered by PRD irrigation. Among the studied cultivars, Arbequina displayed the highest levels for proline, total soluble sugars, phenols and flavonoids, and the lowest ones for MDA and H_2O_2.

The increase in SOD, CAT and POD activity and the reduction and PPO activity under PRD irrigation were more pronounced in 2016 season with highest activities obtained from cultivar Arbequina.

For all the three cultivars, PRD irrigation increased phytohormones concentration in both, roots and leaves and the highest levels were recorded in 2016. In leaves, Arbequina showed the lowest levels of ABA and the highest levels of JA, SA and IAA. PRD irrigation also induces a higher accumulation of ABA, JA and IAA in dried roots than in wetted ones.

Taken together, biochemical mechanisms induced by PRD irrigation were more effective in Arbequina suggesting better protection of their foliar functions compared to other cultivars and its higher adaptability to PRD.

Keywords: *Olea europaea* L., PRD, osmoregulators, secondary metabolites, antioxidants, hormonal response.

1. Introduction

Stressful environmental factors such as water deficiency represents a major constraint limiting crop growth and yield worldwide. Plant responses to water deprivation involve a variety of adaptive mechanisms, which ultimately serve to improve plant function.

These adaptive changes includes mechanisms to avoid water loss (ion homeostasis and osmotic adjustment), mechanisms for the protection of cellular components (qualitative and quantitative changes of pigments), damage repair mechanisms (neutralization of reactive oxygen species) and growth regulation (Frary et al., 2010; Šircelj et al., 2005).

Accordingly, plants have evolved a wide variety of adaptive responses, including morphological, anatomical, physiological, biochemical and molecular, that enable them to adapt to drought (Cochard et al., 2002; Fatma et al., 2013; Pospíšilová, 2003) . These responses, in turn, induce changes in plant metabolism to reduce stress-induced damage.

Under water shortage conditions, stomatal closure represents one of the early responses triggered by plants in order to withstand drought stress and reduce water loss (Ozkur et al., 2009).

As a consequence, the limitation of CO_2 assimilation induces the over-reduction of the components of the electron transport (Bacelar et al., 2007; Ben Ahmed et al., 2009). Hence, leaf cannot dissipate the excess of light energy, which leads to the production of reactive oxygen species (ROS) (Foyer & Noctor, 2005). ROS are mainly represented by superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Reddy et al., 2004).

Excessive generation of reactive oxygen species (ROS) results in the disruption of the normal plant metabolism causing oxidative damage to lipids, proteins, nucleic acids, photosynthetic pigments and enzymes (Mittler, 2002; Ozkur et al., 2009).

Under drought, an over production of reactive oxygen species (ROS) cause important oxidative damage to membrane lipids and elicit lipid peroxidation (Smirnoff, 1993). Malondialdehyde (MDA) level is generally utilized as a marker of oxidative damage (Møller et al., 2007).

In order to overcome oxidative stress and protect themselves from the toxic action of ROS, plants have evolved an efficient antioxidative defense system, involving antioxidant enzymes and molecules (Reddy et al., 2004).

Among the major antioxidant enzymes, superoxide dismutase (SOD) catalyze the disproportionation of $O_2^{\bullet-}$ radicals into H_2O_2 and O_2, and CAT and POD convert H_2O_2 into H_2O (Reddy et al., 2004; Wang et al., 2009; Yang et al., 2008).

The ascorbate–glutathione cycle is also a crucial antioxidant mechanism for H_2O_2 involving different antioxidative enzymes: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) (Foyer & Noctor, 2011).

The commonly non-enzymatic antioxidants consist of water soluble molecules such as ascorbate, glutathione and phenolic compounds, and lipid soluble molecules composed of α-tocopherol and carotenoids (Grace, 2005).

Increase in plant antioxidant enzyme activities is closely related to increased tolerance to several abiotic stressors (Dat et al., 2000). Increased accumulation of antioxidant enzymes in response to water deficit has been found in olive tree (Aganchich et al., 2009; Sofo et al., 2005).

Osmotic adjustment is a well-known adaptive mechanism that many plant species used to withstand drought stress (Chaves et al., 2003; Farooq et al., 2009). This processes leads to the accumulation of low molecular weight osmolytes (organic solutes), such as, sugars, polyols, betaines and proline (Iqbal et al., 2011; Munns & Tester, 2008).

It has been reported that the accumulation of osmoregulators such as proline and sugars is a well-known adaptive mechanism in olive tree against water deficit (Ben Ahmed et al., 2009; Sofo et al., 2004).

Besides their role as a clear marker for environmental stress, they help in preventing membrane damage and stabilizing the structures and the activities of proteins and enzymes (Ben Ahmed et al., 2009; Hessini et al., 2009; Lee et al., 2008).

The mechanisms of stress-response in plants are intricately linked and require various integrated pathways to be activated to overcome the harmful effects of external stresses (Verma et al., 2016).

Owing to their ability to control a wide range of physiological processes, phytohormones have been recognized as one of the major endogenous factors in alleviating adverse effects of abiotic stresses in crop plants by adjusting their metabolism (Aimar et al., 2011). They have pivotal roles in a wide variety of adaptive responses by mediating growth, development, source-sink transitions and nutrient allocation under adverse environmental conditions (Peleg & Blumwald, 2011). Hence, hormone metabolism and signaling processes are potent targets for manipulation to obtain enhanced abiotic stress tolerance (Wani et al., 2016).

Among various phytohormones, ABA is the most studied stress-responsive hormone particularly under drought. It is well established that an overall enhanced drought tolerance was attributed to higher ABA accumulation (Lu et al., 2013). Also, the role of ABA, JA, ethylene, and SA, in mediating various abiotic and biotic stress responses is emerging (Eyidogan et al., 2012). Interestingly, indole acetic acid (IAA) plays an integral role on regulating plant growth during environmental stress (Peleg & Blumwald, 2011).

Olive (*Olea europaea* L.) is one of the most widely cultivated fruit crop in the Mediterranean region. It has traditionally been managed under dry environments. In addition, it is well known for its high tolerance to prolonged drought periods. Recently, irrigation has been introduced into the new intensive olive plantation (Lavee, 2011; Martín-Vertedor et al., 2011).

However, in the Mediterranean regions, where olive is usually cultivated, the availability of water for agricultural sector is generally declining due to the prolonged drought periods and scarce water resources (Giorgi & Lionello, 2008).

This scenario makes the establishment of a sagacious irrigation approach an unavoidable necessity for the future development of modern olive growing and to addressing issues of water shortages in the water-short Mediterranean region.

Current evidence suggests that the implementation of innovative water-saving irrigation strategies that reduce water consumption and improve the water use efficiency (WUE), without detrimental effects on yield and quality of products, could be a major task for dry regions (Fereres & Soriano, 2007).

Possible strategies include partial root zone drying irrigation (PRD), in which approximately half of the root system on either side of tree canopy is wetted at each irrigation event, while the remaining half is left to dry-out to a pre-determined level (Davies et al., 2000, 2002; Dry & Loveys, 1998; Kang & Zhang, 2004).

With recurring decrease of amounts of precipitation and prolonged drought periods in major olive cultivation areas, water deficit become a critical factor for irrigated super high-density (SHD) olive plantation. Therefore, the use of water saving irrigation strategies and the selection of the best adapted varieties is very crucial for a sustainable production and an efficient water use under water scare conditions.

In this respect, the objective of this study is to evaluate the behaviour of three olive cultivars to PRD in a semi-arid region of Tunisia. In an effort to elucidate the adaptive strategies of olive trees to PRD, hormonal and enzymatic responses were accessed. Also, the effects of PRD on biochemical and osmotic traits were evaluated.

2. Material and methods

2.1. Orchard description and experimental conditions

The experiment was performed in the region of Sidi Bouzid, central of Tunisia (lat. 35.015697N; long. 9.430582W; alt. 369 m) on 11-year old, drip irrigated olive trees (*Olea europaea* L.) planted at a high density (2 m × 4 m). The volumetric soil water content was 25% at field capacity and 13% at wilting point.

Four irrigation levels were applied including a control treatment (100% ETC) where trees were supplied at 100% ETc on both root zone sides and three partial root-zone drying irrigation (PRD) treatments irrigated at 100%, 75% and 50% of ETc (100% PRD, 75% PRD and 50% PRD respectively). In PRD treatments, only one side of the root zone was irrigated while the other was left dry and water supply was switched between the two sides every 15 days. Starting from April 2015, PRD irrigation scheduling was applied, and olive trees were irrigated 4−5 times per week.

Irrigation was supplied by two drip lines around each tree, with 1 drip emitters per tree lateral pipes (100% ETC treatments) or 2 drip emitters per tree lateral pipes (100% PRD, 75% PRD and 50% PRD treatments) giving 2 L/h each and located at 80 cm from the trunk (located 0.6 m on either side of the tree row).

Treatments were arranged in a split plot design with three replicates. Treatments were assigned to the main plots and cultivars to the sub-plots.

Irrigations levels were determined based on the recommended FAO56 formula for crop evapotranspiration: $ETc = ETo \times Kc \times Kr$.

Reference evapotranspiration (ET_0) was determined following the FAO Penman-Monteith procedure (Allen et al., 1998), Kc is the crop coefficient and Kr is the coefficient of reduction

associated with the percentage of ground area shaded by the tree canopy (Fereres & Castel, 1981).

2.2. Plant sampling

Leaves were sampled on July 2015 and 2016 from selected olive trees. Fifty to 100 g of fully expanded leaves from the middle portion of nonbearing shoots were collected. Sampled leaves were immediately frozen in liquid N_2 and stored at −80ºC prior to analysis. The frozen leaves were finely ground in liquid nitrogen, and the frozen powder was used to determine proline, malondialdehyde (MDA), H_2O_2, total soluble sugars and pigment concentrations.

