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ORIGINAL PAPER

Physiological and anatomical changes induced by drought in two olive cultivars (cv Zalmati and Chemlali)

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Abstract Photosynthetic gas exchange, vegetative growth, water relations and fluorescence parameters as well as leaf anatomical characteristics were investigated on young plants of two *Olea europaea* L. cultivars (Chemlali and Zalmati), submitted to contrasting water availability regimes. Two-year-old olive trees, grown in pots in greenhouse, were not watered for 2 months. Relative growth rate (RGR), leaf water potential (Ψ_{LW}) and the leaf relative water content (LWC) of the two cultivars decreased with increasing water stress. Zalmati showed higher values of RGR and LWC and lower decreased values of Ψ_{LW} than Chemlali, in response to water deficit, particularly during severe drought stress. Water stress also caused a marked decline on photosynthetic capacity and chlorophyll fluorescence. The net photosynthetic rate, stomatal conductance, transpiration rate, the maximal photochemical efficiency of PSII (F_v/F_m) and the intrinsic efficiency of open PSII reaction centres (F'_v/F'_m) decreased as drought stress developed. In addition, drought conditions, reduced leaf chlorophyll and carotenoids contents especially at severe water stress. However, Zalmati plants were the less affected when compared with Chemlali. In both cultivars, stomatal control was the major factor affecting photosynthesis under moderate drought stress. At severe drought-stress levels, the non-stomatal component of photosynthesis is inhibited and inactivation of the photosystem II occurs. Leaf anatomical parameters show that drought stress resulted in an increase of the upper epidermis and palisade mesophyll thickness as well as an increase of the stomata and trichomes density. These changes were more characteristic in cv. 'Zalmati'. Zalmati leaves also revealed lower specific leaf area and had higher density of foliar tissue. From the behaviour of Zalmati plants, with a smaller reduction in relative growth rate, net assimilation rate and chlorophyll fluorescence parameters, and with a thicker palisade parenchyma, and a higher stomatal and trichome density, we consider this cultivar more drought-tolerant than cv. Chemlali and therefore, very promising for cultivation in arid areas.

Keywords Chlorophyll fluorescence · Gas exchange · Leaf anatomy · *Olea europaea* L. · Water relations · Water stress

Introduction

The evergreen olive tree (*Olea europaea* L.) is one of the most characteristic tree crops from the Mediterranean basin, where plants are frequently subjected to prolonged drought periods (Connor and Fereres 2005). If compared to other fruit tree species, olive tree is able to tolerate the low availability of water in the soil by means of morphological and physiological adaptations acquired in reply to cope

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with drought stress (Connor and Fereres 2005; Bacelar et al. 2007). One of the first responses to drought stress is a reduction in plant growth. The adaptation mechanisms in olive also lead to changes in leaf water status and carbon assimilation (Wang et al. 2003). The maintenance of appropriate plant water status during water deficit is essential for continued growth and this process can be achieved by stomatal regulation (Athar and Ashraf 2005; Ben Ahmed et al. 2007). Thus, stomatal closure significantly decreases transpiration rate and so, contributes to maintaining positive turgor pressure of the cells (Bosabalidis and Kofidis 2002).

Gas exchange and CO_2 assimilation followed chlorophyll synthesis. However, olive leaves reached photosynthetic maturity (chloroplast development) relatively early; this behaviour may represent an advantage in Mediterranean environments, wherein this species has to overcome recurrent hot and dry summers (Marchi et al. 2005). Olive plants maintain a high rate of photosynthesis during long drought stress periods (Dichio et al. 2004). A higher photosynthetic rate under drought is a decisive factor for better drought tolerance in olive cultivars (Bacelar et al. 2007). On the other hand, photosynthetic capacity differed significantly between different degrees of leaf expansion (Marchi et al. 2007). Water deficit induced a parallel decline in assimilation rate and stomatal conductance, as water status decreases (Tognetti et al. 2004), showing that olive trees were able to restrict water loss by modulating stomatal closure at different levels of drought stress (Tognetti et al. 2005). In addition to physiological and environmental variables, endogenous factors (hydraulic and chemical signals) appear to affect stomatal conductance in olive trees (Jones 1998; Moriana et al. 2002). In many works, the C_i/C_a ratio was maintained within a narrow range throughout water treatments, reflecting tight coupling between assimilation rate and stomatal conductance (Moriana et al. 2002; Centritto et al. 2005; Díaz-Espejo et al. 2006), and the reduction in photosynthetic activity in water-stressed olive plants was entirely caused by the diffusional limitations restricting the supply of CO_2 to metabolism in response to soil moisture (Centritto et al. 2003, 2005; Tognetti et al. 2007). On the other hand, as the stress progresses, photosynthetic CO_2 fixation may be limited more directly by non-stomatal factors, such as biochemical constraints (Lawlor and Cornic 2002; Ashraf et al. 2008; Munns and Tester 2008). This limitation causes the over-reduction of the photosynthetic electron chain. Damage to the photosynthetic machinery is mainly due to an imbalance between the capture of light and its use. Chlorophyll fluorescence measurements can give useful information about photosynthetic apparatus integrity and reveal potential water stress-induced damage to PSII (Genty et al. 1989; Angelopoulos et al. 1996; Maxwell and Johnson 2000). Under severe water stress, electron transport to O_2 and increased quenching of excitation energy in the PSII antennae may be unable to dissipate the excess excitation energy in the PSII antennae and photodamage of PSII will result with a possible net loss of D1 protein of PSII reaction centres (Baker 1993; Cornic 1994). Such effects can have significant consequences on the photosynthetic productivity of plants (Long et al. 1994). Tognetti et al. (2004) show a significant change in the ratio of variable to maximal fluorescence yield in dark-adapted leaves, though the PSII photochemical efficiency did not differ significantly between watering regimes, indicating only minor down-regulation of electron transport. However, a different response in olive leaves might be expected at higher level of water deficit (Nogués and Baker 2000).

Because the leaf is the organ most responsive to environmental conditions (Nevo et al. 2000), its structure reflects the effects of water stress more clearly than that of the stem and roots. Olive cultivars well adapted to drought field conditions reveal enhanced sclerophylly, with a high density of foliar tissue and the presence of thick cuticle and trichome layers (Bacelar et al. 2004). On the other hand, changes in cell-wall elasticity can also contribute to turgor maintenance under drought conditions (Patakas and Noitsakis 1999). Changes in leaf anatomical characteristics are known to alter the CO_2 conductance diffusion components from the substomatal cavities to the sites of carboxylation and thus contribute to the maintenance of photosynthetic rates despite the low stomatal conductance (Chartzoulakis et al. 1999; Evans and Loreto 2000). In addition, the photosynthetic capacity of olive plants during leaf development relied greatly on mesophyll properties, more than on leaf mass per area ratio or nitrogen allocation (Marchi et al. 2008).

