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Changes of morphological and physiological markers induced by growth phases in leaves of olive tree (*Olea europaea* L.)

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Mechanisms involved in phase change phenomena (juvenility-maturity) in olive plant (*Olea europaea* L. cv Chondrolia Chalkidikis) were studied at anatomical and ecophysiological levels. Juvenile (JP)-, intermediate-juvenile (IJP) and mature (MP) leaves were obtained from three sources representing the three different stages of plant development including seedlings, two- and three-year-old plants derived from cuttings.
JP leaves were generally smaller, thicker and more rounded compared to IJP- and MP leaves. The densities of stomata and non-glandular hairs (scales) at the abaxial leaf surface were relatively lower in JP leaves. Stomatal conductance (Gs) and photosynthetic rate (Pn) were higher. MP leaves were larger, thinner and more elongated. Stomata and non-glandular hairs were significantly denser. A lower relative volume (%) of the palisade parenchyma cells in the mesophyll of MP leaves was registered. Furthermore, a significant reduction of the Gs and Pn values was also observed in the MP leaves.
IJP leaves revealed an intermediate leaf size as well as intermediate values of almost all of the conducted physiological measurements. No significant difference was found in the relative volume (%) of the mesophyll spongy parenchyma between the JP- and IJP leaves. The higher photosynthetic rate of the JP leaves could be attributed to differences in the leaf anatomical features, especially of the palisade parenchyma cells.

Key Words: Olive (*Olea europaea* L.), phase changes, leaf gas exchange, water relations, leaf anatomy.

INTRODUCTION

A number of changes occur during the ontogeny of the plants. These changes referred to phase changes and have been considered by Hackett and Murray (1996) as a reflection of a change in competence to perceive, or a respond to environmental or endogenous stimuli for flower induction. The phase changes, especially in woody perennial species, have been described for many species (Hackett & Murray, 1993). Little effort has been directed, however, toward the development of practical methods for shortening the juvenile phase (Meilan, 1997) and accelerating the growth, in order to get early flowering.

In many heteroblastic woody species with distinguishable 'juvenile' and 'mature' phases, juvenility as a complex phenomenon is still not fully understood. Zimmerman *et al*., (1985) have defined it as the period during which the plant cannot be induced to flower. The duration of the juvenile phase is quite variable. It reaches up to 30-40 years in *Fagus sylvatica* L. (Meilan, 1997), whereas in olive, the time lapse from seed germination to the first development takes up to l0 years or more (Lavee *et al.,* 1996). However, the study of juvenility duration is highly difficult and still represents a serious obstacle to breeding programs, especially to the selection of improved cultivars (Hansche & Beres, 1980; Poething, 1990). On the other hand, the morphological

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and physiological syndromes of juvenility, as well as their changes could be important markers concerning juvenility duration. Knowing the involving physiological and morphological mechanisms in phase changes of growth, juvenility duration can be properly controlled and shortened.

The investigation in olive of the physiological and anatomical mechanisms involved in the control of hereroblastic phase changes has received little attention. Since the olive tree is of high nutritional importance for humans, especially in the Mediterranean region, and it covers extensive areas in Greece, it has been chosen as a model plant in our study.

The present paper aims to identify the different heteroblastic phases of growth at morphological and anatomical levels, and to evaluate the eco-physiological status of these phases in the olive tree.

MATERIAL AND METHODS

Plant material

The plant material used in our study was obtained from three sources representing three different heteroblastic phases of plant development:

1. **Juvenile phase (JP) (two-year-old seedlings).**
2. **Intermediate-juvenile phase (IJP) (two-year-old plants derived from cuttings).**
3. **Mature phase (MP) (three-year-old plants derived from cuttings).**

Seedlings: Mature olive fruit (*Olea europaea* L. cv. Chondrolia Chalkidikis) were harvested when their colour has changed from green-violet to violet, (20-30 November) from a block of five trees (15 years old) grown at the farm of the Aristotle University of Thessaloniki, Greece.

The exocarp and mesocarp of fruit were removed and the stony endocarp (containing the seed) after several rinses in running water, was air dried for a week and stored in glass containers at room temperature.