A portion of leaf samples were immediately lyophilized and ground to a fine powder in the presence of liquid nitrogen for the determination of enzymatic activities and hormonal analysis. At each sampling, another subsample was dried at 45°C for 48 h in an oven with air circulation, grounded to a fine powder in a hammer mill and then stored in a dry place in the dark for the extraction of total phenolic and flavonoid contents.

2.3. Pigments determination

Fresh leaves (0.2 g) were cut and extracted overnight with 80 % acetone at +4 ºC according to Lichtenthaler & Wellburn (1983). The extract was centrifuged at 10.000 rpm for 5 min and the supernatant was taken for reading spectrophotometrically at λ 470, 645 and 663 nm.

The total chlorophyll and the carotenoid concentrations (mg/g) were determined following equations developed by Lichtenthaler (1987):

Chl a = $12.25\ A_{663} - 2.79\ A_{647}$

Chl b = $21.50\ A_{647} - 5.10\ A_{663}$

Total Chlorophylls = $7.15\ A_{663} + 18.71\ A_{647}$

Carotenoids = $[(1000\ A_{470}) - (1.82\ \text{Chl a}) - (85.02\ \text{Chl b})]/198$

2.4. Proline and total soluble sugars content

Free proline content was assayed according to Troll & Lindsley (1955) amended by Monneveux & Nemmar (1986). Briefly 0.2g of fresh leaf samples were, homogenized in 5 mL 40% (w/v) methanol and the homogenate was placed in water bath at 100°C during 30 min in glass capped tubes. A 1 mL aliquot of the supernatant was mixed with 2 mL acetic acid, 2 mL of the reagent mixture (120 mL distilled water, 300 mL acetic acid and 80 mL orthophosphoric acid), 1 mL ninhydrin solution (25 mg/mL) and incubated in 100°C water bath for 1 h.

After cooling, the reaction mixture was mixed with 4 mL toluene and vortexed for 15−20 min. The toluene fraction containing the chromophore was separated and the absorbance was read spectrophotometrically at 528 nm, using toluene as a blank. Proline concentration was determined using calibration curve as µmol proline g^{-1} FW.

Total soluble sugars content were measured based on the anthrone method (Shields & Burnett, 1960). A total of 100 mg of fresh leaf were left 24 h in 5mL of ethanol 80% in covered glass tubes. The extract obtained was diluted 10 times with ethanol to 80 %. 2 mL of the of the alcoholic extract was mixed with 4 mL of anthrone reagent prepared 4 hours in advance and consisting of 0.2 g of pure anthrone added to 100 mL of sulfuric acid (H_2SO_4). The obtained solution was heated on a water bath at 92°C for 8 minutes. After agitation and cooling of the reagent mixture, the absorbance at 625 nm was determined using ethanol as a blank. Soluble sugars concentration was calculated referring to a glucose solution as a standard curve and expressed as µg g^{-1} FW.

2.5. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content

MDA content was estimated according to the method described by (Heath & Packer, 1968). 0.5 g of fresh leaf was homogenized in 5 mL of 0.1% (w/v) trichlroacetic acid (TCA). After centrifuging at 15000 rpm for 10 min, 1 mL of supernatant was added to 4 mL of 0.5% TBA prepared in 20% TCA solution. The mixture was heated at 95°C for 30 min, cooled immediately in an ice bath and then centrifuged at 10000 rpm for 5 min. The absorbance was determined spectrophotometrically at 532 and 600 nm.

The concentration of MDA was calculated by using a molar extinction coefficient of 156 mM^{-1} cm. MDA equivalents were calculated as follows:

MDA (nmol g^{-1} FW)= [(A532−A600)xVx1000/ Ɛ]xW

V : extraction volume (mL)

Ɛ : The specific extinction coefficient (155 mM^{-1} cm^{-1})

W:Fresh weight (mg)

H_2O_2 levels were estimated using the procedure developed by (Brennan & Frenkel, 1977), with reference to titanium-hydroperoxide reaction. Briefly, 250 mg of frozen leaf samples were homogenized with 5 mL of cold acetone. After 30 min of bath sonication, the homogenate was centrifuged at 6000 rpm for 15 min. 500 µL of supernatant were mixed with 250 µL of titanium reagent (20% titanium tetrachloride ($TiCl_4$) in concentrated HCl, v/v) and 250 µL of 100% ammonia to precipitate the titanium-hydroperoxide complex. The precipitate was repeatedly washed with acetone to remove chlorophyll. After centrifugation at 14,000 rpm for 15 min, the supernatant was discarded, and the precipitate was dissolved in 0.5 ml of 2 M sulphuric acid. The absorbance was read at 415 nm and the H_2O_2 content was reported as µmol g^{-1} fresh weight (FW), on the basis of a standard curve generated with known concentrations of H_2O_2.

2.6. Determination of leaf total phenolic and flavonoid content

Leaf extracts were obtained using methanol as described in Elansary et al. (2016) with some modifications. Dried leaves (5 g) were dissolved in 100 mL of 80% aqueous methanol and then thoroughly mixed under darkness in an orbital shaker for 24 h at 270 rpm. The extract was centrifuged for 10 min at 5000 rpm and the supernatants were then filtered using a filter paper and samples were stored in the dark at −20°C. The extract was concentrated to dryness using a rotary evaporator and was kept for further analyses.

Total phenolic content was determined spectrophotometrically at 760 nm, using the Folin-Ciocalteu reagent method (Škerget et al., 2005) with minor modifications.

In brief, to 0.5 mL of diluted extract, 2.5 mL of Folin–Ciocalteu reagent (diluted 10-fold with ddH_2O) were added. After 3 min, 2 mL of Na_2CO_3 (75 g L^{-1}) were added. The mixture was incubated in a water-bath for 5 min at 50ºC and after cooling at room temperature, the absorbance was measured with a spectrophotometer. As blank, 0.5 mL of ddH_2O was used. The results were expressed as mg of gallic acid equivalents per g of dry weight (mg GAE g^{-1} DW).

Total flavonoid content was determined following the colorimetric method described by Kim et al. (2003) .Four mL of distilled water were added to 1 mL of the extract. Then, 0.3 µL of 5 % sodium nitrite solution was added into the mixture. After 6 min, 0.3 mL of 10% aluminum chloride solution was added. Test tubes were incubated at ambient temperature for 5 min, and then 2 mL of 1 M sodium hydroxide solution were added to the mixture. Immediately, the volume was adjusted to 10 mL with ddH_2O. The mixture was thoroughly vortexed and the absorbance of the pink color developed was measured at 510 nm against a blank. The amount of total flavonoids content was expressed as mg catechin equivalents per g of dry matter (mg−CE g^{-1} DW).

2.7. Enzymes extraction and antioxidant activity assays

For the enzymatic assay, 0.1 g of lyophilized ground leaf tissue were extracted in a bain mill (MillMix20, Domel, Železniki, Slovenija) in 2 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1mM EDTA, 1% (w/v) polyvinyl pyrrolidone (PVP) and 0.1% (v/v) Triton X−100. A K_2HPO_4/KH_2PO_4 buffer (pH 5.5) was used for PPO assay. The extract was centrifuged at 4°C for 10 min at 14,000 rpm. The resultant supernatant was collected and stored at 4 °C until use for the assays of enzymatic activities and protein content.

All procedures for enzyme extraction and determination of enzyme activities were performed at 4 ºC. Total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. All spectrophotometric analyses were conducted on a UV spectrophotometer (Thermo Spectronic Genesys 10 UV, Waltham, MA, USA). Enzyme activity was expressed as U mg^{-1} protein.

Catalase (**CAT**, EC 1.11.1.6) activity was assayed according the method of Aebi (1984). The decline in absorbance at 240 nm was followed for 1 min and the amount of decomposed H_2O_2 was measured. The reaction mixture consisted of 1.9 mL of 100 mM potassium-phosphate buffer (pH 7.0), and 100 µL of enzyme extract. The reaction was started by addition of 1 mL of 30 mM H_2O_2 in a final volume of 3 mL.

An extinction coefficient of 0.039 $mM^{-1}cm^{-1}$ was adopted to express the catalase activity as EU mg^{-1}protein. One unit of CAT activity was defined as the amount of the enzyme that catalyze the decomposition of 1mmol of H_2O_2 in 1 min.

Superoxide dismutase (**SOD**, EC 1.15.1.1) activity was evaluated by monitoring the ability of enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) at 560 nm according to the method of Sun et al. (1988).The reaction mixture (1 mL) consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM nitro blue tetrazolium (NBT), 0.05 mM xanthine, 0.025 unit of xanthine oxidase and 50 µL of enzyme. Xanthine oxidase was added as the last component and absorbance was recorded at 5−s intervals for 1 min with a UV spectrophotometer. One unit of enzymatic activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as units mg^{-1} protein.

The guaiacol peroxidase (**POD**, EC 1.11.1.7) activity was measured based on the oxidation of guaiacol to tetraguaiacol by monitoring the increase in absorbance at 470 nm every 10 s for 1 min as described by Urbanek et al. (1991) with minor modifications. The reaction mixture contained (2 mL) 100 mM phosphate buffer (pH 7.0), 0.1mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and 50µL enzymatic extract.