The aim of this study was to test the hypothesis whether water stress would affect photosynthetic activity in olive by stomatal limitations only or also by non-stomatal factors as damage in the photosystem II and anatomical performance. On the other hand we test the hypothesis of the importance of eventual anatomical adaptations used by this species to cope with drought stress. For this, we have examined the effects of drought on several physiological parameters, such as chlorophyll fluorescence characteristics, photosynthetic pigments, gas exchange parameters, water relationship and growth, as well as, leaf anatomical parameters in two Tunisian olive cultivars (Chemlali and Zalmati) under different irrigation regimes. Another objective of this study was to clarify the response to drought of Zalmati and to compare with that of the famous olive cultivar Chemlali, in order to better understand their differences on water stress tolerance and to find the cultivar that was able to resist better to this abiotic stress.

Materials and methods

Plant material and growth conditions

Two-year-old, own-rooted cuttings of olive cultivars, 'Chemlali' and 'Zalmati', were transplanted in 10-dm^3 pots filled with a mixture of sandy soil and peat (2:1, v/v) in a glasshouse at the Agronomic institute of Chott-Meriam (Tunisia, 35°490′ N, 10°402′ E). Throughout the experimental period, greenhouse temperature was 21–33°C, relative humidity was 65–85% and photosynthetic photon flux (PPF) was about 1,200 μmol m^{-2} s^{-1}. The plants of the two cultivars were homogeneous and had a height of about 120 cm. All of the pots were covered with plastic film and aluminium foil to reduce evaporation from the soil surface and to minimise solar heating of the pot. Plants were watered weekly to field capacity with Hoagland's solution for 2 months before the experiment. After this period, the two different cultivars were exposed to different water regimes. Ten plants of each cultivar were used as controls (well watered (WW)) and irrigated every 2 days to maintain soil water content close to field capacity. An additional 50 plants (10 plants for treatment) from the two cultivars were stressed by withholding water during increasing periods (5, 10, 20, 40 and 60 days). Soil water potential (Ψ_{soil}) in the pots in the drought treatment was about –2.5 MPa at the end of the experiment. During these drought treatments, growth and water relations, photosynthetic pigments contents, gas exchange and chlorophyll fluorescence were measured on five plants of each cultivars under withholding water period (0, 5, 10, 20, 40 and 60 days).

Growth activity and leaf parameters

At the end of the experiment, five plants per treatment of each cultivar were sampled to determine the leaf area (*A*) using Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., England). For the biomass production, the plant material was first cleaned with distilled water. After the water on the plant was absorbed by tissue paper, fresh mass (FM) was measured. The dry mass (DM) was measured after the fresh material was dried at 70°C for 48 h. For the fresh mass at full turgor (TM), it was determined by immersing the leaf petioles in demineralised water for 48 h in the dark at 4°C.

Relative growth rate (RGR), net assimilation rate (NAR), specific leaf area (SLA) and leaf density (D) were calculated in five plants per treatment, as follows:

$$\text{RGR} = [\ln(\text{DM}_2/\text{DM}_1)]/(t_2 - t_1)$$

$$\text{NAR} = [(\text{DM}_2 - \text{DM}_1)/(A_2 - A_1)]/[\ln(A_2/A_1)/(t_2 - t_1)]$$

$$\text{SLA} = [(A_2 - A_1)/(\text{DM}_{L2} - \text{DM}_{L1})]/[\ln(\text{DM}_{L2}/\text{DM}_{L1})/\ln(A_2/A_1)]$$

$$\text{D} = (\text{DM}_L/\text{FM}_L) \times 1{,}000$$

where DM is the total dry mass, *t* is the time, *A* is the total leaf area, DM_L is the total dry mass of leaves, FM_L is leaf fresh mass, and 1, 2 are the beginning and the end of drought period, respectively.

Plant water status

Leaf water potential (Ψ_{LW}) was measured using five fully expanded leaves of similar age and position in the canopy for each treatment. The values of Ψ_{LW} were carried between 8:00 h and 10:00 h using a Sholander pressure chamber (Skye Instruments, Powys, UK). Values of leaf relative water content (LWC) on fully expanded leaves from five plants taken from the mid-section of shoots were determined as:

$$\text{LWC} = (\text{FM}_L - \text{DM}_L)/(\text{TM}_L - \text{DM}_L)$$

where FM_L is leaf fresh mass, DM_L is leaf dry mass and TM_L is leaf fresh mass at full turgor.

Photosynthetic pigment estimation

About 1 g of fresh leaves tissue was used for each extraction. Tissue was homogenised in liquid nitrogen and total pigments extracted in 80% acetone. The absorbance of the extracts was measured on a spectrophotometer (Hitachi U-2000, Krefeld, Germany) at three wavelengths (470, 645 and 663 nm). The concentrations of chlorophylls (*a*+*b*) and carotenoids were calculated according to Arnon (1949) and Mac Kinney (1941), respectively.

Measurements of photosynthetic gas exchange and chlorophyll fluorescence

Net CO$_2$ assimilation rate (P_{n}), stomatal conductance (g_{s}), intercellular CO$_2$ concentration (C_{i}) and transpiration (E) of the seedlings were measured on two mature leaves per plant, obtained from the current year shoot (mid portion) of five plants per treatment (0, 5, 10, 20, 40 and 60 days of withholding water) using a Portable Photosynthesis System (Li-6200, Lincoln, NE, USA). Measurements were made between 8:00 and 9:00 h to avoid photoinhibitory damage potentially resulting from high light stress at midday. Temperature, relative humidity and light intensity during measurement of gas exchange were 23–26°C, 80% of the ambient humidity and 1,000 μmol m^{-2} s^{-1}, respectively.

The CO_2 concentration in the chamber was 360 mmol mol^{-1}. The intrinsic leaf water use efficiency (WUE) was estimated as the quotient of the photosynthetic rate over the stomatal conductance rate (P_n/g_s).

PSII photochemistry was measured on attached leaves at room temperature with a portable fluorometer (PAM-2000, Walz, Germany). After a dark-adaptation of the leaves for at least 30 min, the minimal fluorescence level (F_o) was measured by using modulated light which was low to induce any significant variable fluorescence. The maximal fluorescence level in the dark-adapted state (F_m) was measured by a 0.8-s saturating pulse at 8,000 µmol m^{-2} s^{-1}. To determine the minimal fluorescence level during illumination (F'_o), a piece of black cloth was rapidly placed around the leaf and the leaf-clip holder in the presence of far-red light (7 µmol m^{-2} s^{-1}) in order to fully oxidise the PSII centres. Upon darkening of the leaf, fluorescence dropped to the F'_o level and immediately rose again within several seconds. The maximal fluorescence level during natural illumination (F'_m) was measured at midday with natural sunlight by a 0.8-s saturating pulse at 8,000 µmol m^{-2} s^{-1}. All measurements of F_o and F'_o were performed with the measuring beam set to a frequency of 600 Hz, whereas all measurements of F_m and F'_m were performed with the measuring beam automatically switching to 20 kHz during the saturating flash. By using fluorescence parameters, calculations were made of: (1) the maximal potential PSII efficiency, $F_v/F_m = (F_m - F_o)/F_m$; (2) the efficiency of excitation energy capture by open PSII reaction centres, $F'_v/F'_m = (F'_m - F'_o)/F'_m$ (Demmig-Adams and Adams 1996).