Shortly before the experiments, seeds were carefully liberated from the stony endocarp by using a manual turnstile. The seeds were then soaked in a solution of sterile distilled water and fungicide for 24 h and planted in four traces (100 seeds each) using media of soil, peat-moss and perlite (1:1:1 v). After a month, the germinated seeds were transferred to individual pots (1 litter v) using the above-mentioned media. Then, when the seedlings had reached about 30cm height, they were transferred to pots (9 litter v.).

Cuttings: Fifteen to eighteen cm long cuttings with four leaves at their distal end were taken from sprouts of olive bushes. The cuttings were soaked in a fungicide solution (Benlate 3%) for 10 min. After drying, the bases were refreshed and then dipped for 5 sec in indole-3-butyric-acid (IBA, 4000 ppm). Then, the cuttings were placed in rooting traces (60 × 40 × 15 cm) with peat-moss and perlite as a substrate (1:1 v). The cuttings were maintained under mist for a period of 6 weeks. The rooted cuttings were planted in black plastic bags and transferred into the greenhouse for acclimatization.

At the end of the growing season, the plants were transplanted into big pots of nine-liter volume. During the winter period, the plants were maintained under greenhouse conditions (10-15°C). Meanwhile, the branches were left to grow freely without any further pruning and then transferred and distributed outdoors.

For three years, we were following the same procedure for cutting preparation, and at the end of the third year we came out with plants two and three-year-old representing the intermediate-juvenile and mature phases, respectively. The three year-old-plants were recognized as a mature phase, because of the presence of flowers at the third year, under natural conditions.

Leaf sampling

All measurements were conducted on fully-developed leaves from the middle region of annual shoots.

Leaf morphology and anatomy

Leaf pieces obtained from 10 randomized leaves per treatment, were fixed for 3 h in 5% glutaraldehyde buffered with 0.025 M sodium phosphate (pH 7.2). Samples were then washed in the respective buffer and post-fixed for 5 h in 1% osmium tetroxide similarly buffered. Sample dehydration was carried out in an alcohol series followed by propylene oxide. For light microscopy, the tissue was embedded in Spurr's resin (Spurr, 1969). Sections (1 μm thick) were obtained in a Reichert OM U_2 ultramicrotome, stained with 1% toluidine blue O in borax, and photographed with a Zeiss III photomicroscope.

Morphometric assessments: The morphometric assessments included thickness of adaxial epidermis, upper palisade parenchyma, spongy parenchyma,

lower palisade parenchyma, abaxial epidermis, as well as total leaf thickness. For the assessment of the relative volumes of the leaf histological components, a transparent sheet bearing a square lattice of point arrays, 10 mm apart, was laid over light micrographs of leaf cross-sections (X170). The point-counting-analysis technique was then applied (Steer, 1981).

The densities of stomata and non-glandular hairs on the abaxial leaf surface were determined using leaf paradermal sections.

The leaf area (cm^2) was measured by image analysis, using an Olympus BX 10 TK 1280 E color video camera with a cosmica/pentax 8-48 mmTV zoom lens connected to a quantimet 500MC image processing and analyzing system with associated software (Leica Cambridge Ltd).

Water status measurements

Leaf water potential (Ψ) as well as leaf osmotic potential (π) were measured during the growing season using a Scholander pressure chamber (Turner, 1981; Scholander *et al.,* 1964) and psychrometric techniques (Koide *et al.,* 1991), respectively. The measurements were conducted once a month. For leaf water potential measurement, 6 replicates/treatment were used. Simultaneously to leaf water potential measurements, six leaves/treatment were collected, directly wrapped with Parafilm and stored at -20°C. After 24 hours, leaf disks (0.38 cm^2) were taken from the stored samples for leaf osmotic potential measurement.

Turgor potential (P) was calculated as the difference between leaf water- and osmotic potentials ($P = \Psi - \Psi_{\pi}$).