Peroxidase activity was quantified by the amount of tetraguaiacol formed using the molar extinction coefficient (26.6 mM^{-1} cm^{-1}). Polyphenol oxidase (**PPO**, EC 1.10. 3. 1) activity was determined by measuring the oxidation of catechol as described in Moore & Flurkeys (1990) with slight modification. The increase in absorbance was monitored at 420 nm at 15 sec time intervals. The assay mixture included 100 mM phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 6), 50 mM catechol and 50 µL of the enzymatic extract. Specific activity was expressed as units (UE) min^{-1} mg $protein^{-1}$.One unit of PPO activity was calculated based on absorbance coefficient ε = 2.72 mM^{-1} cm^{-1} and expressed in units (1 U = 1 nmol catechol oxidized min^{-1} mg $protein^{-1}$).

2.8. Hormonal analyses

Hormones were extracted and analyzed following the procedure described by Durgbanshi et al., (2005) , with few modifications. In short, 50 mg of frozen dried leaf/root materiel was extracted with 2 mL of ultrapure water using a mill ball (Mill Mix20, Domel, Železniki, Slovenija). At

the beginning of the extraction procedure, internal standards ($[^2H_6]$-ABA, $[^{13}C_6]$-SA, $[^2H_2]$-IAA and dehydrojasmonic acid) were added to each sample. All following steps were performed at 4°C.

Subsequently, extracts were centrifuged at 15,000 rpm during 15 min and the pH of the supernatants was adjusted to 2.8–3.2 with 30% acetic acid.

Water extracts were partitioned twice against 2 mL of diethyl ether and the organic fractions were recovered and dried under vacuum at room temperature in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The solid residue was resuspended in 500 µL of 10% MeOH solution by gentle sonication, then filtered through a 0.22 µm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain) and 20 µL of each sample were directly injected into an ultra performance liquid chromatography system (Acquity SDS, Waters Corp., Milford, MA, USA).

Chromatographic separations were achieved by a reversed-phase C18 column (Gravity, 50 × 2.1 mm 1.8-µm particle size, Macherey–Nagel GmbH, Germany) using a linear gradient of methanol and water supplemented with 0.01% acetic acid at a flow rate of 300 µL $min^{-}1$.

The MS/MS quantification was performed on a triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column though an orthogonal Z-spray electrospray ion source.

The phytohormones contents were quantified after external calibration against the commercial standards and results were processed using the Masslynx v4.1 software.

2.9. Statistical analysis

Data from each year separately, were subjected to analysis of variance (ANOVA) using the GLM procedure in the SPSS 23.0 statistical software package for the analyses of irrigation treatments, cultivars and their interaction. Significant differences were determined at $P < 5\%$, according to Duncan's multiple range tests. The classification of olive cultivars was provided by Principal Component Analysis (PCA). PCA was carried out using XLSTAT 2014 version.

3. Results

3.1. Pigments, total phenols and flavonoids contents

Table 1

Chlorophyll, carotenoids total phenols and flavonoids content of cultivars Arbequina, Arbosana and Chetoui under control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during two growing seasons (2015 and 2016).

Cultivar	Treatment	Chlorophyll	Carotenoids	Total Phenols	Flavonoids

		mg g^{-1} FW		mg g^{-1} FW		mg GAE g^{-1} DW		mg CE g^{-1} DW	
		2015	**2016**	**2015**	**2016**	**2015**	**2016**	**2015**	**2016**
Arbequina	100% ETC	9,15±0.10^{c}	10,73±0.21^{ab}	2,34±0.11^{c}	3,02±0.04^{d}	51,46±0.83^{b}	50,56±1.13^{a}	18,11±0.42^{a}	30,04±2.49^{a}
	100% PRD	9,05±0.41^{c}	11,41±0.7^{b}	2,35±0.08^{c}	2,88±0.11^{c}	65,35±1.21^{c}	70,19±1.9^{c}	20,72±0.55^{b}	37,69±3.32^{b}
	75% PRD	8,22±0.43^{b}	13,95±0.63^{b}	2,28±0.06^{b}	2,16±0.12^{b}	53,53±2.80^{b}	64,84±0.67^{b}	21,39±0.87^{c}	44,90±1.79^{c}
	50 % PRD	7,38±0.08^{a}	10,63±0.35^{a}	2,07±0.03^{a}	1,80±0.30^{a}	72,57±1.39^{d}	71,01±1.05^{d}	26,71±1.8^{d}	48,67±2.73^{d}
Arbosana	100% ETC	8,89±0.12^{b}	9,04±0.37^{a}	1,84±0.03^{c}	1,95±0.03^{b}	38,43±3.32^{a}	45,29±0.61^{a}	13,62±0.33^{a}	22,61±0.51^{a}
	100% PRD	8,63±0.11^{b}	10,81±0.93^{b}	1,84±0.09^{c}	2,05±0.07^{b}	43,42±0.38^{a}	53,53±0.7^{b}	16,84±1.80^{b}	21,66±0.2^{a}
	75% PRD	6,75±0.25^{a}	12,78±0.31^{b}	1,62±0.04^{b}	1,71±0.13^{a}	42,29±1.69^{a}	52,47±1.4^{b}	18,23±0.15^{c}	23,27±2.88^{b}
	50 % PRD	6,56±0.04^{a}	7,88±0.17^{a}	1,45±0.11^{a}	1,01±0.05^{a}	52,59±0.5^{b}	56,06±1.31^{c}	19,67±2.07^{c}	35,86±1.29^{c}
Chetoui	100% ETC	12,18±0.32^{b}	15,77±0.77^{c}	2,61±0.01^{bc}	2,95±0.15^{c}	48,14±1.90^{b}	77,21±2.29^{a}	20,11±1.11^{a}	39,24±3.81^{a}
	100% PRD	13,12±0.6^{c}	17,33±0.23^{d}	2,86±0.10^{c}	3,00±0.12^{d}	54,77±3.33^{c}	84,66±1c	20,44±0.67^{b}	38,46±1.11^{a}
	75% PRD	13,42±0.29^{c}	14,50±1.10^{c}	2,59±0.10^{b}	2,77±0.10^{c}	48,18±0.31^{b}	81,58±1.03^{bc}	24,82±0.43^{c}	46,23±0.63^{b}
	50 % PRD	11,06±0.54^{a}	14,56±0.52^{c}	2,39±0.06^{a}	2,49±0.05^{b}	57,51±1.70^{c}	89,97±1.41^{d}	27,32±0.28^{d}	59,37±1.59^{c}
Two way ANOVA									
	C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	C*T	0.002	0.001	0.484	0.039	0.027	0.001	0.342	0.039

Values are means ± SE of three replicates. Means followed by different letters within a column indicate significant differences between treatments for each cultivar and year separately at p<0.05, based on Duncan's means test.

Leaf pigment content was significantly affected by PRD irrigation and cultivar (Table 1). In general, the highest pigments content was recorded during the second year (2016) where differences between treatments were more pronounced. PRD irrigation was paralleled by a substantial decline in total chlorophyll in all cultivars studied. In particular, Arbosana reached lowest values of total chlorophyll under 50%PRD (6.56 and 7.88 mg g^{-1} FW, for 2015 and 2016 respectively) if compared to Arbequina (7,38 and 10,63 mg g^{-1} FW), and Chetoui (11.06 and 14.56 mg g^{-1} FW) respectively for 2015 and 2016 seasons. The highest chlorophyll content was observed in Chetoui under 75% PRD (13.42 mg g^{-1} FW) and 100% PRD (17.33 mg g^{-1} FW) for 2015 and 2016 respectively.

Induced water deficit by PRD decreased carotenoids content in the three cultivars mainly during 2016 (Table 1). Hence, the highest carotenoids content was observed under 100% ETC and 100% PRD treatments (3.02; 2.05; 3.00 mg g^{-1} FW), while the lowest carotenoids content was recorded under 50% PRD (1.80; 1.01; 2.49 mg g^{-1} FW) for Arbequina, Arbosana and Chetoui respectively.

Total phenols level in leaves of olive cultivars during 2015 and 2016 seasons were raised with increasing of water deficit intensity induced by PRD irrigation, and its accumulation in 2016 was higher than in 2015 (Table 1). The highest phenols content corresponded to Arbequina and Chetoui cultivars under 50%PRD and the lowest phenols content were observed in Arbosanaa under 100%ETC.

PRD irrigation significantly changed the concentration of flavonoids in leaves ($P \leq 0.01$) (Table 1). Flavonoids levels were increased by PRD irrigation both in 2015 and 2016 years of all olive cultivars. The increments in flavonoids due to water deficit were generally higher in Chetoui and Arbequina.

3.2. Osmotic regulatory compounds

As shown in Fig. 1, the proline content was significantly greater (P< .001) under PRD irrigation with respect to control treatment (100% ETC) and its accumulation was generally higher in 2015 than 2016. This serious accumulation was also significantly influenced by cultivars (P< 0.001) and was more pronounced in Arbequina, whereas Chetoui was characterized by a low proline accumulation compared with the other cultivars (Fig. 1).

During both years, proline content reached its maximum value under 50% PRD when it was 2.28 µmol g^{-1} FW in Arbequina, 1.82 µmol g^{-1} FW in Arbosana and 1.45 µmol g^{-1} FW in Chetoui during 2015 (Fig. 1). However, during 2016 value were 1.17; 0.88; and 0.91 µmol g^{-1} FW for Arbequina, Arbosana and Chetoui respectively.