Leaf anatomy

Anatomical tissue measurements were performed on five healthy, fully expanded mature leaves collected at the end of the experiment from control and water-stressed cultures. Sections were taken from the middle of the leaves to avoid differential thickness due to variations along the leaf. The free-hand sections obtained from fresh mature leaves were cleaned in diluted sodium hypochlorite then rinsed in distilled water and finally stained with stained with 0.1% (w/v) toluidine blue O. Observations were performed under a light microscope (Leitz, Germany). The thickness of whole leaf blade, palisade and spongy mesophyll, upper and lower epidermis were taken with an ocular micrometre and exact values were calculated with a factor derived by comparing ocular with stage micrometres. The stomatal and trichomes density (number per unit leaf area) were measured in artificial replicas of polish (colodium) applied to the abaxial surface of 10 leaves per treatment.

Statistical analysis

The data were subjected to analysis of variance (ANOVA), and comparisons between the mean values of treatments were made by the least significant difference (LSD) test ($P < 0.05$). Statistical analyses were performed using the SPSS statistical package (SPSS 13).

Results

Relative growth rate and leaf water relations

Relative growth rate (RGR) decreased significantly ($P < 0.05$) with increasing water stress in Chemlali cultivar. In contrast, for Zalmati cultivar the significant drop in RGR was observed only at moderate to severe water stress (20–60 days) (Fig. 1a). At the end of the experiment, RGR decreased by 33.8 and 56.2% in Zalmati and Chemlali, respectively, in comparison to their respective plants under well-watered conditions. For both cultivars tested, leaf water relation characteristics (LWC and Ψ_{LW}) were affected by water deficit treatment. The LWC of Chemleli cultivar decreased from 89.1% in well-irrigated olive trees to 49.4% at the end of experiment. The LWC of the Zalmati cultivar dropped from values of 87.2% at the beginning of the experiment to values of 58.8% at the end (Fig. 1b). Thus, the greatest reduction in LWC was recorded in Chemlali plants. Leaf water potential (Ψ_{LW}) in water-stressed plants declined during the stress cycle in both cultivars. The cultivar with the highest relative water content under drought conditions (Zalmati) showed the highest leaf water potential (−4.2 MPa) compared to Chemlali (−6.1 MPa). At the end of the experiment, the reduction in Ψ_{LW} values in Chemlali and Zalmati plants was 80 and 70%, respectively. Thus, compared with the leaf water content, the leaf water potential showed a greater decrease during the water deficit.

Leaf gas exchange parameters

The rate of net CO_2 assimilation (P_n) decreased significantly with water stress period exceeding 5 and 10 days, respectively, in Chemlali and Zalmati cultivars. At the end of the experiment (60 days of withholding irrigation) and compared to the control, Zalmati revealed less reduction in P_n (65.1%) than Chemlali (83.8%) (Table 1). Similar results were found for the transpiration rate (E). After 60 days of water stress, this parameter decreased by 85.4 and 80.6% in Chemlali and Zalmati, respectively, in comparison to their respective plants under well-watered conditions. Moreover, the decrease in E in stressed plants was later in Zalmati than

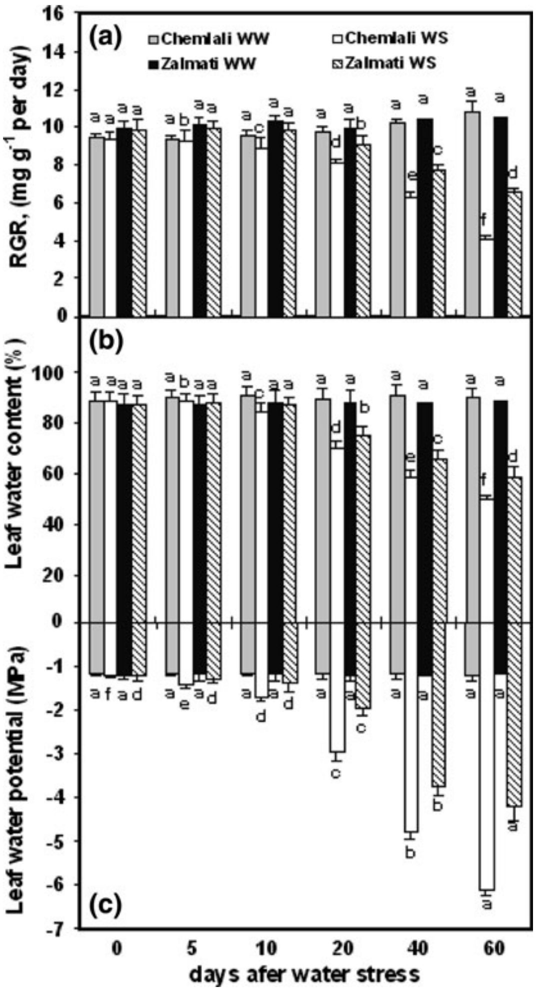

Fig. 1 **a** Relative growth rate (RGR, g g^{-1} per day), **b** leaf relative water content (LWC, %) and **c** leaf water potential (Ψ_{LW}, MPa) in Chemlali and Zalmati olive cultivars treated with increasing drought stress. Bars followed by the same letter do not differ statistically at $P < 0.05$ (LSD Test). Averages of 5 repetitions are presented with bars indicating SE

in Chemlali with respect to control plants (Table 1). The stomatal conductance (g_s) and the intercellular CO_2 concentration (C_i) decreased significantly as water stress developed in both cultivars tested; whereas, C_i was comparatively less reduced than g_s, P_n and E thus, at the end of the experiment C_i declined by approximately 11 and 11.3%, respectively, in Chemlali and Zalmati as compared to the control (Table 1). In the two olive cultivars, water use efficiency was significantly higher in severely drought-stressed plants. A close relationship was found between the rate of CO_2 assimilation and leaf water potential ($r^2 = 0.98$, $P < 0.0001$ in Chemlali; $r^2 = 0.93$, $P < 0.0001$ in Zalmati; Fig. 2a), and between stomatal conductance and leaf water potential ($r^2 = 0.95$, $P < 0.0001$ in Chemlali; $r^2 = 0.95$, $P < 0.0001$ in Zalmati; Fig. 2b). There was a good linear correlation ($r^2 = 0.95$, $P < 0.0001$ in Chemlali; $r^2 = 0.95$, $P < 0.0001$ in Zalmati; Fig. 4a) between photosynthesis and stomatal conductance in both cultivars. In addition, the intercellular CO_2 concentration (C_i) was positively correlated with P_n ($r^2 = 0.66$, $P < 0.0001$ in Chemlali; $r^2 = 0.79$, $P < 0.0001$ in Zalmati; Fig. 4b).