During the procedure, other six leaves/treatment were collected, immediately weighed (FW, g) and then, immersed in distilled water inside a glass tube for 12 h at a temperature of 4-6°C in the darkness. The leaves were blotted dry and the turgid weight (TW, g) was determined. The dry weight (DW, g) was measured after leaves were dried at 80°C for 12 h. RWC was calculated as a percentage by the formula:

$$\% \text{ RWC} = \frac{\text{FW - DW}}{\text{TW – DW}} \times 100.$$

Gas exchange measurements

Ten leaves from each treatment were used monthly for measuring the photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr) during the growing season using a portable IRGA (Li-Cor 6400) device. During the measurements, leaf temperature and photon flux density were maintained at 28 ± 2°C and 1400μmol $m^{-2}s^{-1}$, respectively. Pn, Gs and Tr were expressed on an area base. Water use efficiency (WUE) was calculated by Pn/Tr.

Data analysis

Twenty plants/treatment were distributed in a randomized complete block design (four blocks X five replications). The results were statistically analyzed by using one-way ANOVA for mean comparison among treatments, using the statistical package (SPSS for windows, standard version, and release 8). Significance was determined at $p \leq 0.05$ by applying the Duncan test.

RESULTS

Leaf morphology and anatomy

The leaves of juvenile (JP)-, intermediate-juvenile (IJP)-, and mature (MP) olive phases appeared morphologically quite different with each other (Fig. 1). Thus, JP leaves (Fig. 1A) were shorter and more rounded compared to MP leaves (Fig. 1C), while IJP leaves (Fig. 1B) had intermediate characteristics. Leaf cross-sections disclosed that in JP leaves, the lamina was much thicker (544.90 μm) than that of IJP leaves (477.69 μm) and MP leaves (411.58 μm) (Fig. 2, Table 1). The typical anatomical features of the olive leaf comprised a multiseriate palisade parenchyma with small intercellular spaces and a spongy parenchyma with well-developed aerenchyma (Fig. 2A). At the border between the palisade and spongy parenchymas, vascular bundles occured. Within the mesophyll, numerous elongated sclerenchyma cells were scattered having a varying orientation. The cells of the adaxial epidermis were significantly larger than those of the abaxial one. On the adaxial epidermis stomata did not exist. On the abaxial leaf epidermis, stomata were more numerous in MP leaves compared to JP- and IJP- leaves (Fig. 3, Table 1). The same holds for the non-glandular scales (Figs. 2, 3; Table 1). In JP leaves, the upper palisade parenchyma (facing the adaxial epidermis) was composed of three to four layers of elongated cells, whereas in IJP- and MP- leaves it was ordinary composed of three cell layers (Fig. 2). Furthermore, in MP leaves, the spongy parenchyma was more com-

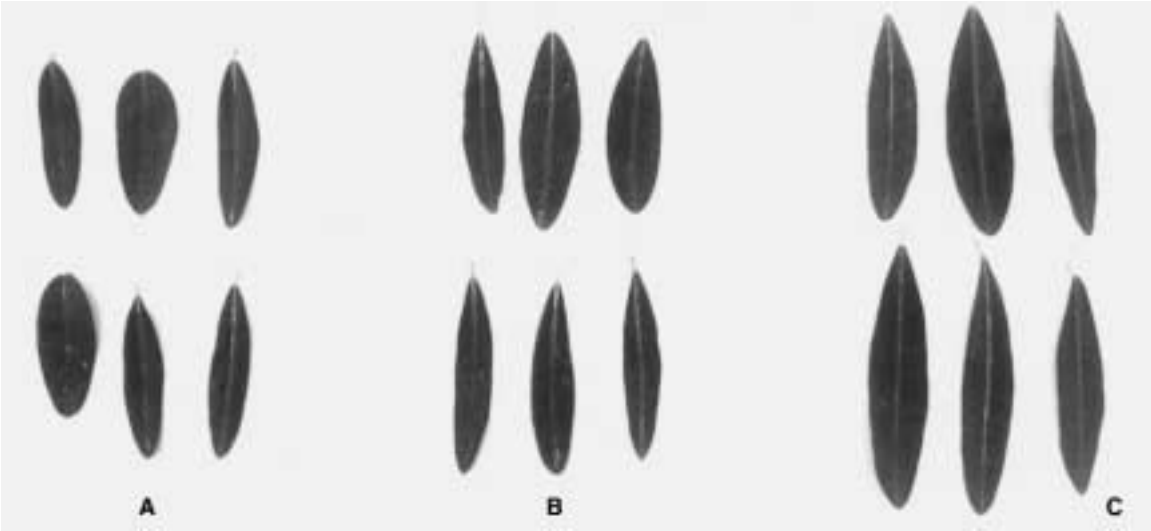

FIG. 1. Comparative leaf morphology in three treatments of olive tree. A. Leaves of juvenile phase (JP), B. Leaves of intermediate-juvenile phase (IJP) and, C. Leaves of mature phase (MP).

