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REGULAR PAPER

Canopy light heterogeneity drives leaf anatomical, eco-physiological, and photosynthetic changes in olive trees grown in a high-density plantation

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Abstract In the field, leaves may face very different light intensities within the tree canopy. Leaves usually respond with light-induced morphological and photosynthetic changes, in a phenomenon known as phenotypic plasticity. Canopy light distribution, leaf anatomy, gas exchange, chlorophyll fluorescence, and pigment composition were investigated in an olive (*Olea europaea*, cvs. Arbequina and Arbosana) orchard planted with a high-density system (1,250 trees ha^{-1}). Sampling was made from three canopy zones: a lower canopy (<1 m), a central one (1–2 m), and an upper one (>2 m). Light interception decreased significantly in the lower canopy when compared to the central and top ones. Leaf angle increased and photosynthetic rates and non-photochemical quenching (NPQ) decreased significantly and progressively from the upper canopy to the central and the lower canopies. The largest leaf areas were found in the lower canopy, especially in the cultivar Arbequina. The palisade and spongy parenchyma were reduced in thickness in the lower canopy when compared to the upper one, in the former due to a decrease in the number of cell layers from three to two (clearly distinguishable in the light and fluorescence microscopy images). In both cultivars, the concentration of violaxanthin-cycle pigments and β-carotene was higher in the upper than in the lower canopy. Furthermore, the de-epoxidized forms zeaxanthin and antheraxanthin increased significantly in those leaves from the upper canopy, in parallel to the NPQ increases. In conclusion, olive leaves react with morphological and photosynthetic changes to within-crown light gradients. These results strengthen the idea of olive trees as "modular organisms" that adjust the modules morphology and physiology in response to light intensity.

Keywords Canopy light distribution · High-density olive orchards · Leaf anatomy · Leaf phenotypic plasticity · Photosynthesis

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Introduction

The evergreen olive tree (*Olea europaea* L.) is one of the major and most characteristic, economically important, crops in the Mediterranean area. Several major technological changes have occurred for the olive industry during the last decades. Olive productivity has increased in the last 20 years, largely due to changes in plantation densities. The traditional rain-fed orchard with low density (<100 olive trees ha^{-1}), intensive tillage, low inputs in fertilizer and pesticides and manual harvest are being substituted by new intensive (200–400 olive trees ha^{-1}) and super high intensive (more than 1,000 olive trees ha^{-1}) drip-irrigated plantations, with reduced tillage, high inputs and mechanical harvesting (Villalobos et al. 2006). The olive orchards with high-density planting system, based on the use of a high number of olive trees (1,250–2,500 trees ha^{-1}), have been recently established and cover currently more than 80,000 ha worldwide (Agromillora Catalana 2007; Connor et al. 2014) of 10 Mha planted to olive, but there is still scarce information on the response of the cultivars (Connor et al. 2014). In these new systems, trees are trained to form hedgerows and pruning can be largely mechanized

(30–40 h ha^{-1} $year^{-1}$ with a mower and final finishing) (Proietti et al. 2012). The aims of a high-density plantation are the easy control of diseases and pests, high early yields (trees are brought into production few years after planting) and low harvest costs (2 operators and 3–4 h ha^{-1}), to maximize short-term profits (Pastor et al. 2007; Connor et al. 2012; Proietti et al. 2012). Arbequina cultivar is considered the best for a high-density planting system, although Arbosana and Koroneiki meet the requirements as well (Proietti et al. 2012; Connor et al. 2014). Design and management of canopy structure are major concerns. One of the main problems of this kind of plantation is to ensure canopy illumination while controlling tree size to allow the harvesting machine to pass over the hedgerows, requiring the latter trees lower than 2.5–3.5 m height and 1.5–2 m width (Proietti et al. 2012). This inconvenience is due to the high vigor of this species (Del Río et al. 2002), especially in areas where the growing season is very long like Tunisia and Spain (Larbi et al. 2011). Therefore, it is important to define canopy height and slope, and alley width to optimize the light interception by the entire canopy (Connor 2006; Connor et al. 2014).

Biomass production is directly related to light interception (Monteith 1977). Olive tree yield is related to the amount of photosynthetically active radiation (PAR) intercepted by the orchard (Mariscal et al. 2000; Villalobos et al. 2006). In olive, it is well established that fruits are formed around the periphery of the canopy that receives high amounts of irradiance (Acebedo et al. 2002). It has been reported by Jackson (1980) that the maintenance of an optimum distribution of irradiance on the constituent foliage is primordial for maximum fruit yield and quality. Furthermore, Connor (2006) indicated that, for maximum productivity, all olive foliage must be illuminated above threshold values for the reproductive sequence critical steps of shoot growth, floral initiation, flowering, fruit formation, and fruit filling. The increase of planting density leads to a greater light interception, mainly during the period of orchard development (Pastor et al. 2007). As the trees grow, however, their canopies occupy more space, and mutual shading may occurs (Pastor et al. 2007; Cherbiy-Hoffmann et al. 2012). Indeed, profiles of radiation incident on olive canopy walls are linear up to 2.5 m, with light interception decreasing from the top to the lower part of the canopy (Connor et al. 2012). It has also been demonstrated experimentally that flowering and fruiting processes can be depressed by shading (Tombesi et al. 1999; Proietti 2000; Gregoriou et al. 2007). Thus, yield and oil concentration increases linearly with mean daily PAR up to 40–60 % of the incident PAR (i.e., 15–25 mol PAR m^{-2} d^{-1}) (Connor et al. 2009; Cherbiy-Hoffmann et al. 2012, 2013). Below that threshold, the fruit becomes the priority sinks for photoassimilates, but olive fruit growth rate and oil concentration is reduced (Cherbiy-Hoffmann et al. 2013). PAR interception above 50 % of the incident PAR does not result in increased yields, because orchard structures generate excessive shading between and within trees (Villalobos et al. 2006).

Very little is known about the effects of shade on other olive tree characteristics (Proietti et al. 1988; Tombesi 1992; Gregoriou et al. 2007; Melgar et al. 2009). Drastic reductions of PAR (below 10 %) increase olive leaf senescence (Proietti et al. 1994). Under more reasonable low to moderate levels of PAR, the partitioning of photosynthates toward fruit growth is favored over vegetative growth (Cherbiy-Hoffmann et al. 2013). Thus, trunk cross-sectional areas and length of non-fruiting branches were markedly reduced in response to shading (Cherbiy-Hoffmann et al. 2013). With regard to leaf traits, long shading increased leaf area (both leaf width and length) and decreased leaf thickness, the latter due to the presence of only 1–2 palisade layers compared to the 3 layers of the control leaves and a reduced length of the palisade cells and spongy parenchyma (Proietti et al. 1988; Gregoriou et al. 2007). A reduction in the number of palisade layers might reduce the capacity of gathering efficiently direct light while not affecting the use of diffuse radiation (Vogelmann and Martin 1993), which can be particularly beneficial for leaves located in the lower part of the canopy in the Mediterranean area where sunlight is rarely a limiting factor. The number of grana and stroma thylakoids in olive leaves increased as shade increased, in line with increases in chlorophyll (Chl) in a fresh weight basis and no Chl changes in an area basis (Proietti et al. 1988; Gregoriou et al. 2007). Shading, on the contrary, decreased photosynthesis (Bongi et al. 1987), stomatal conductance, stomatal and trichome density and leaf mass per area (Proietti et al. 1988; Gregoriou et al. 2007). These changes in olive leaves can be permanent, particularly for those leaves that have emerged under shade (Proietti et al. 1988). When transferred to full sunlight, recovery of photosynthesis in olive trees grown under shade was only small (Gregoriou et al. 2007). A shading-induced reduced photosynthesis might decrease olive leaf sugar and starch (Gregoriou et al. 2007) concentrations and consequently reduce inflorescence bud initiation, leading to a non-fruiting year ("off year"). Olive, by nature, is a strongly alternate bearing tree (Proietti 2000).