Photosynthetic pigments and chlorophyll fluorescence

The water treatment did not significantly affect the Chl ($a+b$) and carotenoid concentrations only after stress period exceeding 5 and 10 days, respectively, in Chemlali and Zalmati cultivars (Table 2). At the end of the experiment, the reduction in Chl ($a+b$) was 56.3 and 39.6% for chemlali and Zalmati, respectively. Similar results were found for carotenoid concentrations, with decreased values by 46.5 and 28.3% in Chemlali and Zalmati, respectively, in comparison to the control.

The study of PSII photochemistry in the dark-adapted leaves showed that there was no significant difference in the maximal quantum yield of PS II (F_v/F_m) between control and mild to moderate water-stressed Chemlali and Zalmati plants (Table 1). At severe water stress (40–60 days), this parameter decreased significantly as compared to control plants and this reduction was greater in Chemlali (F_v/F_m, varying between 0.83 and 0.63) than in Zalmati (F_v/F_m, varying between 0.81 and 0.71). The efficiency of excitation energy capture by open PSII reaction centres (F'_v/F'_m) shows a significant regression at moderate to severe drought stress. The values are varied between 0.62 and 0.35 for Chemlali and between 0.56 and 0.39 for Zalmati (Table 1). There was a good linear correlation between the maximal quantum yield of PSII (F_v/F_m) and leaf water potential ($r^2 = 0.80$, $P < 0.0001$ in Chemlali; $r^2 = 0.66$, $P < 0.0001$ in Zalmati; Fig. 3a), and between the efficiency of excitation energy capture by open PSII reaction centres (F'_v/F'_m) and leaf water potential ($r^2 = 0.97$, $P < 0.0001$ in Chemlali; $r^2 = 0.84$, $P < 0.0001$ in Zalmati; Fig. 3b). On the other hand, the maximal quantum yield of PSII (F_v/F_m) was highly associated with the photosynthetic rate of both cultivar leaves ($r^2 = 0.79$, $P < 0.0001$ in Chemlali; $r^2 = 0.77$, $P < 0.0001$ in Zalmati; Fig. 5a). The efficiency of PSII (F'_v/F'_m) was more correlated with P_n ($r^2 = 0.95$, $P < 0.0001$ in Chemlali; $r^2 = 0.92$, $P = 0.001$ in Zalmati; Fig. 5b) in both cultivars.

Leaf anatomy and characteristics

In leaf paradermal sections of Chemlali and Zalmati cultivars (Table 3) serially cut from the upper to the lower epidermis, the entire leaf lamina, the upper epidermis, the palisade and spongy mesophyll of the stressed leaves appeared to have undergone an increase in thickness.

Table 1 Effects of water stress (WS) periods on net CO_2 assimilation rate (P_n), stomatal conductance (g_s), transpiration (E), internal CO_2 concentration (C_i), and intrinsic water-use efficiency (WUE) in leaves of Chemlali and Zalmati cultivars

Species	WS (days)	P_n (μmol m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol CO_2/mmol H_2O)	F_v/F_m	F'_v/F'_m
Chemlali	0	21.1 ± 0.32a[a]	595 ± 18.1a	10.3 ± 0.15a	320 ± 4.1a	0.035 ± 0.002c	0.83 ± 0.01a	0.62 ± 0.01a
	5	20.5 ± 0.45a	511 ± 20.3b	9.7 ± 0.17b	311 ± 4.5b	0.040 ± 0.002c	0.83 ± 0.01a	0.62 ± 0.01a
	10	18.3 ± 0.41b	434 ± 15.1c	7.8 ± 0.12c	294 ± 3.4c	0.042 ± 0.003c	0.83 ± 0.01a	0.60 ± 0.01b
	20	14.5 ± 0.32c	259 ± 16.9d	5.4 ± 0.15d	290 ± 3.7d	0.055 ± 0.003c	0.83 ± 0.01a	0.56 ± 0.01c
	40	8.2 ± 0.27d	94 ± 11.4e	3.2 ± 0.11e	287 ± 3.1e	0.087 ± 0.011b	0.76 ± 0.01b	0.46 ± 0.01d
	60	3.4 ± 0.15e	12 ± 2.1f	1.5 ± 0.07f	284 ± 13.6f	0.282 ± 0.052a	0.63 ± 0.01c	0.35 ± 0.01e
Zalmati	0	19.2 ± 0.45a	430 ± 7.5a	8.8 ± 0.13a	346 ± 8.2a	0.044 ± 0.002c	0.81 ± 0.01a	0.56 ± 0.01a
	5	19.1 ± 0.39a	404 ± 6.2b	8.7 ± 0.17a	341 ± 7.1b	0.047 ± 0.003c	0.81 ± 0.01a	0.56 ± 0.01a
	10	18.4 ± 0.50a	334 ± 6.4c	8.2 ± 0.16b	342 ± 8.1b	0.055 ± 0.003c	0.81 ± 0.01a	0.56 ± 0.01a
	20	15.1 ± 0.37b	259 ± 5.2d	4.9 ± 0.14c	314 ± 6.5c	0.058 ± 0.003c	0.81 ± 0.01a	0.52 ± 0.01b
	40	11.3 ± 0.34c	105 ± 3.5e	3.8 ± 0.11d	305 ± 6.8d	0.108 ± 0.015b	0.79 ± 0.01b	0.49 ± 0.01c
	60	6.7 ± 0.26d	25 ± 1.3f	1.7 ± 0.12e	307 ± 6.3d	0.266 ± 0.032a	0.71 ± 0.01c	0.39 ± 0.01d
Analysis of variance (F-values)								
Water stress (WS)								
Chemlali		288.8***	3299.5***	1034.3***	412.3***	99.5***	7311.8***	9907.5***
Zalmalti		437.2***	2309.9***	2261.1***	786.5***	292.2***	712.1***	450.3***

Values in each column with the same letter are not significantly different ($P = 0.05$) as described by to LSD test

The probabilities are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and not significant (ns)

[a] Data are mean values ± SE of five measurements

The increase in thickness was determined to be 47.8 and 27.8% for the entire leaf lamina, 19.1 and 20.1% for the upper epidermis, 57.4 and 30.8% for the palisade mesophyll and 44.2 and 27.2% for the spongy mesophyll, respectively, in Chemlali and Zalmati cultivars (Table 3). Zalmati leaves showed a thicker palisade mesophyll (Table 3). In addition, the olive plants grown under water-stressed conditions exhibited a significant increase in the palisade/spongy mesophyll ratio and the Zalmati leaves had the higher values. Analogous were the results as concerns the stomatal and trichome densities of the olive cultivars. In this case, drought stress also resulted in an increase of the number of stomata and trichomes in both varieties. In Zalmati, stomata and trichomes became more numerous per mm^2 of leaf surface by 37.1 and 42.1%, respectively. In comparison, the increase in these two parameters was 34.9 and 35.2% for the Chemlali cultivar (Table 3). In contrast, Zalmati leaves showed a thinner lower epidermis under water stress (WS) conditions. For Chemlali leaves, lower epidermis thickness was not significantly influenced by water availability. In both varieties, leaves developed under water stress conditions had a lower SLA (38.2 and 29.4%, respectively, in Chemlali and Zalmati) and NAR (48.8 and 34%, respectively. in Chemlali and Zalmati) than leaves produced under well-watered conditions (Table 3) associated with an increase in D (35.6 and 33.5%, respectively, in Chemlali and Zalmati).