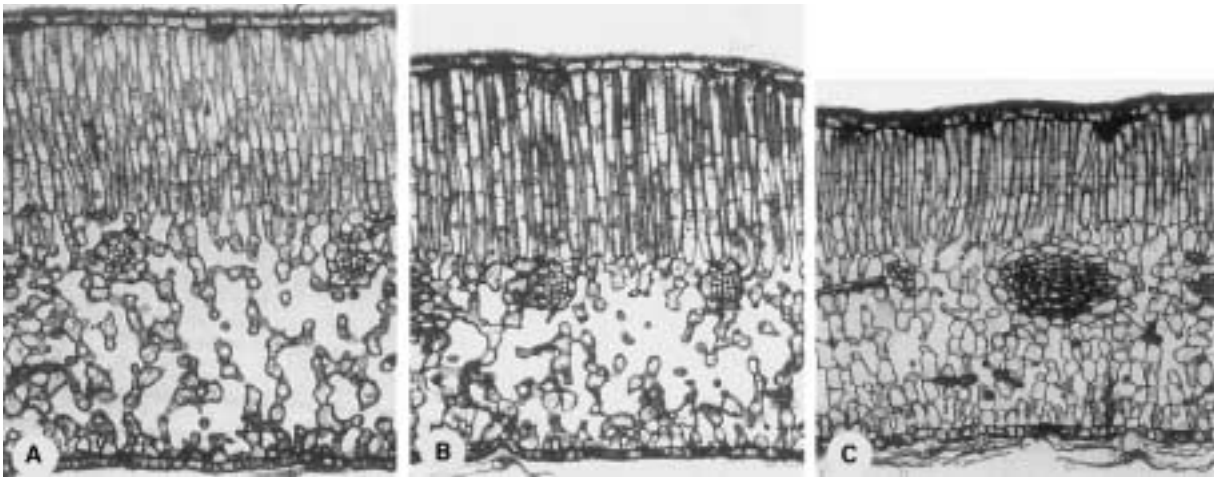

FIG. 2. Comparative leaf anatomy (leaf cross sections) of three treatments X 170 . A. Leaf of juvenile phase, B. Leaf of intermediate-juvenile phase, and C. Leaf of mature phase.

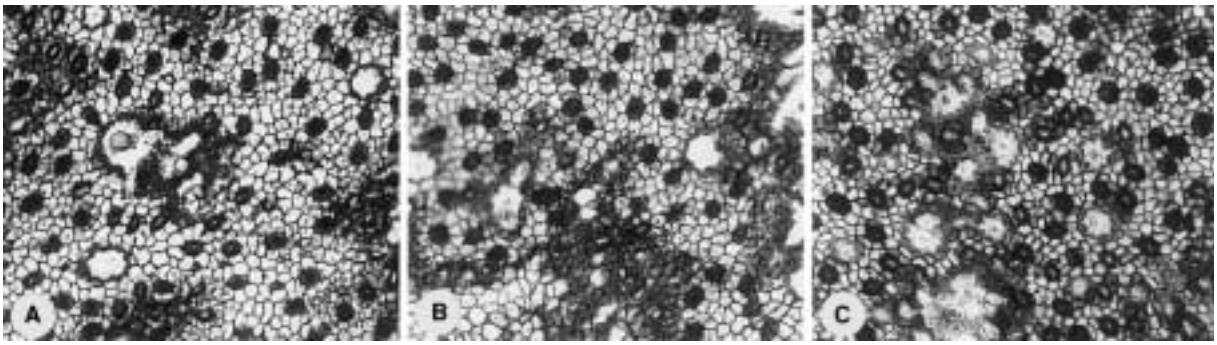

FIG. 3. Densities of stomata and non-glandular hairs (scales) in leaf paradermal sections X 170. A. Leaf of juvenile phase, B. Leaf of intermediate-juvenile phase, and C. Leaf of mature phase.