Acclimation to changing light conditions is achieved through adjustments at different levels, but leaf adjustments, as main organs for gas exchange, play a key role (Givnish 1988). Due to their phenotypic plasticity, leaves have a marked capacity to adjust morphology and physiology in response to different light conditions (Poorter 1999; Yoshimura 2010). To the best of our knowledge, canopy light distribution in olive trees grown in super high intensive-density planting system and its effects is scarcely investigated. In olive tree production, detailed information

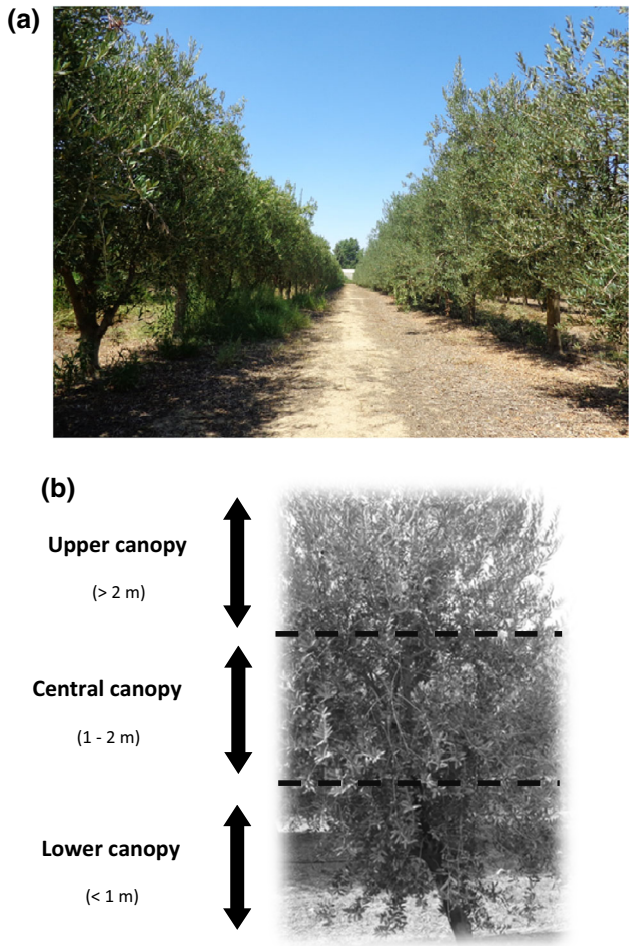


Fig. 1 Orchard under investigation showing the Arbequina (*right*) and Arbosana (*left*) olive tree rows (**a**) and sampling procedure (**b**). Leaves were sampled from three olive canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower one (<1 m)

on the effects of various irradiances on leaf traits and photosynthesis is essential to guide orchard management. The main aim of the present work was to study the effect of a high density plantation frame on canopy light distribution, leaf eco-physiological and anatomical changes, and photosynthesis in an olive (Arbequina and Arbosana cultivars) orchard grown in a Mediterranean area with special emphasis on the possibility of shading effects in the central and lower parts of the canopy when adult trees reach certain height (>2 m).

Materials and methods

Plant material

The experiment was carried out in a high-density olive orchard located in Mornag (northern-east of Tunisia, 36.7°N) with an average rainfall of 450 mm $year^{-1}$. Average mean, maximum, and minimum temperatures were 17.5, 24.2, and 13.9 °C, respectively. Orchard trees planting density was 1,250 trees ha^{-1} and spacing was 2 m between trees and 4 m between rows (alley width). Olive trees of the cultivars 'Arbequina i-18' and 'Arbosana' were planted in alternate rows (i.e., rows in the orchard were Arbequina, Arbosana, Arbequina, Arbosana, etc.; Fig. 1a) with a North–South orientation in 2000 (rows of olive trees in the orchard follow North–South orientation). In 2009 during measurements, tree height averaged 3.9 and 3.6 m for Arbequina and Arbosana, respectively. The soil has a clay-loamy texture, with 28 % total calcium carbonate, 11.2 % active lime, 1.6 % organic matter, pH in water 8.0 and low permeability. Soil surface management was no-till, with weed control performed using herbicides.

In this orchard, the experimental design for olive tree yield purposes was a 4 randomized complete blocks with 5 trees each one for each cultivar. In the case of experiments presented in this report, 3 trees per cultivar and 2 blocks were considered. Each tree was divided into three heights: the lower one (<1 m), the central one (1–2 m) and the upper one (>2 m) (Fig. 1b). On each height, four (quadrants North, East, West and South) and three (canopy interior; 15–20 cm from the trunk, taking into account a canopy of 1–1.2 m diameter, i.e., 50–60 cm of canopy at each side of the trunk) branches were selected and labeled in March 2009. In total, 42 twigs on each height per cultivar were observed during the reproductive season until fruit set stage. Experiments lasted from February to October 2009. The same type of leaves was used in all parameters measured. They were young, fully expanded leaves 3–6 month old (avoiding sampling at the tip and the base of the branch, which are younger and older, respectively).

Light interception

Intercepted PAR by each height of the canopy was estimated using a Skye (Powys, UK) PAR meter. Measurements were taken on each marked shoot each 15 days from March to October 2009. It must be pointed out that measurements were taken under clear and sunny days. To determine the averaged intercepted PAR on each height, three readings were taken with the sensor placed horizontally at the base, the middle and at the tip of each marked branch and the average was taken as the intercepted PAR value. Readings were taken randomly between 9 and 12 h solar time. Also, these PAR measurements and those made during the gas exchange and Chl fluorescence measurements were plotted in light distribution histograms with increments of 125 or 167 (depending on the number of measurements that fell in a given category) from 0 to 2,500 μmol m^{-2} s^{-1} PAR.

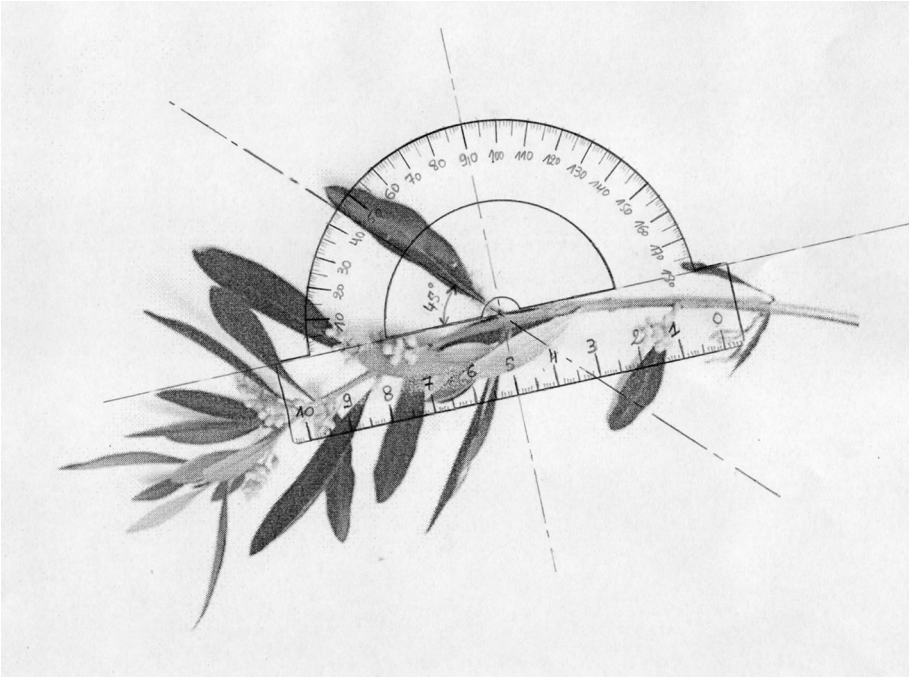


Fig. 2 Example on how measurements of leaf angle were made. It was determined manually using an angle-hook by measuring the insertion angle of leaves on marked branches at each canopy height. Since branches in the lower and upper canopies grow rather horizontally and vertically to the soil, respectively, 0° (and 180°) correspond to leaves growing parallel to the soil in the lower canopy but vertical to the soil in the upper one, whereas 90° corresponds to leaves that grow vertically and horizontally to the soil in the lower and upper canopies, respectively. Measurements (n = 120–126) were made between 8 and 10 h solar time

Leaf area

During the experiment, leaf area was determined using the equation established by Tattini et al. (1995). Completely developed leaves were sampled from the middle part of the marked branch at each height. Length and width of 60 leaves (10 leaves × 3 trees × 2 blocks) were measured. The area of each leaf was calculated according to the following regression equation: $y = 0.735x + 0.125$ ($R^2 = 0.987$), where y is leaf area and x is the product length × width.