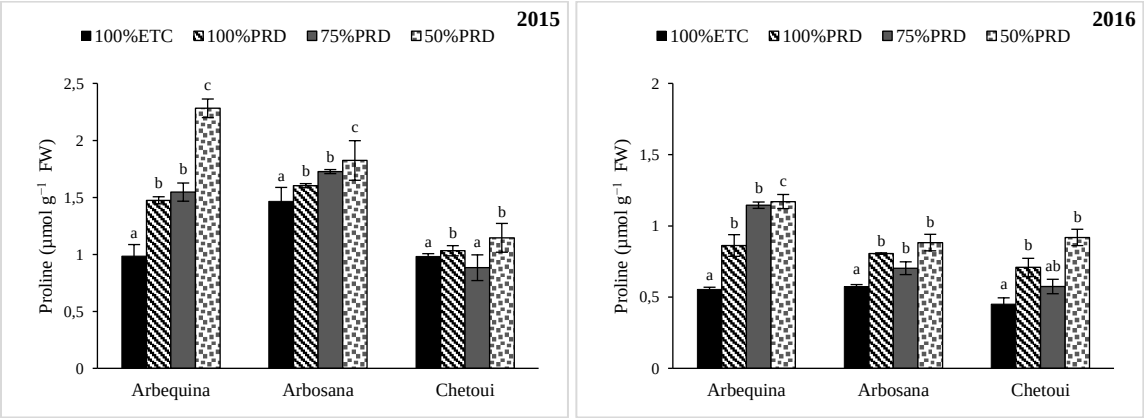


Fig. 1. Proline content in the leaves of Arbequina, Arbosana and Chetoui cultivars under Control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

Total soluble sugars content was influenced by both water regime and cultivar (P< 0.001). In all cultivars, PRD irrigation caused marked significant increase in sugars content and values were higher during 2015 than those of 2016 (Fig. 2).

Arbequina cultivar showed the highest endogenous sugar concentration among the three cultivars, however, Arbosana showed the lowest sugars content compared to other cultivars (Fig. 2). Furthermore, the maximum values belonged to 50% PRD and was 449.36 µg g^{-1} FW and 301.5 µg g^{-1} FW respectively for 2015 and 2016 years.

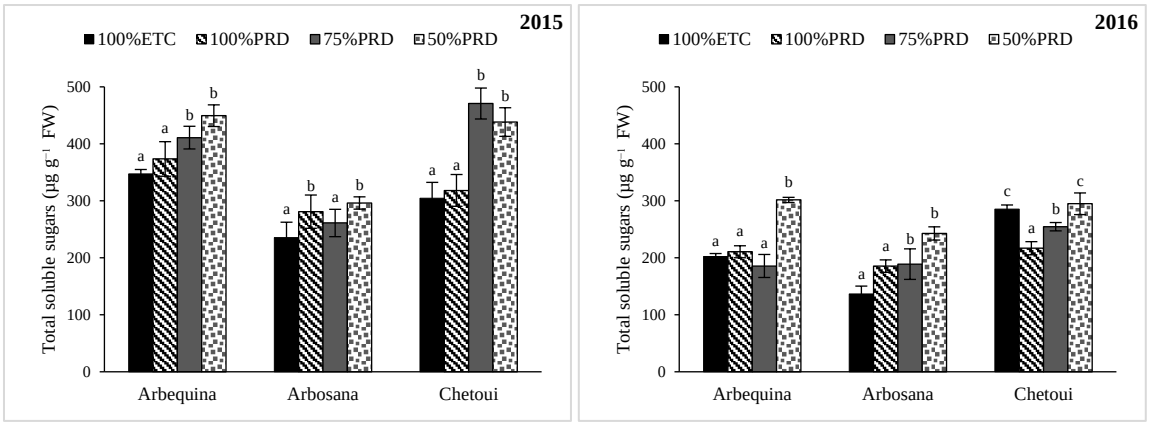


Fig. 2. Total soluble sugars content in the leaves of Arbequina, Arbosana and Chetoui cultivars under Control (100%ETC) and PRD irrigation treatments (100% PRD, 75% PRD,50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

3.3. Oxidative stress indicators

MDA content in leaves of olive cultivars were significantly affected by water regime. The levels of MDA (Fig. 3) increased strongly in trees subjected to PRD irrigation during both years. Remarkably levels were higher in 2016 than 2015. 50% PRD induced the accumulation of MDA compared to control (100%ETC) and the levels were significantly higher in Chetoui (44 and 125 nmol g^{-1} FW) and Arbosana (34 and 131 nmol g^{-1} FW) compared to Arbequina (28 and 72 nmol g^{-1} FW) respectively for 2015 and 2016.

Furthermore, during 2015, The 50%PRD induced an increase of 90.49%, 143.99% and 34.39% of malondialdehyde levels compared to 100%ETC in Arbequina, Arbosana and Chetoui respectively. Howerver, for 2016 the increase was of 40.16%, 33.34% and 87.95% in Arbequina Arbosana and Chetoui, respectively.

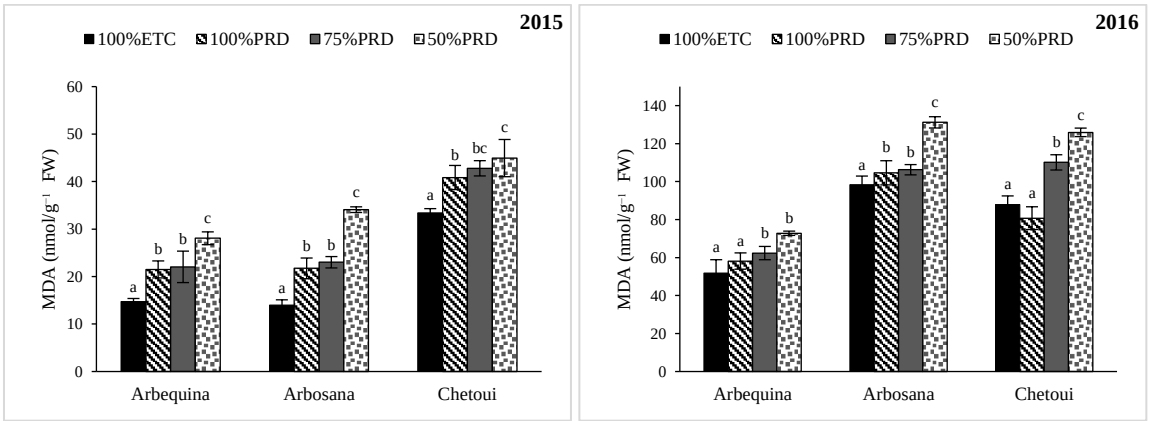


Fig. 3. MDA content in the leaves of Arbequina, Arbosana and Chetoui cultivars under Control (100%ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

Significant differences were found between irrigation treatments and cultivars in the amount of H_2O_2 mainly in 2016 samples ($P<0.01$) (Fig.4). The PRD irrigation led to a gradual increase in the levels of H_2O_2 contents in all cultivars. In fact, 50%PRD induced the highest H_2O_2 levels regardless of olive cultivars. Among cultivars, leaves of Chetoui had the highest H_2O_2 content (6.01 and 10.25 µmol g^{-1} FW, respectively for 2015 and 2016) followed by Arbosana (5.24 and 8.77 µmol g^{-1} FW, respectively) and Arbequina (3.36 and 5.04 µmol g^{-1} FW respectively).

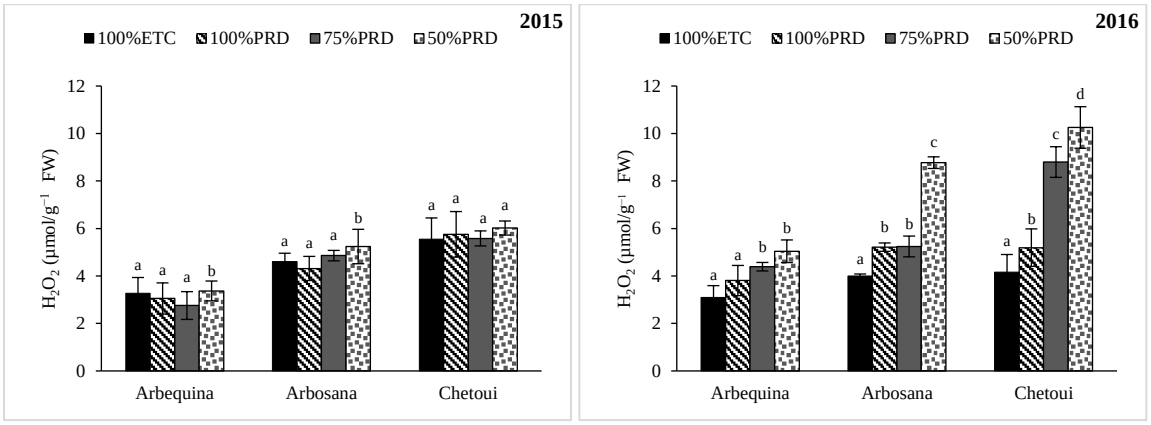


Fig. 4. H_2O_2 content in the leaves of Arbequina, Arbosana and Chetoui cultivars under Control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

3.4. Enzymatic responses

The results indicated that enzyme activities were affected by irrigation treatment, cultivar and their interactions. Furthermore, the activity of antioxidant enzymes was considerably higher in 2016 ('On' year) than 2015 ('Off 'year) independently of the cultivar and PRD treatment (Table 2).

As shown in Table 2, the activity of **CAT** enzyme was higher in all PRD treatments than control (100%ETC) for all studied cultivars in both years. Compared to control trees, **CAT** activity under 50%PRD was increased by 70.17%, 61.98% and 65.50% in Arbequina, Arbosana and

Chetoui cultivars, respectively in 2015. This increment was 94.82%, 62.42% and 66.13% in 2016, respectively in Arbequina, Arbosana and Chetoui cultivars.

PRD irrigation increased the **SOD** activity of olive cultivars in both years with a larger extent in Arbequina and Chetoui than Arbosana, especially for the 50% PRD treatment (Table 2). Interestingly, under 50% PRD , **SOD** activity increased by 72.32 % and 64.39% for Arbequina, by 64.13% and 30.08% for Arbosana and by 65.60 and 53.64% for Chetoui in 2015 and 2016 respectively.