Discussion

Drought stress reduced growth activity of the two olive cultivars (Fig. 1a; Tables 3), as is observed by other plants species (Ramesh 1999; Liu and Stützel 2004; Degu et al. 2008). Generally, when water is not restricting growth, plants invest a considerable fraction of photoassimilates in the expansion of photosynthetic tissues, maximising light interception and, as a consequence, growth (Dale 1988). In addition, the maintenance of RGR during drought can be considered a general measure of drought tolerance (Tschaplinski et al. 2006). In both the olive cultivars, the reduced relative growth rate was associated with decreases of specific leaf area (SLA) and net assimilation rate (NAR) (Table 3). Thus, the decrease in leaf area per unit leaf mass (SLA) by producing leaves of high tissue density, provided less surface area and therefore lower light harvesting capability per unit of investment in leaf mass (Morse et al. 2002). Similar results were reported in *Ficus benjamina*, *Vigna unguiculata* and three olive cultivars (Cobrançosa, Madural and Verdeal Transmontana) (Veneklaas et al. 2002; Anyia and Herzog 2004; Bacelar et al. 2006). The decrease in NAR under drought conditions suggests that water availability affects net photosynthetic rate. Thus, high-density leaf tissue leads to a decrease in the fractional volume of intercellular spaces and tends to decrease the

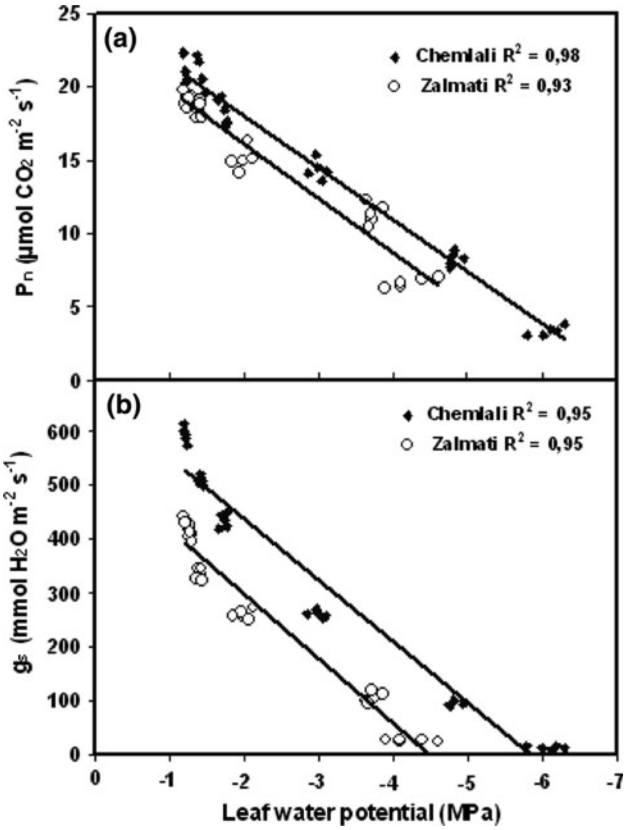

Fig. 2 **a** Relationships between leaf water potential (Ψ_{LW}) and net CO_2 assimilation rate (P_n), and **b** stomatal conductance (g_s) at Chemlali and Zalmati olive cultivars cultivated under increasing drought stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

diffusion component of CO_2 conductance (Chartzoulakis et al. 2000; Mediavilla et al. 2001).

Table 2 Effects of water stress periods on photosynthetic pigment contents in leaves of Chemlali and Zalmati cultivars

Cultivars	WS (days)	Photosynthetic pigments (mg g^{-1} FW)	
		Chl *a*+*b*	Carotenoids
Chemleli	0	14.2 ± 0.15a[a]	2.34 ± 0.09a
	5	14.1 ± 0.17a	2.32 ± 0.07a
	10	12.9 ± 0.14b	2.29 ± 0.08b
	20	11.3 ± 0.18c	2.01 ± 0.09c
	40	8.1 ± 0.15d	1.59 ± 0.06d
	60	6.2 ± 0.09e	1.25 ± 0.07e
Zalmati	0	13.1 ± 0.18ab	2.15 ± 0.08a
	5	13.2 ± 0.21a	2.13 ± 0.07a
	10	12.9 ± 0.25b	2.16 ± 0.06a
	20	11.7 ± 0.22c	2.07 ± 0.09b
	40	9.8 ± 0.19d	1.75 ± 0.06c
	60	7.9 ± 0.17e	1.54 ± 0.07d
Analysis of variance (*F*-values)			
Water stress (WS)			
Chemleli		4738.6***	2781.4***
Zalmalti		738.6***	565.1***

Values in each column with the same letter are not significantly different ($P = 0.05$) as described by to LSD test

The probabilities are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and not significant (ns)

[a] Data are mean values ± SE of five measurements

Since leaves are the main organs of internal water removal, water-stressed olive plants undertake leaf anatomical alterations in order to save water. In fact, Zalmati leaves showed a thicker entire leaf lamina, upper epidermis and palisade mesophyll than Chemlali leaves. A thicker upper epidermis (including upper cuticle) and a thicker palisade parenchyma in this cultivar may enhance survival and growth under water stress (WS) conditions by improving water relations and providing higher protection for the inner tissues (Bacelar et al. 2004). In order for olive leaves to cope with water stress, the size of the epidermal and mesophyll cells was reduced. According to Oertli et al. (1990), the small size of the epidermal cells contributes to a significant resistance against cell collapsing due to arid conditions. Another remarkable leaf anatomical feature observed in both olive cultivars grown under water stress was a significant increase in the number of the epidermal and mesophyll cells (data not shown). Bosabalidis and Kofidis (2002) showed that the increase in the number of epidermal cells per leaf surface unit reflects a better control of water loss through cuticular transpiration. An important role associated with the survival of the plants grown under drought conditions is played by the leaf stomata. In the present study, the stomatal density increased significantly with water stress and Zalmati cultivar indicates the highest percentage of promotion. Bosabalidis and Kofidis (2002) showed that the rise of the density of stomata contributes to a better control of transpiration. On the other hand, leaves developed under water deficit have a higher trichome density than leaves produced under WW conditions. This feature is thought to increase water use efficiency by increasing leaf boundary layer resistance (Savé et al. 2000) and decreasing transpirational water loss (Baldini et al. 1997). Bongi and Palliotti (1994) observed that trichomes are a barrier to the diffusion of CO_2 and H_2O, and that they lower the boundary layer conductance in the air surrounding the stomata. From this conclusion we can say that Zalmati is more protected against water loss than Chemlali, as indicated by the highest trichome density.