Leaf angle

Leaf angle was determined manually using an angle-hook by measuring the insertion angle of leaves on marked branches at each canopy height. Figure 2 illustrates how angle measurements were made. In these measurements, the angle refers to the position of the leaf with respect to the branch. Since branches in the lower and upper canopies grow rather horizontally and vertically to the soil, respectively, 0° (and 180°) correspond to leaves growing parallel to the soil in the lower canopy but vertical to the soil in the upper one, whereas 90° corresponds to leaves that grow vertically and horizontally to the soil in the lower and upper canopies respectively. Measurements (n = 120–126) were made between 8 and 10 h solar time.

Leaf morphology and surface characteristics

Leaf surfaces by scanning electron microscopy (SEM)

Leaf surfaces were observed in a Hitachi S-3400N microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) using secondary and back-scattered electrons (SEM–BSE). Secondary electron images (1,280 × 960 pixels) were obtained at 15 kV, with a beam current from 6.9 to 7.5 nA. In order to properly observe the leaf surface characteristics including stomatal parameters, leaf trichomes from the lower, abaxial face were removed with adhesive tape (sticking and gentle pull).

Light microscopy

Representative areas (25 mm^2) from the middle region of the leaflet blades adjacent to main veins were embedded in 5 % agar and sectioned 50-mm thick with a microslicer (Leica VT 1000S). Transversal sections were placed on microscope slides and images (2,592 × 1,994 pixels) were taken on an inverted microscope (Leica DMIL LED) equipped with a CCD camera (Leica).

Fluorescence microscopy

Fresh leaf pieces were submerged in ethanol with the aim of removing as much as possible ethanol-extractable compounds, such as Chl. Fluorescence images of leaf transversal slices (2,592 × 1,994 pixels; x- and y-axis resolution of 360 × 270 μm) were taken with an inverted microscope (DM IL LED, Leica Microsystems GmbH) equipped with a fluorescence kit (340–380 nm excitation wavelength and 425 nm cut-off filter; A1 filter cube, Leica Microsystems GmbH) and a CCD camera (DFC 240C, Leica Microsystems GmbH). Without the interference of

the UV-induced red fluorescence of Chl, the blue-green fluorescence of the cell wall-bound hydroxycinnamic acid derivatives is magnified (Morales et al. 1996; Cerovic et al. 1999), allowing for an easier visualization of the different cell layers across the leaves.

Image data analysis

Thickness of total sections, including different plant tissues (adaxial epidermis, palisade parenchyma, spongy parenchyma, and abaxial epidermis), was obtained by image analysis (Photoshop CS3 software) of light and fluorescence microscopy images, whereas SEM images were used to obtain stomata data (pore length and stomatal density). Five measurements [mean values ± standard error (SE)] from 4 images of 4 different leaves ($n = 20$) were carried out. Differences among treatments in olive leaves were analyzed using one-way ANOVA analyses, followed by a post hoc multiple comparison of means using the Duncan's test ($P < 0.05$). SPSS 15.0 statistical software was used to conduct these analyses.

Gas exchange parameters

Leaf gas exchange measurements were performed on fully expanded leaves using a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA). Measurements (30 replications per height; 5 replications × 3 trees × 2 blocks) took place between 9 and 13 h solar time. The replications were measured randomly, in order to cover as such as possible the daily variability. Measurements were done on cloudless, sunny days. The photosynthetic active radiation (1,500 μmol m^{-2} s^{-1} PAR) was supplied with a 6400-02B light-emitting-diode (LED) light source. The temperature, CO_2 concentration, and relative humidity (RH) inside the leaf cuvette were set to 25 °C, 400 μmol mol^{-1}, and 60 ± 1 %, respectively.

Modulated chlorophyll fluorescence analyses

Modulated Chl fluorescence measurements were made in attached leaves with a pulse–amplitude modulation PAM 2100 portable fluorometer (H. Walz, Effeltrich, Germany). Only developed leaves were used in these experiments. 30 leaves per height and variety (5 replications (4 quadrants and interior) × 3 trees × 2 blocks) were used to determine Chl fluorescence. Measurements were taken randomly between 9 and 12 h solar time on sunny, cloudless days and using the incident sunlight reaching the leaves after crossing the tree canopy as actinic illumination. F_O was measured after 30 min of darkness by switching on the modulated light at 0.6 kHz; PPFD was below 0.1 μmol m^{-2} s^{-1} at the leaf surface. F_M was measured at 20 kHz with 1 s pulse of 6,000 μmol photons m^{-2} s^{-1} of white light. The experimental protocol for the analysis of the Chl fluorescence quenching was essentially as described by Genty et al. (1989) with some modifications. These ones involved the measurements of F_O and F'_O, which were measured in presence of far-red light (7 μmol photons m^{-2} s^{-1}) in order to fully oxidize the photosystem II (PSII) acceptor side (Belkhodja et al. 1998). The dark adapted, maximum potential PSII efficiency was calculated as F_V/F_M (Morales et al. 1991). The actual (Φ_{PSII}) and the intrinsic ($\Phi_{exc.}$) PSII efficiency was calculated as $(F'_M - F_S)/F'_M$ and F'_V/F'_M, respectively (Genty et al. 1989; Harbinson et al. 1989). Photochemical quenching (qP) was calculated as $(F'_M - F_S)/F'_V$ according to van Kooten and Snel (1990). Non-photochemical quenching (NPQ) was calculated as $(F_M/F'_M) - 1$, according to Bilger and Björkman (1990). The fraction of light absorbed by PSII that is dissipated in the antenna (D, identical to $1 - \Phi_{exc.}$) was estimated as in Demmig-Adams et al. (1996).

Photosynthetic pigment composition

Leaf disks were taken from the same area of the leaves in which gas exchange and modulated Chl fluorescence were measured. Disks (1 cm diameter; 0.785 cm^2 area) were cut with a calibrated cork borer, wrapped in aluminum foil, immediately frozen and stored at −20 °C. Leaf pigments were later extracted with acetone in the presence of Na ascorbate. Pigments extracts were thawed on ice, filtered through a 0.45 μm filter and analyzed by an isocratic high performance liquid chromatography (HPLC) method (Larbi et al. 2004). Three samples per tree were taken from each height, every sample composed of three disks, each one from a different leaf.

Statistical analyses

Statistical analyses were carried out with the SPSS Base 8.0 software, SPSS 15.0 in the case of image data analyses (Chicago, IL, USA). One factor ANOVA was run to assess if changes observed within different positions in the olive trees were statistically significant.

Results

Canopy light interception

The average light interception from March to October decreased significantly from the upper canopy to the central and lower ones in both cultivars (Fig. 3). Indeed, from the upper part of the canopy to the central and lower one, PAR decreased by 16–33 and 36–52 % for Arbequina and Arbosana cultivars, respectively (Fig. 3). In Arbequina, the light