TABLE 1. Morphometric assessments on leaf components in three treatments of olive tree cv. Chondrolia Chalkidikis. (n=12; ± SD, standard deviation; for each row, values with different letters were significantly different at $p \leq 0{,}05$ as determined by Duncan test)

Leaf components	Treatments		
	Juvenile phase (JP)	Intermediate-juvenile phase (IJP)	Mature phase (MP)
Thickness (μm):			
Adaxial epidermis	19.89±1.68[a]	18.14±1.17	18.27±1.24
Upper palisade parenchyma	217.10±7.13[a]	208.65±11.70[b]	140.08±3.08[c]
Spongy parenchyma	270.08±10.34[a]	213.85±18.00	206.70±7.02
Lower palisade parenchyma	20.48±4.14	20.80±7.52	29.38±3.94[a]
Abaxial epidermis	17.36±1.09	16.25±1.54	17.16±0.59
Total thickness (without hairs)	544.90±14.08[a]	477.69±6.96[b]	411.58±6.54[c]
Relative volumes (%):			
Adaxial epidermis	4.07±0.45	3.69±0.50	4.21±0.56
Upper palisade parenchyma	39.93±1.05b	44.93±2.14[a]	35.20±1.31[c]
Spongy parenchyma	47.92±1.88[a]	42.64±1.46	48.98±1.05[a]
Lower palisade parenchyma	4.49±0.83	4.75±0.56	7.17±0.51[a]
Abaxial epidermis	3.60±0.74[b]	4.01±0.51[ab]	4.45±0.30[a]
Stomatal density (No.mm^{-2})	377.89±45.92	389.67±67.48	450.78±20.34[a]
Non-glandular hair density (No.mm^{-2})	50.67±9.96	74.11±19.35	170.11±33.02[a]

pact and contained more sclerenchyma cells. More compact was also the lower palisade parenchyma (facing the abaxial epidermis) of MP leaves. The values of the relative volumes (%) of the leaf histological components in the three kinds of leaves are given in Table 1.

Leaf water relations and gas exchange parameters

The relationship between leaf water potential (Ψ) and relative water content (RWC) (Fig. 4) revealed that leaves of juvenile phases (JP) had lower values of Ψ at the same value of RWC compared to those of intermediate-juvenile (IJP)- and mature (MP) phases. A greater change was observed in RWC along with the growing season in JP, whereas IJP and MP had intermediate and smaller changes, respectively.

JP maintained a higher turgor potential at the same values of Ψ (Fig. 5), whereas IJP and MP presented intermediate and lower turgors, respectively.

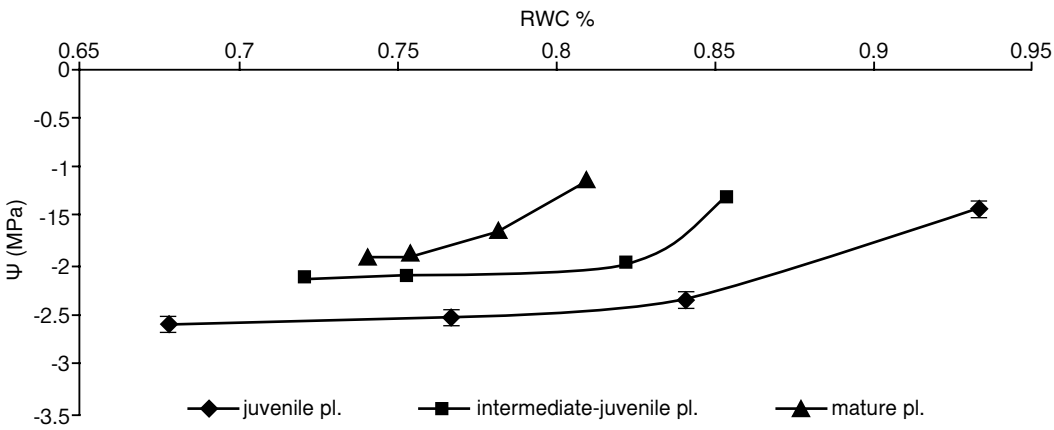

FIG. 4. Changes of leaf water potential (Ψ) in relation to relative water content (RWC) in three treatments of olive tree. Bars represent the standard error of the mean of six replicates. (Figures with no visible error bars condense very small standard error).