In the same way, POD activity was also significantly increased by PRD irrigation treatments, but at different levels among olive cultivars. Under 50%PRD treatment the increase in POD activity was about 95.18%, 28.78% and 75.84% during the first year and about 167.65%, 93.91% and 58.92% during the second year in Arbequina, Arbosana and Chetoui, respectively, in comparison to control. The lowest enzymes activities were recorded during the first year under well irrigated treatment (100%ETC) for all studied cultivars (Table 2).

Decreasing trends in PPO activity is shown in table 2 for the three cultivars with PRD irrigation. Compared to control, the relative reduction of PPO activity in 50% PRD tress was 58%, 59%, 29% and 35%, respectively in Arbequina, Arbosana and Chetoui in 2015. During 2016, the reduction was 12%.52% and 22% for Arbequina, Arbosana and Chetoui, respectively.

Table 2

Catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (POD) and polyphenoloxidasse (PPO), activities in the leaves of Arbequina, Arbosana and Chetoui cultivars under Control (100%ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods.

Cultivar	Treatment	Enzymes activitiy (Units.mg^{-1} protein)							
		CAT		SOD		POD		PPO	
		2015	2016	2015	2016	2015	2016	2015	2016
Arbequina	100%ETC	2,85±0.58^{a}	3.48±0.59^{a}	5.13±0.74^{a}	7,78±0.59^{a}	1,87±0.27^{a}	4,37±0.86^{a}	6,12±0.73^{b}	8,22±0.58^{a}
	100%PRD	3,08±0.55^{ab}	4.50±0.34^{a}	7,10±0.85^{b}	7,56±0.86^{a}	1,99±0.50^{ab}	5,14±0.39^{a}	5,44±0.91^{b}	7,00±1.41^{a}
	75%PRD	4,30±0.52^{bc}	5,51±0.67^{b}	8,13±0.65^{c}	10,14±0.68^{b}	2,35±0.4^{bc}	6,82±0.55^{b}	5,45±0.80^{ab}	6,72±1.22^{a}
	50%PRD	4,85±0.63^{c}	6,78±0.16^{b}	8.84±0.64^{c}	12,79±1.07^{c}	3.65±0.34^{c}	11,69±1.15^{c}	2,72±0.56^{a}	3,30±0.81^{a}

Arbosana	100%ETC	1,71±0.21^{a}	1,73±0.31^{ab}	2,37±0.22^{a}	6,88±0.59^{a}	1,32±0.41^{a}	2,63±0.34^{a}	8,29±1.06^{b}	8,60±0.42^{b}
	100%PRD	1,79±0.34^{ab}	1,28±0.16^{a}	2,78±0.26^{ab}	6,20±0.09^{a}	1,59±0.24^{b}	4,58±0.68^{b}	8,43±1.02^{b}	13,50±2.4^{b}
	75%PRD	2,27±0.48^{c}	2,75±0.76^{b}	3,07±0.10^{bc}	8,48±0.87^{b}	1,61±0.20^{bc}	4,99±1.73^{b}	5,46±1.22^{ab}	7,38±1.42^{b}
	50%PRD	2,77±0.63^{c}	2,81±0.46^{b}	3,89±0.44^{c}	8,95±0.67^{b}	1,70±.0.17^{c}	5,10±0.12^{c}	4,42±1.14^{a}	5,65±1.13^{b}
Chetoui	100%ETC	2,29±0.19^{a}	3,78±0.38^{a}	3.75±0.55^{a}	6.58±0.30^{a}	1,78±0.17^{a}	6,67±0.87^{a}	8,84±0.68^{b}	13,82±0.35^{b}
	100%PRD	3,35±0.58^{ab}	4,89±0.34^{a}	5.44±0.68^{b}	7.32±0.48^{a}	1,93±0.27^{ab}	7,23±0.55^{a}	7,85±0.88^{b}	10,40±1.09^{b}
	75%PRD	3,55±0.89^{bc}	6,09±0.48^{b}	5.72±0.52^{bc}	9.02±0.86^{b}	2,92±0.24^{bc}	10,52±1.13^{b}	5,33±1.35^{ab}	10,74±1.57^{b}
	50%PRD	3.79±0.31^{c}	6,28±0.41^{b}	6.21±0.0.16^{c}	10.11±0.83^{c}	3,13±0.48^{c}	10,60±0.43^{b}	5,20±0.74^{a}	10,30±1.22^{b}
Two way ANOVA									
	C	<0.001	<0.001	<0.001	0.002	0.001	<0.001	0.115	0.307
	T	0.009	<0.001	<0.001	<0.001	0.001	<0.001	0.276	0.213
	C*T	0.845	0.303	0.414	0.414	0.192	0.024	0.053	0.183

Values are means ± SE of three replicates. Values not followed by the same letter within a column and for each cultivar indicate significant differences between treatments at $p \leq 0.05$, based on Duncan's multiple range test. (n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.5. Hormonal responses

The endogenous content of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and indole-3-acetic acid (IAA) in leaves (Figs. 5−8) of the three studied cultivars was affected differently depending on the water regime during the two years of study. For leaf endogenous ABA concentrations, the differences among treatments were marked mainly in Arbosana and Chetoui cultivars. Arbosana exhibited the highest values under 50%PRD, reaching 26.9 ng g^{-1} FW in 2015 and 35.9 ng g^{-1} FW in 2016. In Arbequina, this increase was only appreciable in 50% PRD treatment, with values 2.38-fold higher than control during the first year, whereas during the second year this treatment showed values 1.53-fold higher than control (Fig.5).

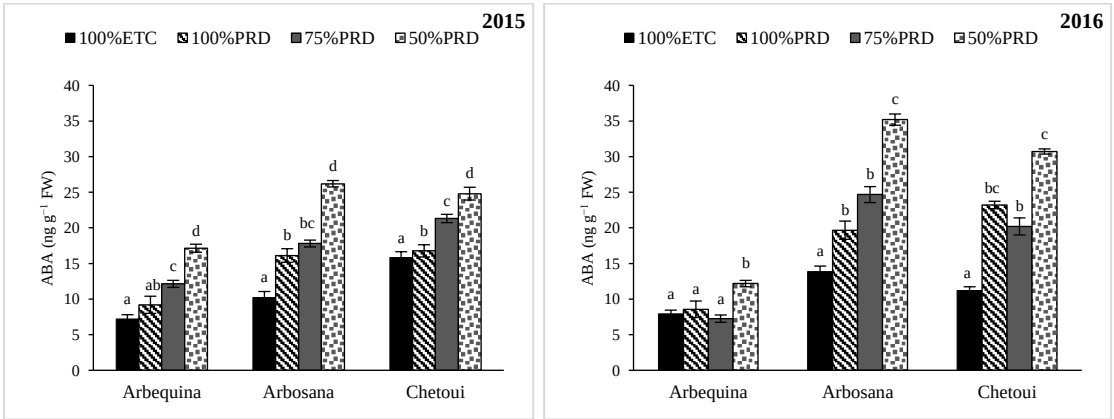


Fig. 5. Endogenous abscisic acid (ABA) levels in leaves of Arbequina, Arbosana and Chetoui cultivars subjected to Control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

PRD irrigation treatments induced an increase in SA concentration in leaves of all cultivars, with higher values in 2016. Regardless of the treatment, Arbequina exhibited the highest SA

content in either the control or PRD treatments. The SA level in Arbequina, Arbosana and Chetoui showed a significant increase under 50%PRD (Figure 6). The increase during 2015 was 29.77% , 119.43% and 100.59%, and the increase during 2016 was 53.10%, 150.32% and 70.93% compared with control, respectively (figure 6).

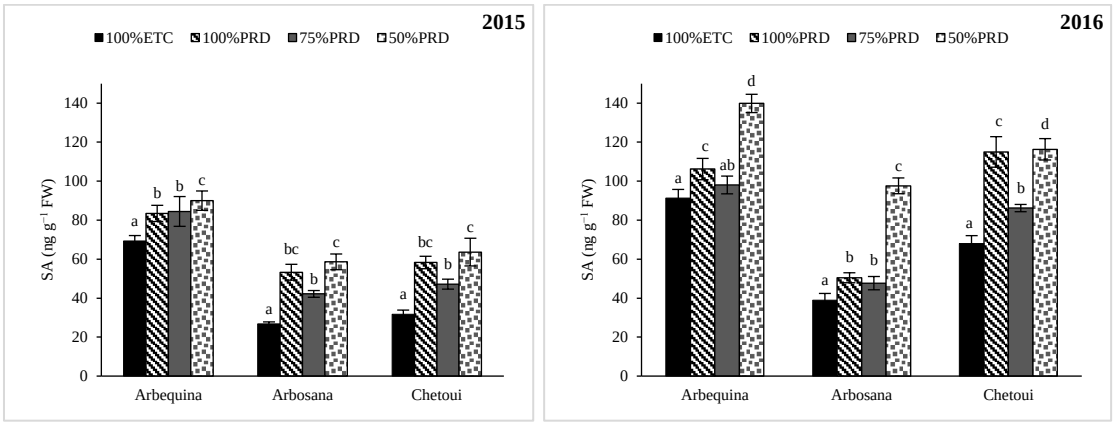


Fig. 6. Endogenous salicylic acid (SA) levels in leaves of Arbequina, Arbosana and Chetoui cultivars subjected to Control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

The increase in JA content under PRD irrigation was observed in the three cultivars and for both years, with highest values recorded under 50% PRD. This treatment induced increases in JA content by 282% and 37% for Arbequina, by 131% and 149% for Arbosana and by 88% and 83% related to control treatment (100% ETC) in 2015 and 2016, respectively (Fig. 7).