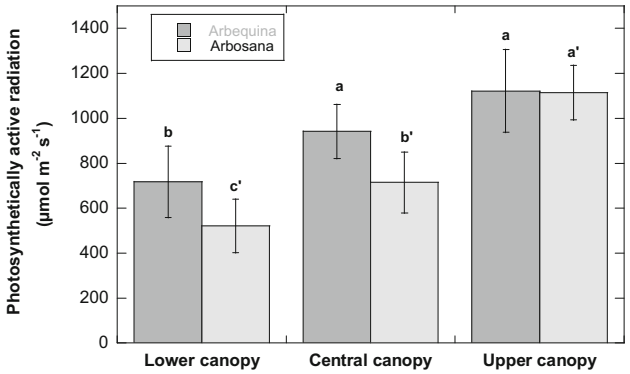


Fig. 3 Photosynthetically active radiation (PAR) measured in the three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower part (<1 m) in the Arbequina (*dark gray bars*) and Arbosana (*light gray bars*) cultivars. Three readings were taken with the sensor placed horizontally at the base, the middle and the tip of each marked branch each 15 days from March to October 2009, measuring at the North, East, West, and South (one branch each) and at the canopy interior (15–20 cm from the trunk, taking into account a canopy of 1–1.2 m diameter, i.e., 50–60 cm of canopy at each side of the trunk) (three branches). Data are mean ± SE ($n = 15$). *Different letters* between zones indicate significant differences ($P < 0.05$)

intensities monitored during the gas exchange and Chl fluorescence measurements, ranged from 36 to 2,051 (lower canopy), from 45 to 2,161 (central canopy) and from 117 to 2,201 (upper canopy) µmol m^{-2} s^{-1} PAR. In Arbosana, they ranged from 25 to 2,210 (lower canopy), from 53 to 2,371 (central canopy) and from 130 to 2,800 (upper canopy) µmol m^{-2} s^{-1} PAR. These values are the minimum and maximum light intensities found in the different heights of the canopy during the above-mentioned measurements, but the PAR environment is better represented by the light distributions (Fig. 4). Light followed a Gaussian distribution (upper canopies of both Arbequina and Arbosana cultivars; Fig. 4c, f) but the central and lower canopies did not (Fig. 4a, b, d, e). A generally shaded environment sometimes interrupted by strong sunflecks could explain the bimodal light distribution, particularly found in the lower and central canopies of both cultivars (Fig. 4a, b, e) with the exception of the Arbosana lower canopy (Fig. 4d).

Leaf angle

Leaves in the top of the trees (upper canopy) were close to the branches (parallel to sunlight) in comparison to leaves of the branches from the lower canopy, where leaves were more perpendicular to sunlight in order to intercept the maximum possible radiation (Fig. 5a). Indeed, leaf angle increased significantly from 22° in the upper canopy to 70–80° in the central and 110–165° in the lower canopy in the Arbequina and Arbosana cultivars (Fig. 5a). It should be reminded here that branches in the lower and upper canopies grow rather horizontally and vertically to the soil, respectively. Therefore, as previously mentioned, an angle close to 0° corresponds to leaves growing parallel to the soil in the lower canopy but vertical to the soil in the upper one, whereas 90° corresponds to leaves that grow vertically and horizontally to the soil in the lower and upper canopies, respectively.

Leaf area

Leaf area was significantly higher in the lower canopy of Arbequina cultivar (5.76 cm^2), when compared to the rest of the sampled leaves (ca. 4.73 cm^2) (Fig. 5b). In Arbosana, leaf area sampled from the lower and central canopy was 6 and 9 % higher than in the leaves sampled from the upper canopy (Fig. 5b).

Leaf tissue structure and stomatal density

Leaf anatomical characteristics of both cultivars varied significantly with their position into the canopy (Fig. 6; Table 1). Indeed, we observed differences for most of the anatomical parameters studied (e.g., leaf tissue thickness, spongy and palisade parenchyma) (Table 1). This study shows that the leaf tissue thickness decreased significantly in the lower as compared to the central and upper canopy for both cultivars (Table 1). The major changes were observed in the palisade and spongy parenchyma in both cultivars. In both cultivars, palisade parenchyma thickness decreased by 7–9 and 28–24 % in the central and lower canopy, respectively, when compared to the upper one (Table 1). Spongy parenchyma thickness decreased significantly (15–17 %) only in the lower in comparison to the central and upper canopy in both cultivars (Table 1). In contrast, the intercellular spaces of the spongy parenchyma increased in leaves from the central and lower canopy when compared to the upper one (Fig. 6). However, the thickness of the upper (adaxial) and lower (abaxial) epidermis and the pore length did not vary in leaves sampled from the different heights of the canopy in both cultivars (Table 1). Stomatal density was higher in Arbequina leaves sampled from the lower canopy, whereas it did not change significantly in Arbosana (Table 1).

The fluorescence properties under UV (240–380 nm) excitation and detection in the whole visible spectrum (using a 425 nm cut-off filter) of transversal leaf slices from Arbequina and Arbosana at the different canopy positions are also shown in Fig. 6. Both the mesophyll of Arbequina (Fig. 6c, i, o) and Arbosana (Fig. 6f, l, r) were dominated by a blue fluorescence emission, irrespective of canopy position (Fig. 6). The abaxial and adaxial epidermal cells emitted blue and red fluorescence, with trichomes showing orange fluorescence at the top, blue at the base and red in the insertion with the epidermis (Fig. 6). The

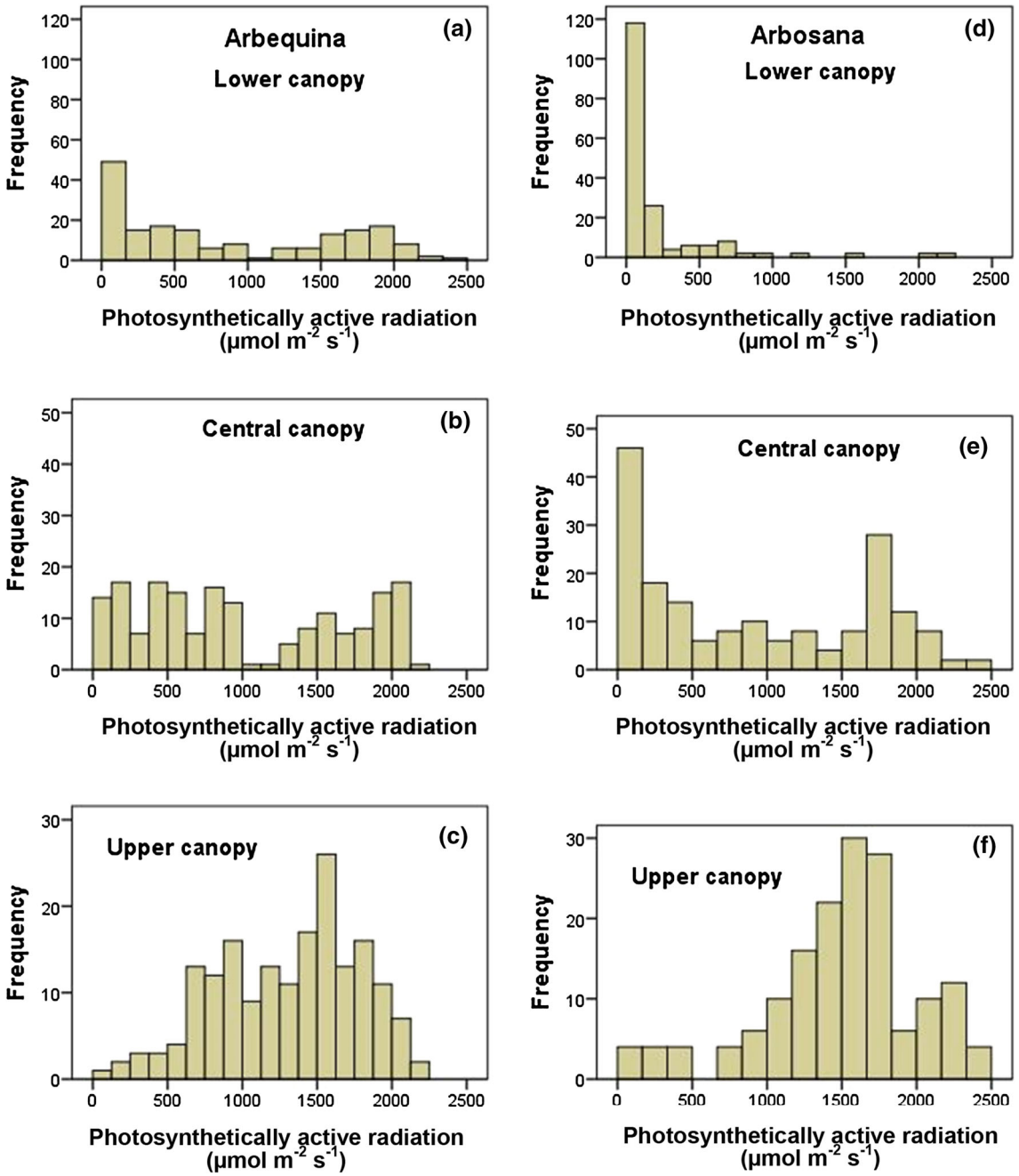


Fig. 4 Distribution of the photosynthetically active radiation (PAR) intensities found in the Arbequina (**a**–**c** $n = 178$–180) and Arbosana (**d**–**f** $n = 162$–180) in the three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower part (<1 m). Data are plotted in frequency histograms with increments of 125 or 167 from 0 to 2,500 µmol m^{-2} s^{-1} PAR (depending on the number of measurements that fell in a given category). Three readings were taken with the sensor placed horizontally at the base, the middle and the tip of each marked branch each 15 days from March to October 2009, measuring at the North, East, West and South (one branch each) and at the canopy interior (15–20 cm from the trunk, taking into account a canopy of 1–1.2 m diameter, i.e., 50–60 cm of canopy at each side of the trunk) (three branches), and those made during the gas exchange and Chl fluorescence measurements were also included

most remarkable difference between cultivars was a higher blue fluorescence emission in the mesophyll of Arbosana when compared to that of Arbequina, being such differences more clearly distinguishable in the palisade than in the spongy parenchyma (Fig. 6).