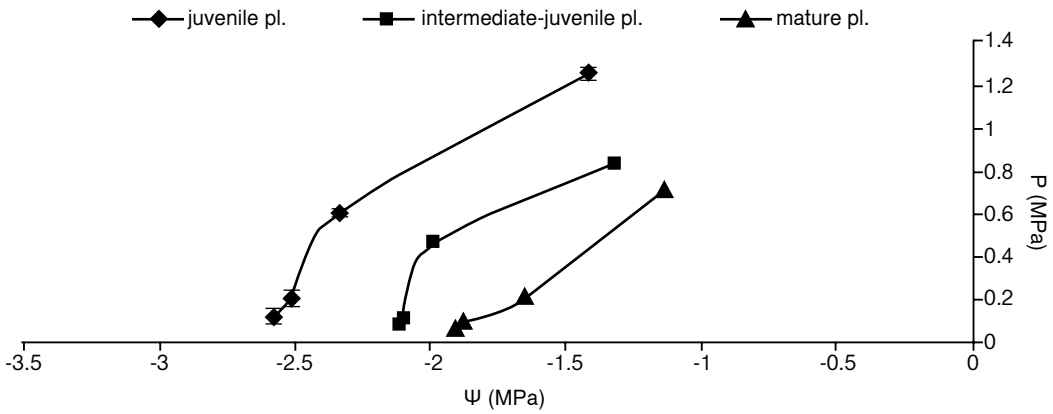

FIG. 5. Changes of leaf water potential (Ψ) in relation to turgor potential (P) in three treatments of olive tree. Bars represent the standard error of the mean of six replicates. (Figures with no visible error bars condense very small standard error).

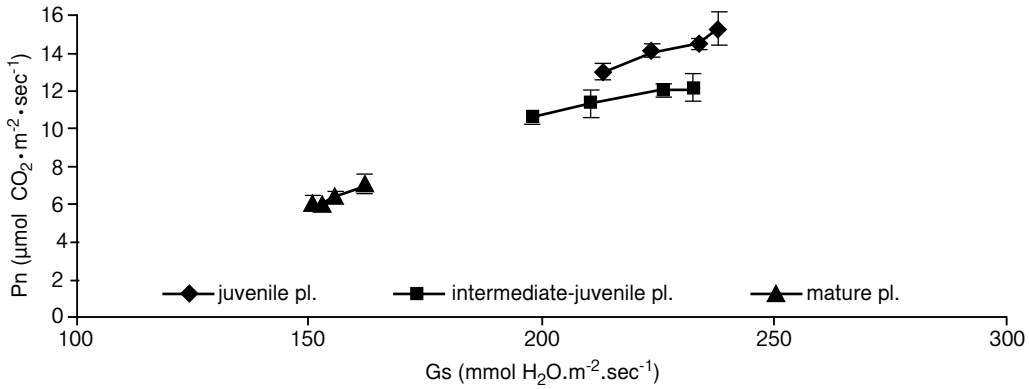

FIG. 6. Relationship between stomatal conductance (mmol $H_2O.m.^{-2}sec^{-1}$) and photosynthetic rate (µmol $CO_2.m.^{-2}sec^{-1}$) in three treatments of olive tree. Bars represent the standard error of the mean of 10 replicates.