In our study, the reduction in SLA under WS conditions was associated with an increase in D (Table 3). Among cultivars, Zalmati had the lowest SLA and the highest D (Table 3). According to Witkowski and Lamont (1991), variations in leaf density, manifested as variations in the dry mass to fresh mass ratio, may be the result of

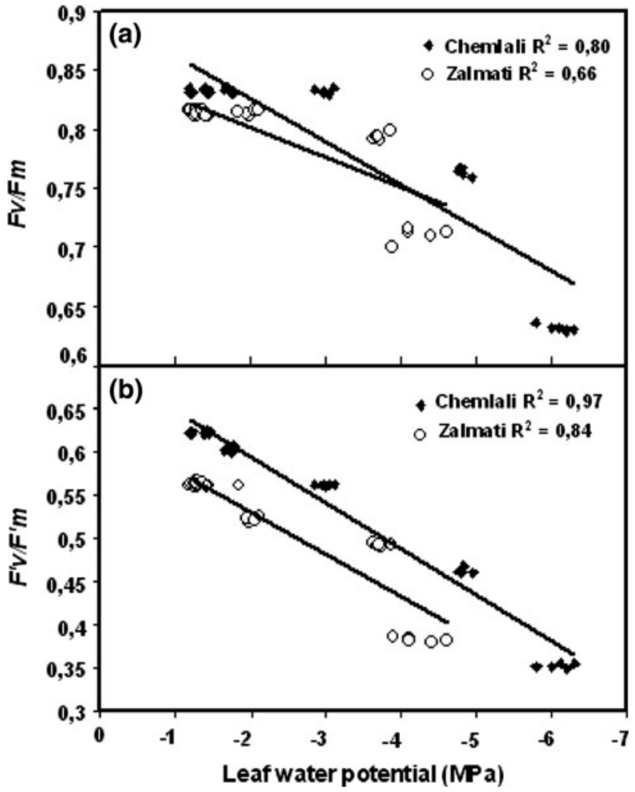

Fig. 3 **a** Relationships between leaf water potential (Ψ_{LW}) and the maximum efficiency of PSII photochemistry (F_v/F_m), and **b** the efficiency of excitation capture by open PSII reaction centres (F'_v/F'_m) at Chemlali and Zalmati olive cultivars cultivated under increasing drought stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

differences in thickness and density of the cuticle and cell walls, inclusions in the cells (starch grains, crystals), and the abundance of air spaces, sclereids, fibre groups and vascular bundles. Leaves with a high D are better able to survive a severe drought because of a higher resistance to physical damage by desiccation (Mediavilla et al. 2001). Leaves with a high D are also more stable mechanically than leaves with low D and this may be the fundamental cause of their longer life span (Niinemets 2001). The highest D in Zalmati plants corroborate the anatomical study, which revealed a thinner spongy parenchyma for this cultivar, and is probably related to the small volume of mesophyll intercellular spaces, presenting another anatomical strategies developed by the olive leaves in order to reduce transpiration.

Changes in photosynthetic activity may be an important step for enhancing olive cultivation, particularly in arid regions suffering from limited water resources. Reductions in photosynthetic performance under water stress have also been observed by Tognetti et al. (2005), Bacelar et al. (2006) and Ben Ahmed et al. (2009). In this study, net photosynthetic rate (P_n) of Chemlali and Zalmati plants was unaffected by mild water stress (up to 5 and 10 days of water stress, respectively). At moderate and higher water stress conditions we observed significant reduction in P_n. In addition, as drought stress intensified, stomatal conductance (g_s) and transpiration rate (E) decreased in both cultivars. Following the drought stress, Zalmati showed a lower reduction in these photosynthetic parameters (P_n, g_s and E) in response to water deficit when compared with

Table 3 Anatomical variables and properties of the two olive cultivars under two water regimes (irrigated and stressed)

Parameters	Chemlali		Zalmati	
	Irrigated	Stressed	Irrigated	Stressed
UE (µm)	33.4 ± 2.5d[a]	39.8 ± 1.2b	35.2 ± 0.9c	42.3 ± 1.2a
PM (µm)	324.6 ± 21d	510.7 ± 25b	409.1 ± 34c	535 ± 38a
SM(µm)	285 ± 14d	411.6 ± 19a	312 ± 23c	397 ± 25b
PM/SM (%)	113.6 ± d	124.2 ± c	131.5 ± b	134.1 ± a
LE (µm)	25.1 ± 1.9a	24.8 ± 1.0a	23.1 ± 1.5b	21.8 ± 1.7c
LT (µm)	667.8 ± 47d	986.7 ± 59b	779.7 ± 51c	996.1 ± 48a
Trichomes density (number mm^{-2})	105 ± 7d	142 ± 12b	123 ± 8.5c	175 ± 15a
Stomatal density (number mm^{-2})	375 ± 31d	506 ± 28b	432 ± 41c	594 ± 57a
SLA (10^{-2} m^2 g^{-1})	4.71 ± 0.9a	2.91 ± 0.6c	3.94 ± 1.1b	2.78 ± 0.7d
NAR (g m^{-2} day^{-1})	5.10 ± 0.9b	2.61 ± 0.9d	5.32 ± 0.9a	3.51 ± 0.9c
D (g kg^{-1})	451 ± 3.1d	612 ± 6.4b	483 ± 5.2c	645 ± 7.1a

UE upper epidermis, *LE* lower epidermis, *PM* palisade mesophyll, *SM* spongy mesophyll, *PM/SM* palisade/spongy mesophyll ratio, L_T leaf thickness, trichomes density, stomatal density, *SLA* specific leaf area, *NAR* net assimilate rate, D leaf tissue density

[a] Data are means values ± SE of ten measurements. Values in each line with the same letter are not significantly different (P = 0.05) as described by to LSD test

Chemlali. Several experiments have demonstrated some level of genetic variation in gas exchange responses to water stress between olive cultivars (Chartzoulakis et al. 1999; Tognetti et al. 2002). According to Bongi and Palliotti (1994), our results confirm the great sensitivity of leaf photosynthesis to drought. The intrinsic water use efficiency (WUE) increased significantly at severe water stress in both cultivars tested. In our experiments, chlorophyll (*a*+*b*) and carotenoid contents remained unaffected up to 5 and 10 days of WS in Chemlali and Zalmati, respectively then decreased at higher drought levels (Table 2). Despite these photosynthetic pigments in Chemlali, this cultivar showed a greater reduction of this parameter under water stress compared to Zalmati. This decrease in chlorophyll at decreasing leaf water potentials can be attributed to the sensitivity of this pigment to increasing drought stress. Similar results were reported for chlorophyll (*a*+*b*) and carotenoid contents of three olive cultivars (Cobrançosa, Madural and Verdeal Transmontana) (Bacelar et al. 2006).