Leaf gas exchange

Both Arbequina and Arbosana cultivars had photosynthetic rates (Fig. 7a) similar to those previously reported for these cultivars (Kchaou et al. 2013), which indicate good

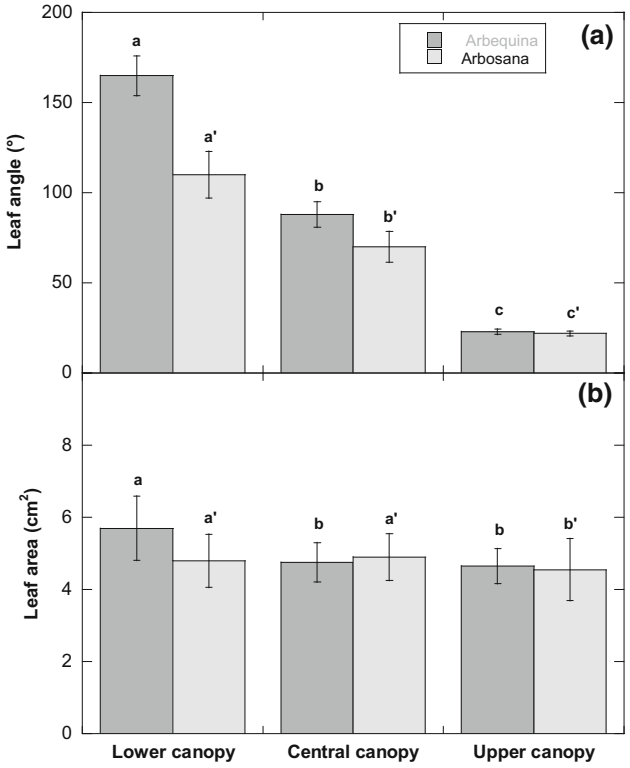


Fig. 5 Leaf angle (°) (**a**) and leaf area (cm^2) (**b**) in the three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower part (<1 m) in the Arbequina (*dark gray bars*) and Arbosana (*light gray bars*) cultivars. Data are mean ± SE ($n = 120$–126 and 60 for angle and area, respectively). *Different letters* between zones indicate significant differences ($P < 0.05$) (see legend to Fig. 2 for further details on how angle measurements were made and interpretation)

adaptation to the orchard environment. Photosynthetic rates decreased significantly and progressively from the upper to the central and the lower canopy in Arbequina and Arbosana, although the difference was not significant between the upper and the central canopy of Arbosana cultivar (Fig. 7a). Decreases were 39 and 62 (Arbequina) and 19 and 43 % (Arbosana) in the central and lower canopy when compared to the upper one, respectively (Fig. 7a). Transpiration rates and stomatal conductance decreased significantly from the upper to the central and the lower canopy in Arbequina, whereas in Arbosana stomatal conductance and transpiration rates decreased significantly only in the lower canopy when compared to the central and the upper one (Fig. 7b, c). Both transpiration rates and stomatal conductance were 50–43 and 57–63 % lower in the central and the lower canopy when compared to the upper one in Arbequina. In Arbosana, decreases were 37 and 35 % for transpiration rates and stomatal conductance, respectively (Figs. 7b, c). The lowest sub-stomatal CO_2 concentrations were monitored in the central canopy in Arbequina and in the upper canopy in Arbosana (Fig. 7d).

Chlorophyll fluorescence parameters

The F_V/F_M values were not influenced by the position of the leaves into the canopy in the two cultivars investigated (Fig. 8a). Actual (Φ_{PSII}) and intrinsic ($\Phi_{exc.}$) PSII efficiencies were significantly increased from the upper canopy to the central and lower ones in both cultivars (Fig. 8b, c). Photochemical quenching (qP) values were increased in leaves from the central and the lower canopy when compared to the upper one in both cultivars (Fig. 8d). However, NPQ values decreased significantly and progressively in leaves from the upper canopy to the central and then the lower canopy in both cultivars (Fig. 8e). It should be noted here that many of the Chl fluorescence measurements were made with an incident PAR close to the low values shown in Fig. 4.

Leaf pigment composition

Table 2 shows the leaf pigment composition in the different heights of the canopy for Arbequina and Arbosana. Leaf Chl concentration was similar in all heights of the canopy both in Arbequina and Arbosana (increases of Chl in the Arbequina upper canopy were not statistically significant). The Chl *a*/*b* ratio did not change in Arbequina, whereas in Arbosana it decreased significantly in the lower and central canopy, when compared to the upper one. The leaf concentrations of neoxanthin, violaxanthin + antheraxanthin + zeaxanthin (V + A + Z) pigments pool, β-carotene and lutein per area were higher in the upper than in the central and lower canopy in Arbequina. In Arbosana, however, only β-carotene and the V + A + Z pool per area were significantly higher in leaves from the upper when compared to the central and the lower canopy. When expressed on a Chl basis, only V + A + Z cycle pigments clearly increased significantly from the lower to the upper canopy, although the difference was not significant between the central and the lower canopy in Arbosana. The de-epoxidated forms Z and A increased significantly in leaves from the upper canopy when compared to the central and the lower ones in both cultivars.

Discussion

The results of this study contribute to understand olive canopy light distribution and the photosynthetic behavior of olive trees in high-density planting systems. Detailed studies scaling photosynthesis from the leaf to the canopy level are necessary taking into account that canopy photosynthesis is the result of combining the photosynthesis of all the leaves located at different heights within the canopy (Ceulemans and Saugier 1991).