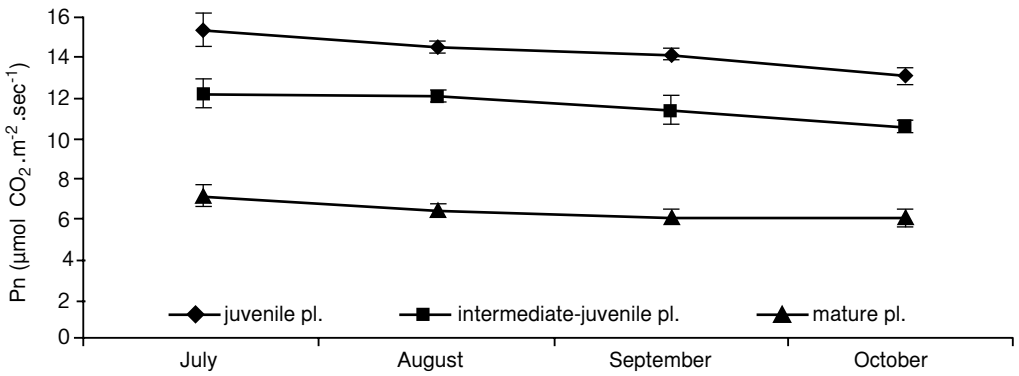

FIG. 7. Seasonal changes in photosynthetic rate (µmol $CO_2.m.^{-2}sec^{-1}$) in three treatments of olive tree. Bars represent the standard error of the mean of 10 replicates.

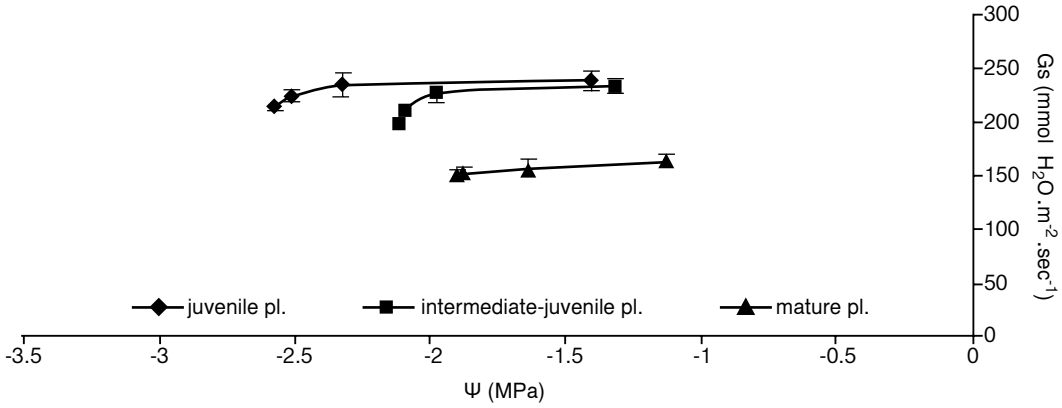

FIG. 8. Changes of leaf water potential (Ψ) in relation to stomatal conductance (Gs) in three treatments. Bars represent the standard error of the mean of six replicates. (Figures with no visible error bars condense very small standard error).

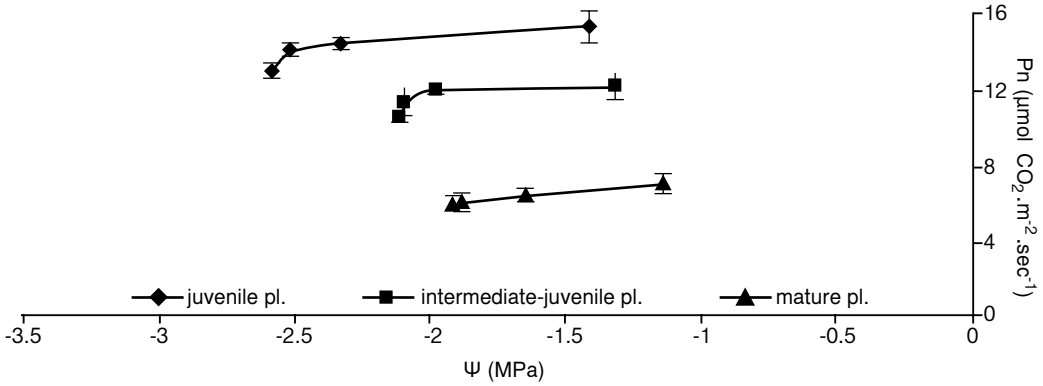

FIG. 9. Changes of leaf water potential (Ψ) in relation to photosynthetic rate (Pn) in three treatments. Bars represent the standard error of the mean of six replicates. (Figures with no visible error bars condense very small standard error).