The water status of the olive trees has been put in evidence by leaf water potential (Ψ_{LW}) and leaf relative water content (LWC) parameters, so the LWC rate decreases significantly for the Chemlali cultivar from the mild stress (10 days of WS); while for Zalmati, the effect of the drought stress appeared during the moderate and severe stress. On the other hand, the comparison of the leaf water status between the two cultivars showed that leaf water potential of Chemlali (−6.2 MPa) decreased more under water deficit than did Zalmati. Olive leaves tolerate an extremely low water potential (−10 MPa) with an unimpaired capacity for rehydration (Rhizopoulou et al. 1991). A significant relationship was determined between P_n, g_s and Ψ_{LW} (Fig. 2a, b) in both cultivars, which shows that the classical response of net photosynthetic rate to decreasing leaf water potential and leaf relative water content whether induced by decreasing the water supply to the roots and thence to the leaves by soil drying. The net photosynthetic rate and stomatal conductance of higher plants leaves are known to decrease as LWC and leaf water potential decrease (Lawlor and Cornic 2002). Under water stress, a good correlation is often observed between leaf water potential and stomatal conductance (Giorio et al. 1999), and may indicate a control of g_s through a hydraulic feedback mechanism (Jones 1998). Moreover, the highly significant correlation between P_n and g_s ($r^2 = 0.95$ for Chemlali and Zalmati; Fig. 4a) suggests that stomatal diffusion was the primary factor limiting photosynthesis under water stress. Centritto et al. (2005) indicated that stomatal conductance (g_s) plays a strong control over photosynthetic assimilation (P_n) and the diffusional limitations restricting the supply of CO$_2$ to metabolism were still higher in drought-stressed olive plants than in control. Similar results have also been documented for other olive cultivars (Bacelar et al. 2006; Guerfel et al. 2009), and for other plant species, such as grapevines (Escalona et al. 1999). According to Chaves (1991), this close relationship between P_n and g_s is also a common feature of drought-adapted species.

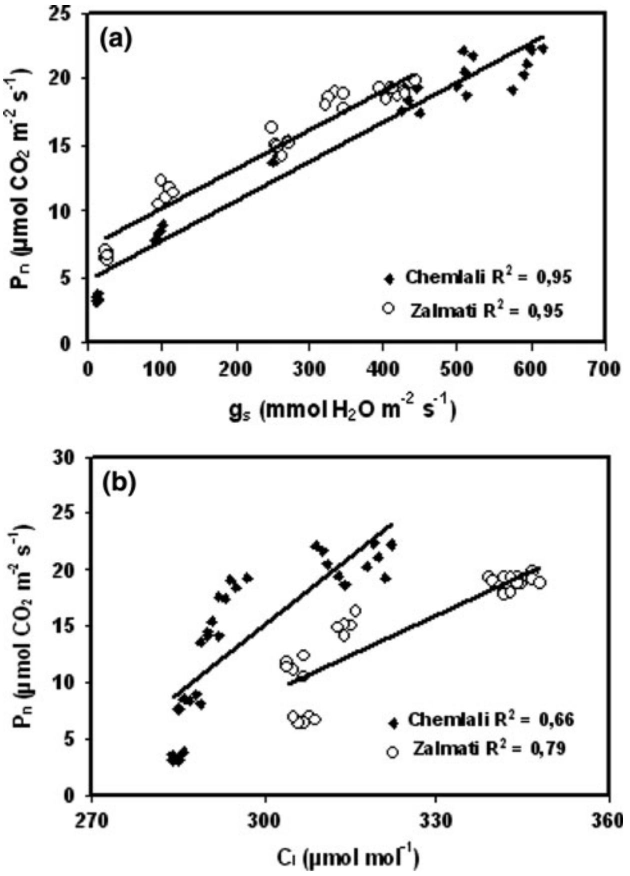

Fig. 4 **a** Relationships between net CO$_2$ assimilation rate (P_n) and stomatal conductance (g_s), and **b** internal CO$_2$ concentration (C_i) at Chemlali and Zalmati olive cultivars cultivated under increasing drought stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

For both olive cultivars tested, reductions in P_n and g_s were accompanied by reductions in C_i (Table 1). In addition, this decline in C_i was relatively higher correlated with P_n in Chemlali ($r^2 = 0.79$, Fig. 4b) than in Zalmati ($r^2 = 0.66$, Fig. 4b). This would indicate that for these olive cultivars stomatal conductance is the dominating factor which limits assimilation, irrespective of any metabolic impairment (Flexas and Medrano 2002). In contrary, the much greater reduction in g_s compared with C_i indicates that photosynthetic apparatus can play a major role in limiting photosynthesis when olive is submitted to prolonged water deficit. Bongi and Palliotti (1994) in 4-month-old olive submitted to water deficit under field conditions found a similar reduction in C_i and a much smaller reduction in g_s indicating a primary role for stomata in limiting photosynthesis. The results of this study on 2-year olive trees indicate that water deficits affect the potential rate of photosynthesis and, conversely, its strong effect on the diffusional limitations of photosynthesis may be one of the major mechanisms involved in the increase of WUE in water-stressed plants that resulted being 70% higher than that of control plants (Wahbi et al. 2005). Similarly, it has been recently shown in salt-stressed olive plants that the decrease in mesophyll conductance to CO_2 diffusion was just as rapid and reversible as for g_s, and that the low chloroplast CO_2 concentration set by both low stomatal and mesophyll conductances, i.e. the so-called diffusional limitations, were the main limitations of photosynthesis (Centritto et al. 2003; Loreto et al. 2003). On the other hand, the measurements of photosynthesis without estimating the mesophyll conductance to CO_2 can lead to significant underestimation of diffusional limitations and may give misleading indications on the response of photosynthesis to environmental stresses.