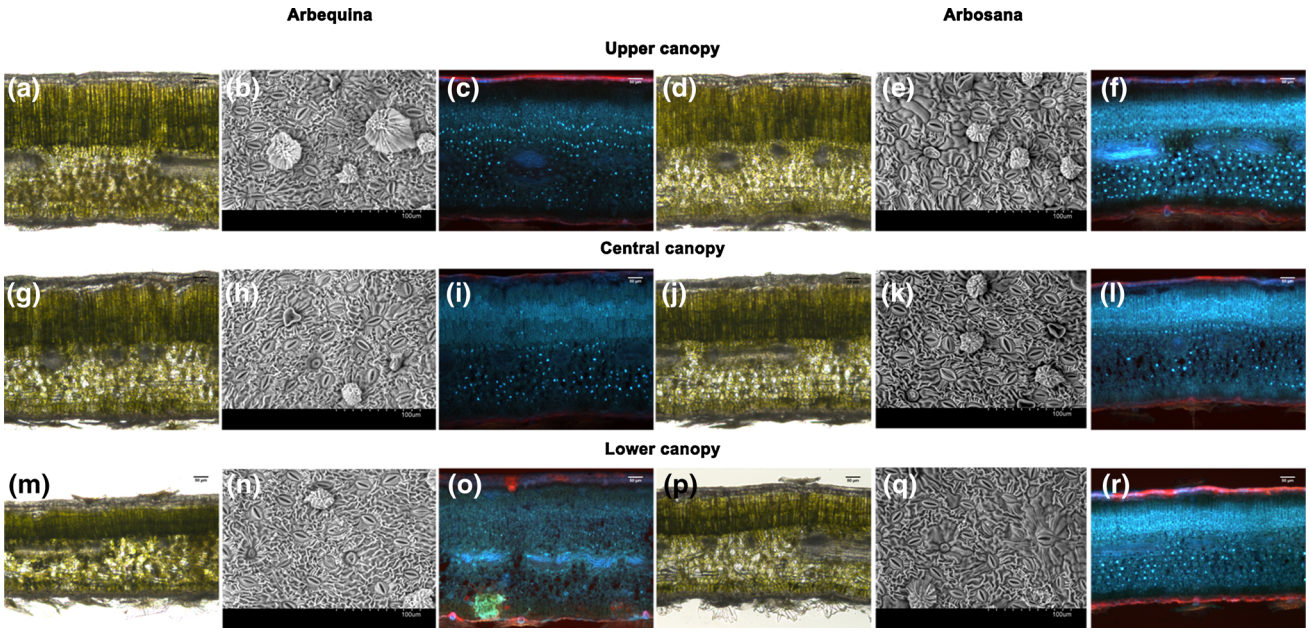


Fig. 6 Light microscopy images of leaf transversal sections (**a**, **g**, **m**, **d**, **j**, **p**), SEM images of lower, abaxial epidermises (**b**, **h**, **n**, **e**, **k**, **q**) and fluorescence microscopy images of leaf transversal sections (**c**, **i**, **o**, **f**, **l**, **r**) under UV (240–380 nm) excitation and detection in the whole visible spectrum (using a 425 nm cut-off filter). Red and blue signals are fluorescence from Chl and other unknown compound(s) (*red*) and hydroxycinnamic acid derivatives and NAD(P)H (*blue*). Most of the trichomes were removed to observe properly the leaf surface. Sampling was made at the upper (>2 m; **a**–**f**), the central (1–2 m; **g**–**l**) and the lower canopy (<1 m; **m**–**r**) in the Arbequina (**a**–**c**, **g**–**i**, **m**–**o**) and Arbosana (**d**–**f**, **j**–**l**, **p**–**r**) cultivars

Table 1 Thickness of the different leaf layers, and stomata density and size in the leaves of three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m), and a lower part (<1 m) in olive (cultivars Arbequina and Arbosana) trees ($n = 20$; five measurements from 4 images, each from a different leaf)

Leaf anatomy parameters	Arbequina			Arbosana		
	Lower canopy	Central canopy	Upper canopy	Lower canopy	Central canopy	Upper canopy
Total section (μm)	417.2 ± 19.5a	489.0 ± 3.1b	508.0 ± 3.3b	408.3 ± 7.2a	486.0 ± 7.9b	490.0 ± 3.8b
Adaxial epidermis (μm)	30.3 ± 0.9a	29.2 ± 0.6a	29.2 ± 1.0a	27.4 ± 1.1a	29.2 ± 0.8a	27.6 ± 0.9a
Palisade parenchyma (μm)	171.8 ± 12.9a	205.0 ± 1.8b	225.6 ± 4.0b	135.3 ± 3.1a	174.7 ± 5.1b	187.9 ± 4.8c
Spongy parenchyma (μm)	190.7 ± 7.4a	229.6 ± 3.1b	226.7 ± 2.7b	219.7 ± 5.7a	257.5 ± 4.6b	248.6 ± 2.7b
Abaxial epidermis (μm)	26.1 ± 0.8a	28.2 ± 0.8a	27.6 ± 0.7a	26.6 ± 0.8a	27.4 ± 1.1a	27.3 ± 0.8a
Pore length (μm)	12.2 ± 0.1a	12.6 ± 0.1bc	13.0 ± 0.2c	12.9 ± 0.1a	12.8 ± 0.2a	12.7 ± 0.2a
Stomatal density (stomata mm^{-2})	558.5 ± 11.8a	500.4 ± 11.3b	509.3 ± 19.1b	641.4 ± 18.0a	689.4 ± 15.4a	642.5 ± 21.8a

Light and fluorescence microscopy images were used to obtain thickness data, whereas stomata data were obtained from SEM images (Fig. 6). Data are mean ± SE. For each cultivar, different letters in the same row indicate significant differences ($P < 0.05$)

Optimally illuminated canopies are those that receive irradiance over a threshold value at the base of the canopy. The illumination patterns on olive canopy walls are affected by row height, wall slope and alley, and row widths (Connor 2006). When canopy height exceeds 3 m, alley width must range 4.9–7.9 m to provide adequate illumination at the canopy base (Connor 2006). In our case, the orchard under investigation had 4 m row width (alley width was <2 m) with tree height averaging 3.88 m ± 0.10 (Arbequina) and 3.55 m ± 0.16 (Arbosana). As a consequence, there was a decrease of light interception at the base of the canopy when compared to the upper one. The lower light interception by the Arbosana canopy base could be explained by the trial design in which Arbosana was planted in lines between Arbequina. In a trial with 5 m wall height, Pastor et al. (2007) reported decreases up to 90 %. Connor and Fereres (2005) reported that the threshold irradiance for olive trees should be higher than 800 μmol m^{-2} s^{-1} PAR. In the lower part of the canopy in Arbequina and Arbosana, the average PAR was below the proposed threshold value.