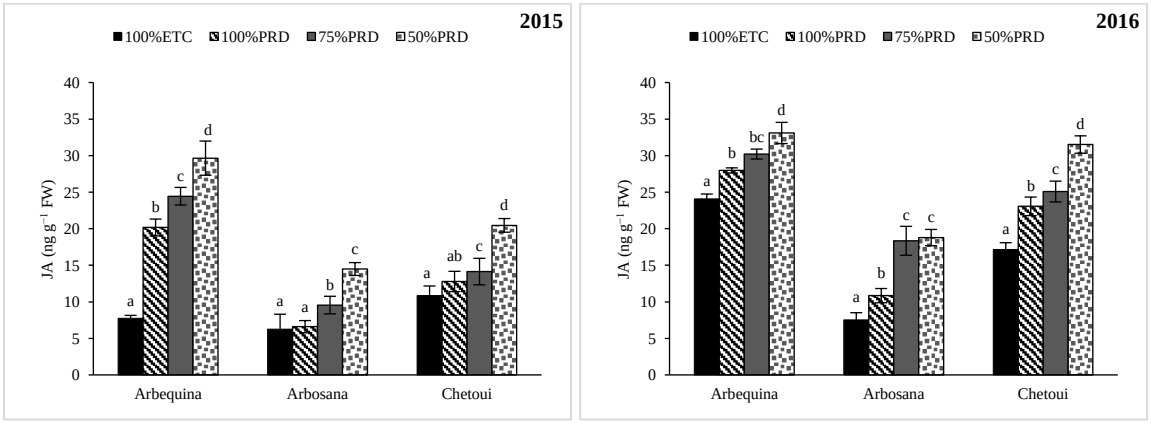


Fig. 7. Endogenous jasmonic acid (JA) levels in leaves of Arbequina, Arbosana and Chetoui cultivars subjected to Control (100% ETC) and PRD irrigation treatments (100% PRD, 75%

PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

IAA content was significantly increased in all studied cultivars under PRD irrigation. The highest values were obtained in Arbequina under 75%PRD and 50% for the first and the second year respectively (Fig. 8). Compared with control (100% ETC), ABA level increased significantly in Arbequina by 34.56% in 2015 and by 57.21% in 2016 under 75% PRD and 50% PRD, respectively.For Arbosana and Chetoui, the accumulation of IAA under 50% PRD was elevated up to 28.84% and 54.44% in 2015 and up to 35.32% and 97.57% in 2016, compared with control.

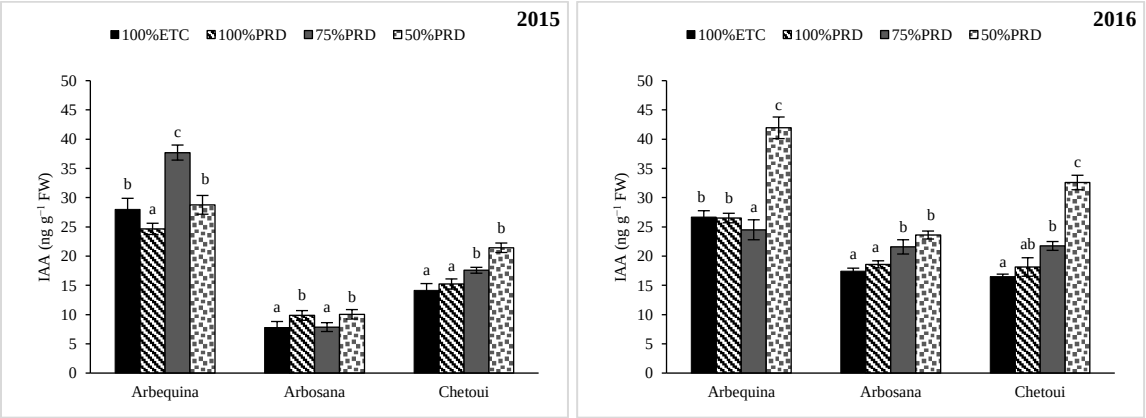


Fig. 8. Endogenous indole-3-acetic acid (IAA) levels in leaves of Arbequina, Arbosana and Chetoui cultivars subjected to Control (100%ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods. Vertical bars represent ± standard error of the mean (n = 3). Different letters within cultivars are significantly different at $p \leq 0.05$.

In root tissue, PRD irrigation induced changes in endogenous phytohormones concentration in all cultivars. Additionally, the pattern of accumulation of phytohormones was different between dry and wet sides of the roots in all treatments (Table 3).

The highest contents of ABA in roots were detected on the dry side in all treatments and cultivars, but this increase was more marked in the roots of Arbosana (20.46 ng g^{-1} FW under 50%PRD) than in Arbequina (8.57 ng g^{-1} FW under 100% PRD) and Chetoui (9.88 ng g^{-1} FW

under 75% PRD) Table 3). The control treatment (100% ETC) showed the lower values of ABA throughout the experiment, about 1.98 ng g^{-1} FW, 6.58 ng g^{-1} FW and 1.32 ng g^{-1} FW for Arbequina, Abosana and Chetoui respectively

The concentration of SA in roots was distinctly affected by the root status (dry or wet) and the water regime (Table 3). In the wet side, SA concentration was higher in control trees than in PRD irrigated trees. By contrast, in the dry side , the SA level was higher in PRD treatments and 100 % PRD considerably enhanced the SA concentration more strongly in Arbosana (20.12 ng g^{-1} FW) as compared to Arbequina (15.48 ng g^{-1} FW) and Chetoui (16.29 ng g^{-1} FW).

PRD irrigation maintained higher JA accumulation in roots as compared to control treatment in all cultivars. JA concentration reached maximum values in dry roots, and it increased markedly in all PRD irrigation treatments (Table 3). Roots of Arbosana and Chetoui had a higher JA level than the roots of Arbequina. Under 50%PRD, JA reached values of 39.99 ng g^{-1} FW, 59.25 ng g^{-1} FW and 105.39 ng g^{-1} FW in wet roots. However, in dry roots values was 41.89 ng g^{-1} FW, 103.40 ng g^{-1} FW and 157.98 ng g^{-1} FW for Arbequinaa Abosana and Chetoui, respectively.

Regarding the endogenous ABA concentrations in roots, significant differences were found among cultivars and treatments (Table 3). The concentration of IAA in roots was lowest in the wet roots, and it increased significantly in dry roots with highest values under 50% PRD irrigation treatment for Arbequina (35.25 ng g^{-1} FW), Arbosana (49.25 ng g^{-1} FW) and Chetoui (46.58 ng g^{-1} FW).

Table 3

Endogenous abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and indole-3-acetic acid (IAA) levels in roots of Arbequina, Arbosana and Chetoui cultivars subjected to Control (100% ETC) and PRD irrigation treatments (100% PRD, 75% PRD, 50% PRD) during 2015 and 2016 experimental periods.

Cultivar	**Treatment**	**Root ABA** (ng g^{-1} FW)		**Root SA** (ng g^{-1} FW)		**Root JA** (ng g^{-1} FW)		**Root IAA** (ng g^{-1} FW)	
		Wet root	**Dry root**	**Wet root**	**Dry root**	**Wet root**	**Dry root**	**Wet root**	**Dry root**
Arbequina	100% ETC	1,98±0.12^{a}		29,72±0.30^{c}		37,54±0.58^{b}		18,50±1.28^{a}	
	100% PRD	5,31±0.20^{c}	8,57±0.77^{a}	19,25±0.38^{c}	15,48±0.58^{c}	38,63±0.62^{b}	41,19±0.68^{b}	20,14±1.01^{b}	25,25±0.69^{a}
	75% PRD	5,28±0.53^{c}	7,98±0.16^{b}	7,59±0.33^{a}	7,31±0.20^{a}	35,92±0.35^{a}	39,97±0.67^{a}	23,74±0.98^{c}	27,25±0.58^{b}
	50 % PRD	3,93±0.19^{b}	7,96±0.14^{b}	17,20±0.43^{b}	14,86±0.26^{b}	39,99±0.21^{c}	41,89±0.57^{b}	34,59±0.51^{d}	35,25±0.50^{c}
Arbosana	100% ETC	6.58±0.56^{b}		19,50±0.46^{b}		64,56±3.55^{b}		15,28±0.60^{a}	
	100% PRD	6.50±0.29^{a}	9.21±1.20^{a}	31,87±0.98^{c}	20,12±0.68^{bc}	63,39±5.10^{b}	95,34±2.58^{a}	25,74±0.76^{b}	30,25±0.58^{a}
	75% PRD	6.59±0.30^{b}	11.91±0.21^{b}	13,51±0.42^{a}	9,61±0.48^{a}	81,71±2.30^{c}	100,12±3.58^{b}	40,60±1.26^{c}	45,25±1.37^{b}
	50 % PRD	14.6±1.21^{c}	20.46±1.21^{c}	20,40±0.88^{b}	19,70±0.27^{b}	59,25±3.73^{a}	103,40±4.46^{b}	39,61±0.72^{c}	49,25±1.32^{c}
Chetoui	100% ETC	1.32±0.07^{a}		26,35±0.40^{c}		46,43±5.26^{a}		26,67±0.92^{b}	
	100% PRD	2.00±0.18^{b}	4.62±0.38^{a}	23,92±1.03^{b}	16,89±0.19^{c}	49,36±2.17^{a}	99,41±3.17^{a}	26,51±0.89^{b}	32,25±2.52^{b}
	75% PRD	1.32±0.08^{a}	9.88±0.32^{b}	9,25±0.25^{a}	8,56±0.56^{a}	130,01±2.69^{c}	175,25±3.21^{c}	24,50±1.90^{a}	28,25±2.08^{a}
	50 % PRD	3.95±0.23^{c}	8.56±0.57^{b}	24,89±0.72^{b}	14,18±0.58^{b}	105,39±3.52^{b}	157,98±3.95^{b}	41,94±1.47^{c}	46,58±0.80^{c}
Two way ANOVA									
	C	<0.001	<0.001	<0.001	0.083	<0.001	<0.001	<0.001	<0.001
	T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	C*T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