To test the functionality of the photochemical apparatus, Chemlali and Zalmati plants were treated with increasing drought stress (up to 60 days of WS). For both cultivars studied, we found that the significant changes in their maximal efficiency of PSII photochemistry (F_v/F_m) were observed only at severe water stress. In agreement with the results of Angelopoulos et al. (1996) showing that during the development of water stress a gradual decline of the ratio F_v/F_m occurred. Recently, Boussadia et al. (2008) show that F_v/F_m was reduced significantly from 0.83 in control non-stressed Koroneiki olive cultivar to 0.61 in plants submitted to severe water stress. In addition, increasing drought stress resulted in subsequent decrease in the photochemical efficiency of open PSII reaction centres (F'_v/F'_m) (Table 1). In this fact, the two ratios F_v/F_m and F'_v/F'_m decreased relatively slightly for Zalmati as compared to Chemlali in severe drought stress. Declining F_v/F_m values implies that photochemical conversion efficiency is altered and could indicate the possibility of photoinhibition (Krause and Weis 1991; Ranjbarfordoei et al. 2006). In our study, photoinhibition was more pronounced and occurred earlier in Chemlali than in Zalmati. On the other hand, these decreases in F_v/F_m and F'_v/F'_m can be ascribed as a down-regulation of PSII that reflect the protective or regulatory mechanism to avoid photodamage of photosynthetic apparatus (Demmig-Adams 1990; Demmig-Adams and Adams 1992). A considerable decrease in F'_v/F'_m could be associated with an increase in energy dissipation in the PSII antennae (Demmig-Adams et al. 1995; Demmig-Adams and Adams 1996). The data show that variations in F'_v/F'_m are more sensitive to water stress compared to changes in F_v/F_m.

A significant relationship was determined between F_v/F_m, F'_v/F'_m and Ψ_{LW} (Fig. 3a, b) in the two cultivars especially in Chemlali ($r^2 = 0.80$ and 0.97, respectively). Comparable trends were observed in several perennial plants, including olive (Demmig-Adams and Adams 1992; Lu and Zhang 1998; Kasraoui et al. 2006). Regression analyses among net photosynthetic rate (P_n) and the maximal efficiency of PSII photochemistry (F_v/F_m) on the one hand and between P_n and the efficiency of PSII (F'_v/F'_m) on the other hand (Fig. 5a, b), show that a significant correlation between these parameters was found in Chemlali ($r^2 = 0.79$ and 0.95, respectively) and in Zalmati ($r^2 = 0.77$ and 0.92, respectively), suggesting that these parameters behave in a parallel way under water stress. The highly significant relationship between P_n and F'_v/F'_m confirmed the idea that limited carbon assimilation by the decrease in stomatal conductance is viewed as an important protective mechanism under drought. This down-regulation of photosynthesis resulted from the thermal dissipation of excessive excitation energy in the chloroplast, as shown by the increase of non-photochemical quenching (Boussadia et al. 2008). The fact that leaf chlorophyll fluorescence declined simultaneously with leaf chlorophyll and

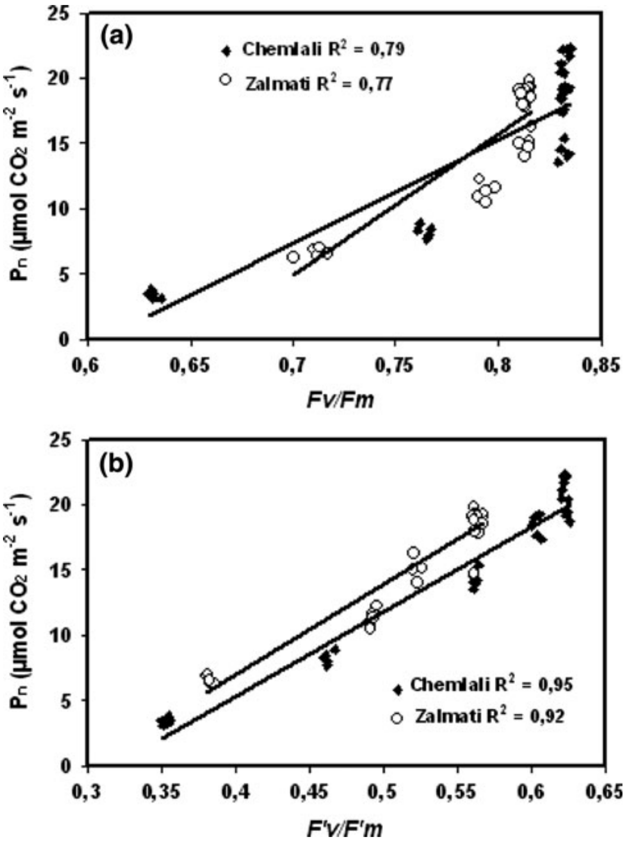

Fig. 5 **a** Relationships between dark-adapted fluorescence (F_v/F_m) and net CO_2 assimilation rate (P_n), and **b** between light-adapted fluorescence (F'_v/F'_m) and net CO_2 assimilation rate (P_n) at Chemlali and Zalmati olive cultivars cultivated under increasing drought stress. An average of 5 repetitions and confidence interval was calculated at the threshold of 95%

carotenoid content, suggests that the reduction in photosynthesis particularly at severe drought stress may be due to damages in the photosynthetic apparatus rather than to factors affecting stomatal closure. In agreement with the view that non-stomatal factors are responsible for the decrease in photosynthetic activity in olive cultivars subjected to severe drought stress, an imbalance between the photochemical activity of PSII and the electron requirement for photosynthesis is promoted leading to an overexcitation and, subsequently, photoinhibition (Epron et al. 1992; Angelopoulos et al. 1996). However, the photosynthetic apparatus in olive trees is quite resistant to mild and moderate drought stress and that stomata are the main limiting factor to carbon uptake (Kaiser 1987; Cornic et al. 1992; Boussadia et al. 2008).

In summary, we have investigated the changes in physiological and morphological parameters in two olive cultivars grown under increasing drought stress. Our results show that water stress (1) caused a significantly lower growth rate (2), affect plant water relations, as expressed by a significantly lower leaf water potential and leaf relative water content, (3) caused a significantly decrease in leaf gas exchange parameters (P_n, g_s and E), (4) induced a significant decrease in photosynthetic pigment contents (Chl a+b and carotenoids) and chlorophyll fluorescence and (5) finally, caused leaf anatomical changes, such as, (i) a significantly increase in upper epidermis and palisade mesophyll thickness, (ii) and a significantly increase in stomatal and trichome density. From the results, it can be concluded that the two olive cultivars respond to water stress by developing many morphological and structural adaptations to reduce water loss at the leaf level. The results obtained in the present study indicate that the reduction in photosynthetic rate in both olive cultivars tested was mostly linked to factors affecting stomatal closure in mild and moderate drought conditions. However, in severe drought stress this reduction may be due to damages in the photosynthetic apparatus (non-stomatal factors). Based on the behaviour of Zalmati plants, which showed a lower reduction in gas exchange parameters, photosynthetic pigment contents and chlorophyll fluorescence, a higher water use efficiency, a thicker palisade parenchyma, and a higher stomatal and trichome density, we believe that this cultivar can withstand water stress more effectively than Chemlali plants and may be promising for cultivation in arid areas like the south of Tunisia. For Chemlali cultivar, the absence of indications of drought sensitivity (shedding of leaves) under the WS regime reinforces the idea that this cultivar can be cultivated in semiarid regions, and classified as mild-risk cultivar for planting in dry areas.

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