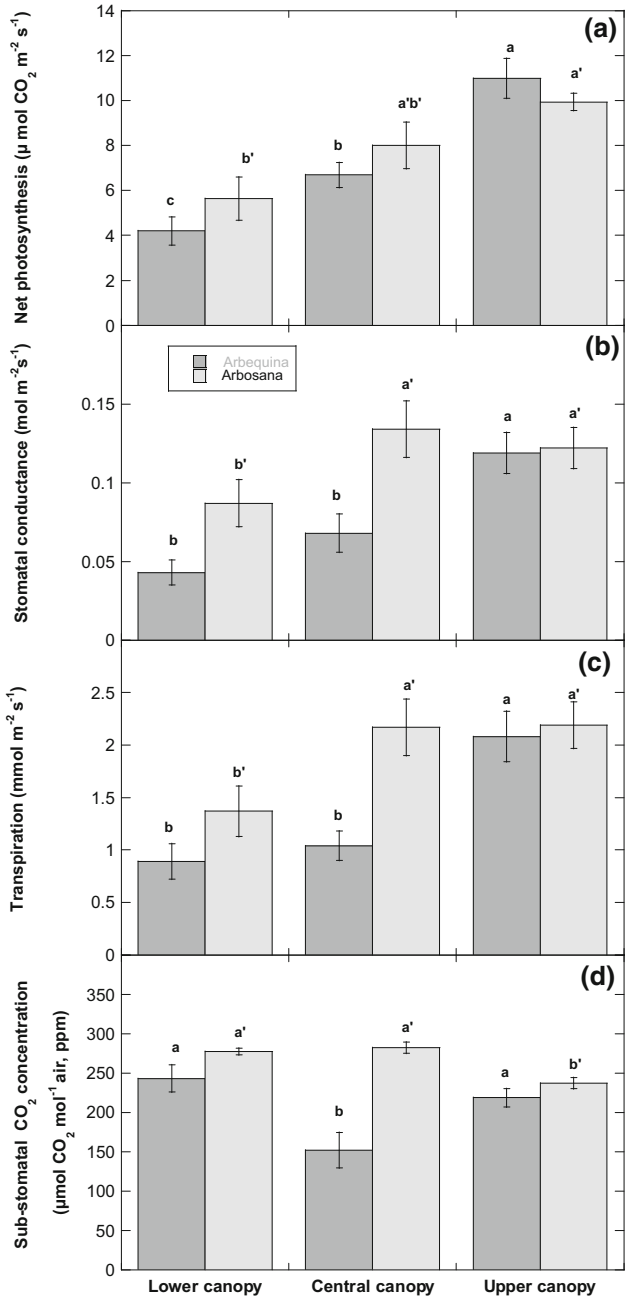


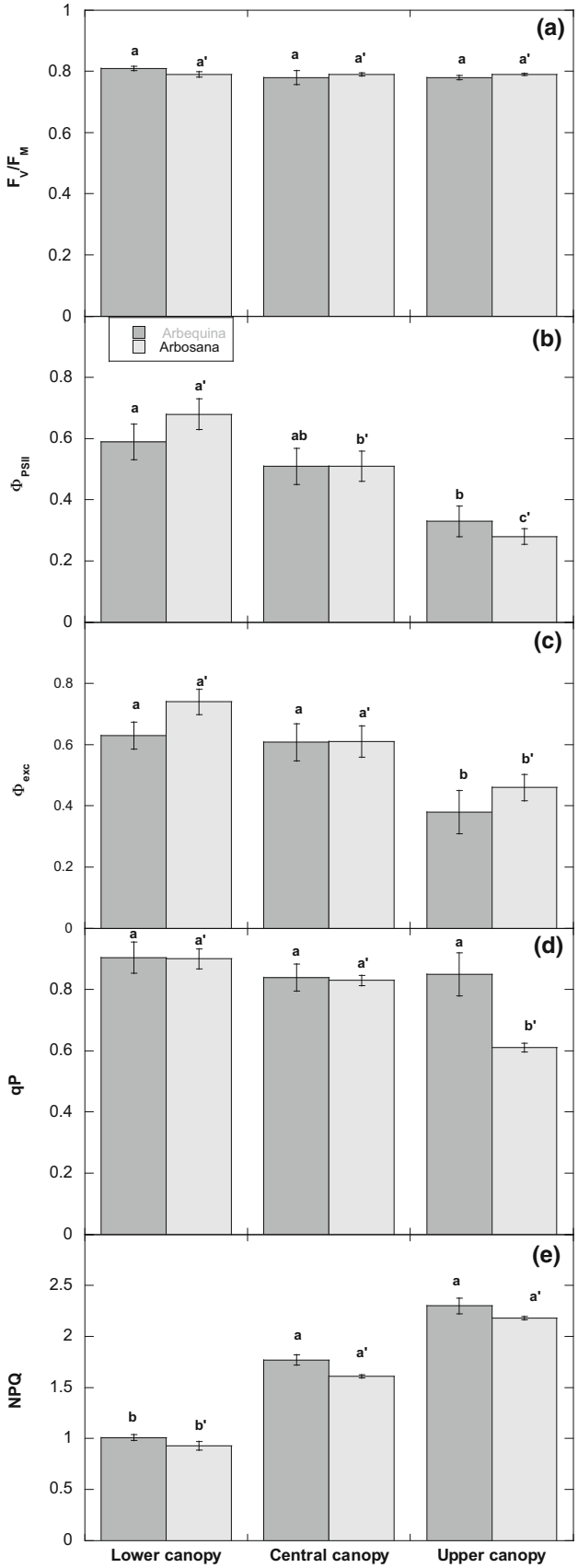
Fig. 7 Net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}) (**a**), stomatal conductance (g_S, mol m^{-2} s^{-1}) (**b**), transpiration (E, mmol m^{-2} s^{-1}) (**c**) and sub-stomatal CO_2 concentration (C_i, μmol CO_2 mol^{-1} air, ppm) (**d**) in the three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower part (<1 m) in the Arbequina (*dark gray bars*) and Arbosana (*light gray bars*) cultivars. Measurements ($n = 30$) took place between 9 and 13 h solar time, with a photosynthetic active radiation of 1,500 μmol m^{-2} s^{-1}, at 25 °C, 400 μmol CO_2 mol^{-1} and relative humidity of 60 ± 1 %. Data are mean ± SE. *Different letters* between zones indicate significant differences ($P < 0.05$)

Olive leaves showed phenotypic plasticity, responding with light intensity-induced morphological and photosynthetic changes. An increased leaf area is considered as a common adaptation to low irradiance (Marler et al. 1994) in order to capture more light (Yang et al. 2014) and may have been caused by an increase in the contents of auxins and gibberellins of the leaves grown under low irradiance (Salisbury and Ross 1978). Leaf area increased significantly in both cultivars in the central and the lower parts of the canopy, in line with previous reports (Tombesi and Cartechini 1986; Proietti et al. 1988; Gregoriou et al. 2007).

In both cultivars, photosynthetic rates decreased progressively from the upper canopy to those receiving low PAR. Between the upper and the central canopy heights, this impaired photosynthesis can be at least in part ascribed to stomatal behavior. Indeed, stomatal conductance, transpiration and sub-stomatal CO_2 concentration decreased in Arbequina, but not in Arbosana. Previous studies reported that olive leaf photosynthesis and stomatal conductance decreased under prolonged shade (Proietti et al. 1988; Gregoriou et al. 2007). The significant decrease in photosynthesis, parallel to decreases in stomatal conductance and transpiration, between the upper and the lower canopy can be related to the well-known reduced photosynthetic capacity of shade-grown leaves (Boardman 1977; Leong and Anderson 1984b) and not to the low PAR (Gregoriou et al. 2007). Olive leaf, despite growing in areas of high sunlight, has a relatively low light saturation point when compared to other fruit trees (Higgins et al. 1992). All photosynthesis measurements were made at saturating light intensity (olive tree leaves have a light compensation point of and saturate at 53 and 900–1,000 μmol m^{-2} s^{-1} PAR, respectively; Higgins et al. 1992; Gregoriou et al. 2007).

These decreases in photosynthesis cannot be ascribed to the amount and morphology of stomata. Olive leaves have stomata mainly in the abaxial epidermis and stomatal density varies among olive cultivars (Leon and Bukovak 1978; Bongi et al. 1987; this work). Stomatal density did not vary significantly among the different canopy heights in Arbosana, and even increased in the Arbequina lower canopy. Our results of stomatal density are similar to those previously reported for 4 olive cultivars (Roselli et al. 1989), higher than those reported for Manzanillo, Koroneiki, Chemlali and Zalmati cultivars (Leon and Bukovak 1978; Gregoriou et al. 2007; Boughalleb and Hajlaoui 2011) and much higher than those reported by Bongi et al. (1987). Stomata pore length did not change in Arbosana and was only 6 % smaller in the lower than in the upper canopy of Arbequina.

In contrast, the intercellular spaces within the spongy mesophyll were increased in leaves from the central and lower canopy when compared to the upper one. This change may alter the CO_2 mesophyll conductance (from the sub-stomatal cavities to the sites of carboxylation in the chloroplasts), restricting photosynthesis (Boardman 1977;



◀**Fig. 8** F_V/F_M (**a**), Φ_{PSII} (**b**), $\Phi_{exc.}$ (**c**), qP (**d**) and NPQ (**e**) in the three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m) and a lower part (<1 m) in the Arbequina (*dark gray bars*) and Arbosana (*light gray bars*) cultivars. Measurements ($n = 30$) took place between 9 and 12 h solar time, under sunlight and with the photosynthetically active radiation close to the low values shown in Fig. 4. Data are mean ± SE. *Different letters* between zones indicate significant differences ($P < 0.05$)

Syvertsen et al. 1995; Sagardoy et al. 2010). Contrary to this, leaf thickness decreased in the central and lower canopy of both cultivars when compared to the upper one, due to the reduction of both palisade (from 3 to 2 cell layers) and spongy parenchyma. Similar effects have been reported in citrus (Syvertsen and Smith 1984), peach (Nii and Kuriowa 1988), carambola (Marler et al. 1994), olive (Gregoriou et al. 2007) and bamboo (Yang et al. 2014). Olive leaf photosynthesis has been negatively correlated to leaf thickness (Kchaou et al. 2013).

One aspect that is usually neglected when investigating photosynthesis and photoprotection is the angle with which leaves are being sunlight illuminated. Obviously, leaves with orientations more perpendicular to sunlight will absorb more light than those with more parallel orientations. On the other hand, a first strategy of photoprotection can be to place leaves with parallel orientation to sunlight, avoiding an excess of incident sunlight. In *Heliconia*, a change in leaf orientation from 10 to 80° from the horizontal plane decreases incident PAR by 41 % and increases dark-adapted F_V/F_M from 0.63 to 0.72 (He et al. 1996). Msallem (2002) found that leaf angle in optimal conditions was around 60° for adult olive trees. In our trial, the leaf position of the lower canopy tended to maximize light interception under low incident PAR conditions. On the contrary, the position of the leaves of the upper canopy tended to minimize light absorption.