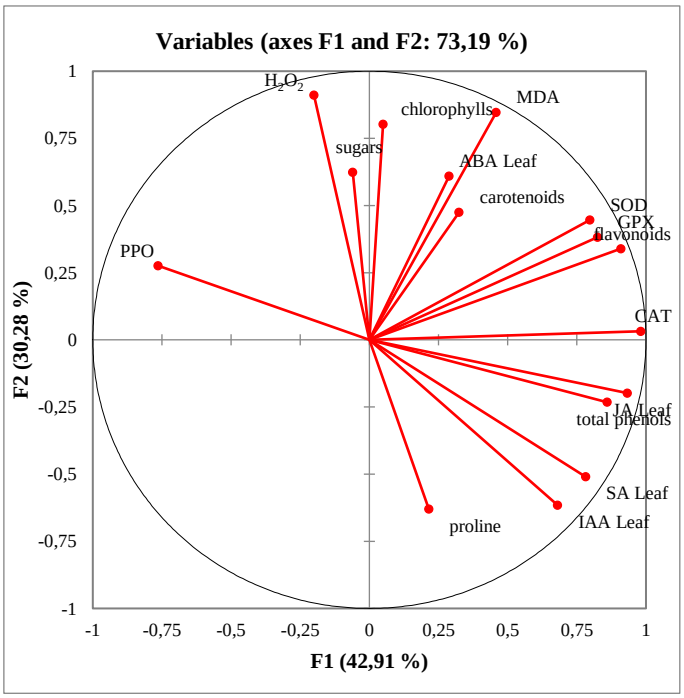
Values are means ± SE of three replicates. Values not followed by the same letter within a column and for each cultivar indicate significant differences between treatments at $p \leq 0.05$, based on Duncan's multiple range test. (n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.6. Principal component analysis (PCA)

Principal component analysis (PCA) was performed basing on selected parameters. Biochemical parameters were loaded into two major principal components (PC1 and PC2), explaining 73% of the total variances (Fig. 9). Most of the examined parameters were discriminated by PC1, and thus explained by the larger proportions of variances (42.91%,); while the lower proportions of variances (30.28%) were indicated by PC2 (Fig. 9).

As regards the distribution of variables (Fig. 9) we have to see that the PC1 was highly and positively correlated with $[JA]_{leaf}$, total phenols, flavonoids, CAT, SOD, POD, and was negatively correlated with PPO. Additionally, PC2 received the main positive contribution from MDA, H_2O_2, chlorophylls, $[ABA]_{leaf}$ and sugars concentrations and also a negative contribution from proline and $[IAA]_{leaf}$.

Chetoui cultivar under 100%PRD, 75% PRD and 50% PRD showed high concentrations of H_2O_2, MDA, sugars, chlorophylls, $[ABA]_{leaf}$, and thus were located in the positive side of both PC1 and PC2. On the contrary, the cultivar Arbequina received 100%PRD, 75% PRD and 50% PRD showed high concentrations of proline, $[IAA]_{leaf}$, $[SA]_{leaf}$ and low concentrations of H_2O_2, MDA, sugars, chlorophylls, ABA leaf, being located on the positive side of PC1 but on the negative side of PC2 (Fig. 9). Arbosana cultivar under the four irrigation treatments showed different concentrations and were located on the negative side of PC1.



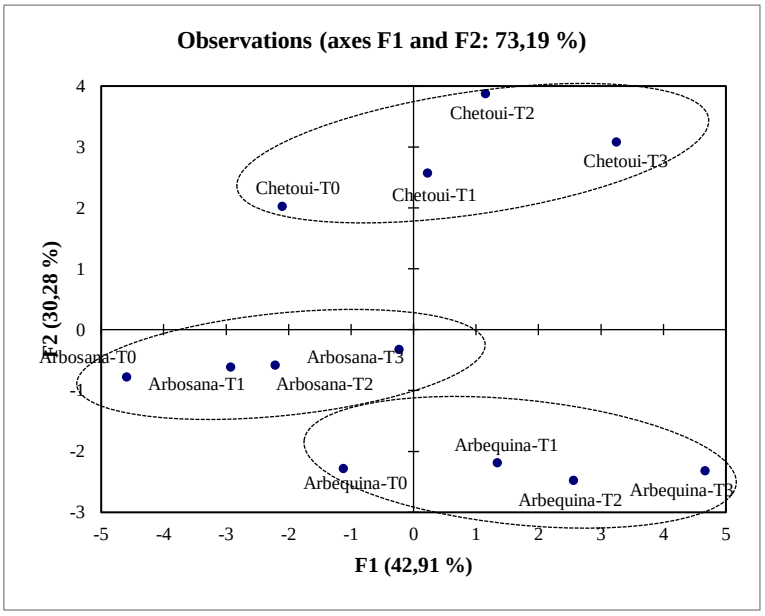


Fig. 9. Principal component loading plot and scores of principal component analysis (PCA) of biochemical traits and hormones contents of olive cultivars (Arbequina, Arbosana and Chetoui) submitted to 100%ETC (T_0), 100% PRD (T_1), 75% PRD (T_2) and 50% PRD (T_3) treatments.

4. Discussion

4.1. Osmotic regulatory substances, pigments and oxidative stress indicators

Under water stress conditions, plants can accumulate compatible solutes such as proline and soluble sugars to facilitate water absorption and preserve hydration of protoplast (Ashraf & Foolad, 2007). The accumulation of these substances, known as osmoregulation, improve plant performance by avoiding the negative effects of water decline. Proline accumulation in plants is considered a general marker of the drought tolerance (Ben Ahmed et al., 2009; Liu et al., 2011). Proline play a key role under full stress conditions, since it regulates the osmotic potential and avoids cell dehydration (Ashraf & Foolad, 2007). Also due to its role as an antioxidant, proline maintains the cellular redox balance and acts as radical scavenging contributing to limit membrane damage and maintaining its integrity (Ashraf & Foolad, 2007; Bacelar et al., 2006).

Our results showed that, the accumulation of proline was higher under 50%PRD in all cultivars. Stikic et al. (2003) also reported that proline accumulation was significantly higher in leaves of PRD irrigated tomato as compared to the leaves of fully irrigated plants.

It has been well established that the accumulation of osmolytes such as proline is a common adaptative response in olive tree under water deficit conditions (Sofo et al., 2004).

Besides this, Arbequina accumulated higher amounts of proline in leaves in response to PRD irrigation than the two other cultivars demonstrating the higher ability of this cultivar to deal with water deficit. This result matches previous works on olive (Ben Ahmed et al., 2009; Ennajeh et al., 2006), in which tolerant cultivars accumulate higher amounts of proline. Zandalinas et al. (2016) also reported a higher proline accumulation in tolerant than in sensitive rootstock of citrus.

Accordingly, these observations suggest that Arbequina is more tolerant to water deficit induced by PRD irrigation. Interestingly, it has been reported that the accumulation of this osmolyte is strongly correlated with tolerance to drought stress (Hoekstra et al., 2001; Sivritepe et al., 2008). Accumulation of soluble sugars also enables osmotic adjustment under water deficit conditions (Chakhchar et al., 2015). In our study, leaf total soluble sugar (TSS) content increased under PRD irrigation in all studied cultivars. Higher soluble sugar content under PRD irrigation was also reported by Stikic et al. (2003). Results also show that Arbequina presented the highest amount of TSS followed by Chetoui and Arbosana under 50% PRD treatment. These finding agree with previous reports indicating that a higher TSS accumulation improves the plants drought tolerance (Karimi et al., 2018).

Water stress induce the generation of ROS that can cause oxidative damage in plants. This metabolic imbalance can be estimated by MDA and H_2O_2 levels.

Increased MDA and H_2O_2 levels under water deficit stress have been reported in olive (Petridis et al., 2012). Accordingly, the level of MDA and H_2O_2 was mostly raised under 50%PRD and the increase rates were lower in Arbequina than in Chetoui indicating that Arbequina was less subjected to oxidative damage. The increased rate of MDA and H_2O_2 has been correlated with membrane damage, and the extent of this damage is directly linked to susceptibility of olive to adapt to water deficit and is commonly used as an indicator of stress tolerance (Bacelar et al., 2007). This significant correlation has been also reported by Boughalleb & Mhamdi (2011) in olive under drought stress.

Our results showed that the water deficit, induced by PRD irrigation, significantly reduced the total chlorophylls content, mainly for Arbosana cultivar with lowest values under 50% PRD. This response reduces the amount of photons absorbed by leaves which enhances the photoprotective and antioxidant leaf capacity of leaves and allows trees to survive under stressed conditions (Doupis et al., 2013).

Carotenoids play a major role in preventing photo-oxidative damage by stabilizing the photochemical processes under limited water availability. Moreover, it has been reported that better plant tolerance to water deficit stress is associated with higher carotenoids content (Abbasi et al., 2014). In the present study, we observed that under 50% PRD Arbequina and Chetoui had the most carotenoids content showing their water stress resistance capacity compared to Arbosana.

Our study findings showed that total phenols and flavonoids contents increased in response to PRD irrigation. Among cultivars, Arbequina and Chetoui had the highest values of total phenols, while Arbosana had the lowest values. Petridis et al. (2012) suggested that higher phenols accumulation in olive was associated with water stress resistance which confirms our results.