One of the leaf characteristics most affected by shading, or by prolonged reductions in incident light, is pigment composition and pigment-related photoprotection. No significant changes in Chl $a + b$ were observed contrary to other results found under prolonged olive leaf shade where increased (Gregoriou et al. 2007; Melgar et al. 2009). In line with reports that indicate increases of Chl *a*/*b* ratio in sun-grown leaves (Leong and Anderson 1984a; Demmig-Adams et al. 1995; Gregoriou et al. 2007), the Chl *a*/*b* ratio increased in the upper canopy of Arbosana (but not Arbequina) when compared to the rest of the tree. In both cultivars, V + A + Z concentration decreased significantly in the central and the lower canopy, when compared to the upper one. Sun-grown leaves typically exhibit a larger total pool size of the xanthophyll cycle components as compared to shade-grown leaves (Demmig-Adams and Adams 1996) including olive leaves (Melgar et al. 2009).

Table 2 Concentration of photosynthetic pigments in leaves of three canopy zones differing in light interception: an upper one (>2 m), a central one (1–2 m), and a lower part (<1 m) in olive (cultivars Arbequina and Arbosana) trees

	Arbequina			Arbosana		
	Lower canopy	Central canopy	Upper canopy	Lower canopy	Central canopy	Upper canopy
Total Chl	548.1 ± 39.2a	536.2 ± 34.8a	640.5 ± 28.4a	454.8 ± 33.5a	502.0 ± 30.3a	492.9 ± 25.8a
Chl *a*/*b*	2.85 ± 0.23a	3.10 ± 0.17a	3.07 ± 0.13a	2.83 ± 0.09a	3.25 ± 0.10a	4.11 ± 0.11b
β-Carotene	54.8 ± 1.6a	53.9 ± 3.4a	73.4 ± 6.2b	51.1 ± 4.0a	59.4 ± 4.4b	64.3 ± 4.3b
Lutein	64.8 ± 3.0a	71.6 ± 6.9ab	80.0 ± 7.7b	61.3 ± 3.7a	65.9 ± 5.5a	59.7 ± 3.2a
Neoxanthin	27.9 ± 2.3ab	25.9 ± 1.8a	35.2 ± 3.3b	24.5 ± 1.7a	25.8 ± 2.6a	22.3 ± 1.1a
V + A + Z	24.9 ± 1.7a	29.2 ± 2.2a	40.9 ± 1.8b	21.1 ± 1.8a	25.8 ± 1.7a	34.1 ± 2.2b
Z + A/V + A + Z	0.056 ± 0.034a	0.143 ± 0.046b	0.163 ± 0.020b	0.083 ± 0.006a	0.127 ± 0.004b	0.194 ± 0.005c
Lutein/Chl	118 ± 7a	134 ± 10a	163 ± 5b	135 ± 2a	130 ± 6a	132 ± 2a
V + A + Z/Chl	46 ± 6a	55 ± 5ab	64 ± 3b	47 ± 4a	47 ± 2a	69 ± 4b

Total Chl (Chl $a + b$), β-carotene, lutein, neoxanthin and violaxanthin + antheraxanthin + zeaxanthin (V + A + Z) are given in mmol pigment m^{-2}. Lutein/Chl and V + A + Z/Chl are given in mmol pigment mol^{-1} Chl. Data are means ± SE ($n = 3$). For each cultivar, different letters in the same row indicate significant differences ($P < 0.05$)

The PAR reduction in the central and the lower canopy did not affect the maximum potential PSII efficiency in dark-adapted leaves (F_V/F_M). Decreases in Φ_{PSII} in leaves from the upper canopy with respect to the lower one were due to both decreases of the intrinsic PSII efficiency ($\Phi_{exc.}$) and qP. These decreases in Φ_{PSII} were associated to increases of energy dissipation as indicated by the increase of NPQ (Demmig-Adams et al. 1995; Melgar et al. 2009; Sofo et al. 2009) and thermal energy dissipation (D, calculated as $1 - \Phi_{exc.}$; Demmig-Adams et al. 1996) in both cultivars. Leaves from the upper canopy dissipated 54–60 % of the light absorbed by the PSII antenna, whereas in leaves from the lower canopy dissipation decreased to 25–36 %. The increase of NPQ, indicative of energy dissipation in the pigment bed, is often observed when high concentrations of Z (and A) occur (Demmig-Adams and Adams 1992; Gilmore and Yamamoto 1993), but high NPQ levels have been also observed with low Z (and A) concentrations (Peguero-Pina et al. 2013). Increases in NPQ and D in sun leaves from the upper canopy of the tree were associated to significant, but no so large, increases of the conversion of V into A and Z.

Fluorescence microscopy images were useful not only to investigate leaf anatomy (number of palisade cell layers, leaf thickness, etc.) but also revealed differences between Arbequina and Arbosana cultivars. Within the mesophyll, the blue fluorescence emission of the palisade parenchyma was more intense in Arbosana than in Arbequina. Under UV excitation, the blue fluorescence emission has been reported to originate from hydroxycinnamic acid derivatives (Cerovic et al. 1994, 1999; Morales et al. 1996) and NAD(P)H (Cerovic et al. 1994, 1998; Morales et al. 1994). Therefore, the most plausible possibility is that differences found were due to cultivar-dependent hydroxycinnamic acid derivatives and/or NAD(P)H concentration (or composition in the former case). It is unlikely that differences between cultivars in blue fluorescence were related to cell wall thickness. The specific leaf weight per area (SLW) was 22 and 15 mg dry weight cm^{-2} (not shown) in Arbequina and Arbosana, respectively, just the contrary to what expected if blue fluorescence were related somehow to SLW.

Olive trees are well suited for testing whether within-plant variation represents a strategy for optimizing light absorption. Due to the mismatch between sun position and leaves orientation, the potential of light gathering by a particular leaf is reduced most of the time (Granado-Yela et al. 2011). Maximum values of light absorption of a given leaf occur when placed perpendicular to sunlight, an "anecdotic" diurnal situation that changes seasonally due to solar azimuth (Granado-Yela et al. 2011). However, trees are spatially heterogeneous with interconnected populations of leaves that face diverse light environments under field conditions. Olive trees are modular organisms with groups of leaves located in different positions within the tree that may, or may not, play similar or identical physiological roles. Our results strengthen the idea of olive trees as modular organisms that adjust the modules (lower, central and upper canopy) morphology and physiology in response to light intensity, in line with previous results (Granado-Yela et al. 2011). The diverging photosynthesis in different positions of the canopy might thus maximize overall performance, with the upper leaves contributing largely to the tree C balance when light conditions are optimal and the lower ones ensuring a more stable photosynthetic performance throughout the year (see discussion of optimization theories and canopy modules in de Casas et al. 2011; Granado-Yela et al. 2011) and adjusting their

photosynthetic traits to maximize the light use in shaded areas (Zhang et al. 2007; Huang et al. 2011). Aspects such as light gathering, C fixation, and C accumulation/export should be considered in order to integrate resources allocation at the scale of tree crown (Granado-Yela et al. 2011). Thus, the profitability of producing a new module may respond to light availability, construction costs and the spatio-temporal integration within the productive processes at the whole olive tree level (Granado-Yela et al. 2011).

From our results, in summary, it can be inferred that a bad canopy management in a high-density olive orchard may lead to a reduced PAR exposure of leaves at the central and mainly the lower canopy, which causes several leaf morphological and anatomical changes, decreases in photosynthesis and potentially in olive fruit yield. Indeed, olive fruit yield was differently distributed among the lower, central, and upper canopy, contributing with 16, 30, and 54 %, respectively to the total tree yield (not shown), in line with recently published data (Connor et al. 2012). Thus, productivity (Connor et al. 2012) and oil content (Cherbiy-Hoffmann et al. 2012) in hedgerows olive orchards can be related to patterns of solar radiation intercepted by the canopy walls, emphasizing the importance of the upper canopy to achieve high olive yields.

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