PRD irrigated trees showed a significant higher accumulation of total phenols and flavonoids relative to control trees. The ability of trees to maintain higher phenols content may allow trees to adapt to drought stress.

Total phenols and flavonoids content of Arbequina was much higher than in the other cultivars and highest values were recorded under 50%PRD (Table 1). Therefore, the drought tolerance of Arbequina may be related to these metabolic changes.

4.2. Enzymatic responses

The generation and accumulation of reactive oxygen species (ROS) under stress conditions induce biochemical disturbance and oxidative damage. The damaging effects of excessive ROS production is counteracted by different defense mechanisms, including antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO).

In this study, The PRD irrigation induced a high activity of SOD, CAT and POD in all cultivars while the activity of PPO decreased.

SOD is a potent enzymatic antioxidant involved in the tolerance process and play an important role in the mechanisms of defense against ROS (Sayfzadeh et al., 2011). The highest SOD activity was observed under 50% PRD. Analogous results have been found by Ben Ahmed et al. (2009) and Boughalleb & Mhamdi (2011) in olive plants under drought stress**.**

Among cultivars, Arbequina exhibited the highest activities, indicating intra-specific variability in protection levels against water deficit stress.

According to our results, the highest CAT activity was observed in Arbequina. Hence, appears to play a key role in the drought tolerance exhibited by Arbequina; and may also explain the better performance of this cultivar under water deficit induced by PRD.

Our results are further strengthened by the increased POD activity exhibited by Arbequina under 50% PRD.

According to our results, all olive cultivars exhibited increases in POD activity under PRD. This increase was enough to overcome the excess of H_2O_2 production only in cultivar Arbequina since the levels of H_2O_2 were reduced in this cultivar.

Accordingly, under PRD irrigation, POD and CAT enzymes controlled the levels of H_2O_2 that positively reflected in the lower MDA content under PRD irrigation. These results are in agreement with those of others (Sofo et al., 2005) who indicated that CAT and POD are involved in the reduction of H_2O_2 levels and membrane damage in olive tree under drought stress.

All olive cultivars exhibited decreases in PPO activity under PRD irrigation .This confirms the results obtained by Sofo et al. (2008), who argued that the activity of PPO in olive is lower under drought stress.

The observed decrease in PPO activity suggests that PRD irrigation improves the antioxidant role of phenols by the inhibition of polyphenol oxidase activity and consequently the maintenance of the phenol compounds pool in the reduced state as reported by Sofo et al. (2008) under drought stress. This hypothesis is further strengthened by the increase of total phenol content exhibited by Arbequina trees under PRD treatments.

4.3. Hormonal Changes

Plant hormones are essential molecules able to modify plant physiology and modulate metabolic plant responses in rapid response to stress conditions, an imperative requirement to ensure their survival.

From the studied hormones, ABA is commonly known as a key hormone in the response to drought stress (Zhang et al., 2006). ABA has been shown to form part of a complex signalling network, which mediates the activation of many physiological responses induced by drought stress. Overall, ABA plays vital roles in the drought tolerance mechanisms, as in our study, the ABA content in all cultivars was higher under PRD irrigated trees as compared to control treatment. This result may contribute to the decreased stomatal conductance, decreased transpiration rate and increased water use efficiency for studied cultivars under PRD irrigation

(Abboud et al., 2019), which would be beneficial to improve adaptation to water deficit conditions.

In our experiment 50% PRD irrigated tree undergo more severe water deficit condition than other treatments, higher foliar ABA concentration in this treatment could be attributed not only to the relative higher sap flow from the root system, but also to 'in situ' ABA synthesis in leaves themselves (Wilkinson & Davies, 2002). Leaf cells are known to rapidly synthesize ABA as their water potential significantly decreases (Sauter et al., 2001). Thus, greater leaf ABA accumulation under 50% PRD treatments could be a result of a combination of ABA higher transportation rates from drying roots and 'in situ' synthesis in leaves (Soar et al., 2004). Dbara et al. (2016) also found higher ABA accumulation in the leaves of olive tree cv. Chetoui under PRD irrigation.

Other hormones such as SA, JA and IAA can play direct or indirect roles in the response of plant to abiotic stress (Arbona & Gómez-Cadenas, 2008; Brossa et al., 2011; Gómez-Cadenas et al., 1996; Mahouachi et al., 2007).

Regarding JA and its metabolically active derivatives (jasmonates), there is increasing evidence that they are also crucial signaling molecules involved in many plant responses to biotic and abiotic stresses (Brossa et al., 2011).

In our work, ABA and JA hormones seem to have a synergistic interaction in response to water deficit stress, which is according to other studies (Brossa et al., 2011). JA may interact with ABA synthesis under water deficit conditions (Bandurska et al., 2003; de Ollas et al., 2013) and this interaction could regulate stomatal closure (Acharya & Assmann, 2009).

In reaction to PRD irrigation, a significant increase in the endogenous content of SA was reported in all studied cultivars with higher levels observed in Arbequina.

Our results also showed an increase on IAA content in trees subjected to PRD irrigation, which explained that under moderate water deficit IAA promotes water uptake into the protoplasts (Pustovoitova et al., 2003). IAA and ABA are probably involved in turn in the process of drought adaptation and perform phase specific functions (Pustovoitova et al., 2003) since a rise in both hormones was observed with increased water stress level. In fact, IAA is considered for some authors as the most representative 'water deficit signal' (De Diego et al., 2012).

The results of this work indicate that Under PRD irrigation, ABA, JA and IAA concentrations in roots generally increased with PRD irrigation, in a higher extent in dried roots compared to wetted ones.

Under our experimental conditions, leaf ABA accumulation occurred at higher rates in 50%PRD treatments resulting in significant higher ABA concentrations in both years. Taking

into consideration that dried roots consist the primary source of leaf ABA, higher ABA leaf concentration obtained in 50%PRD treatment could be attributed to higher ABA production in roots. Thus, it would be expected that roots ABA concentration would be higher in roots of PRD irrigation treatments.

Importantly, several studies presented evidence that under drought conditions ABA is transported from the root system to the leaves where it is accumulated in the guard cells apoplast (Wilkinson & Davies 2010). Thus, the lower ABA level observed in roots compared with leaves indicates the presence of an efficient root-shoot translocation of ABA under PRD irrigation.

In our study, we detect a higher accumulation of IAA in roots mainly under 50%PRD. This accumulation may be attributed to a greater root development aiming to explore the soil and to improve water uptake under water-stressed conditions. Accordingly, our results are in agreement with the literature data, suggesting that IAA plays essential role in promoting root growth (Seo et al. 2009).

Additionally, our results revealed a higher ABA accumulation in roots under PRD irrigation, and suggest that interactions of ABA with auxin could potentially play a key role in regulating root growth under water stress. These results are in agreement with the studies of Xu et al. (2012) suggesting that ABA is directly implicated in the regulation of auxin levels in the root growth zone resulting in maintaining root development under water deficit conditions.

Our results show also that**,** SA concentration in olive roots decreased mainly in Arbequina which agrees with previous works indicating that water deficit triggers changes in SA concentrations and this hormone plays an important role in stomatal closure, thus favoring drought tolerance (Miura et al., 2013).

Overall, under PRD irrigation, studied cultivars have a great ability to cope with water deficit conditions by triggering a network of interactive signaling pathways.

4.4. Principal component analysis (PCA)

In sum, on the basis of a PCA analyze investigating biochemical traits and hormonal response in the three olive cultivars under PRD irrigation, a notorious separation between cultivars have been shown (Fig. 9). In addition, PCA revealed close relationships among biochemical parameters showing the involvement of different mechanisms in olive in response to PRD irrigation. Indeed, a negative relationship was established between proline and MDA and H_2O_2, which were found positively associated with sugars. This behavior constituted an adaptive strategy to protect cellular processes against membranes damage and to conserve water in cells (Sofo et al., 2008). Nevertheless, regarding antioxidants, different levels of susceptibility were

detected between the three cultivars, being Arbosana most sensitive. In Arbequina and Chetoui CAT, SOD and POD contribute more to oxidative damage protection (Fig. 9). Similar results were disclosed in many several previous investigations (Bacelar et al., 2006; Boughalleb & Hajlaoui, 2011) and indicated better protection against oxidative stress.

In additions, the negative correlation between SA, JA and IAA leaf concentration, with H_2O_2 and MDA contents suggest that hormonal mechanism take place to protect olive trees from oxidative stress damage. Accordingly, the studied olive cultivars were able to prevent oxidative damage by regulating synthesis and accumulation of hormones and antioxidants.

5. Conclusion

In this study, we revealed that the three investigated olive cultivars showed a clear difference in their response to PRD irrigation. The PRD irrigation triggered several biochemical adjustments in olive cultivars. These metabolic changes assisted olive cultivars to overcome water deficit stress induced by PRD irrigation. Thus, PRD irrigation lead to an enhanced activation of secondary metabolites with antioxidant properties such as phenols and flavonoids that mitigate the damaging effects of stress. Our results also demonstrated that Arbequina exhibited higher chlorophyll and carotenoids contents, higher SOD, CAT and POD activities and lower contents of MDA and H_2O_2 content than Arbosana and Chetoui.

Others metabolic changes, including proline and total solubles sugars accumulation seem to be more effective in Arbequina since it displays a better protection of leaf function compared to the other cultivars.

Besides, we argue that improving olive adaptation to water deficit is attributed to enhancing phytohormones levels for controlling water loss and eventually improving WUE.

Based on the data obtained, it is clear that Arbequina can be a candidate cultivar in super intensive production in semi-arid regions due to the higher efficiency of the compatible solute and pigments accumulation along with higher hormonal levels and effective activation of antioxidant machinery under the highest level of water stress induced by PRD irrigation.

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