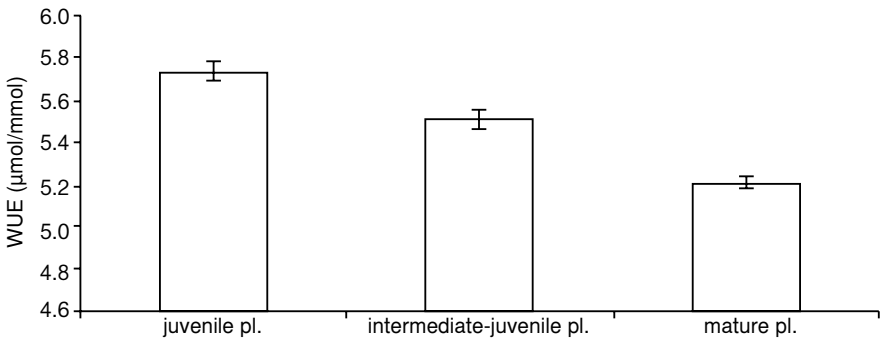

FIG. 10. Water Use Efficiency (WUE) in three treatments of olive tree at the lower values of Ψ. Bars represents the standard error of the mean of 6 replicates.

In all treatments, the turgor pressure (P) decreased with respect to leaf water potential.

The seasonal changes of stomatal conductance in relation to photosynthetic rate (Fig. 6) remained very low overall the growing season. However, the leaves of JP presented higher values of stomatal conductance compared to those of MP. A similar pattern presented the seasonal changes of photosynthetic rate (Fig. 7).

The seasonal changes of Gs and Pn in relation to Ψ revealed that, JP maintained also higher values of Gs and Pn at the same value of Ψ. On the contrary, MP had lower values and IJP had intermediate values, respectively (Figs 8 and 9). JP showed significantly higher values of water use efficiency (WUE) compared to those of IJP and MP plants (Fig. 10).

DISCUSSION

It is well documented that, the photosynthetic rate (Pn) in the xeromorphic species, including olive tree, can be affected by plant water status, stomatal conductance (Chartzoulakis *et al.,* 2002), leaf anatomy (Karabourniotis *et al.,* 1994), or by a combination of the above factors.

The lower values of Ψ at the same values of RWC observed in JP plants compared to other treatments, suggest that JP plants induced a mechanism of positive water balance in order to maintain higher turgor potential and consequently higher stomatal conductance. Indeed, the relation between turgor potential and Ψ suggests that JP leaves maintain a higher turgor compared to IJP and MP leaves, probably either through the osmotic adjustment or the high elasticity (During, 1984; Xiloyiannis *et al*., 1988; Schultz & Matthews, 1993; During & Dry, 1995; Patakas *et al*., 1997; Chartzoulakis *et al.,* 1999). Thus, the higher turgor potential of JP plants might explain their higher values of stomatal conductance at the same values of Ψ, especially compared to stomatal conductance of MP plants (Vitagliano & Sebastiani, 2002; Chartzoulakis *et al.,* 2002). The higher stomatal conductance of JP plants indicates that these plants could present a higher photosynthetic rate. In fact, the seasonal changes of Pn have verified the above assumption. However, since JP plants seem to have a higher photosynthetic rate at the same stomatal conductance compared to the other treatments, this suggests that Gs is neither the only nor the main factor affecting the photosynthetic rate in the leaves of olive tree (Evans *et al.,* 1994; Patakas *et al.,* 2003). Therefore, it could probably be assumed that the differences in Pn between the two juvenile phases are attributed to the leaf turgor potential and/or to the differences in the internal leaf architecture.

The higher amount of mesophyll palisade parenchyma and the thicker leaves in JP plants may shorten the path through which CO_2 is diffused to the carboxylation sites (Syvertsen *et al.,* 1995; Klich, 2000; Patakas *et al*., 2003) increasing thereby the Pn. On the other hand, the thicker cutinized outer walls of the epidermal cells in the same leaves may also reflect a better control of water loss through cuticular transpiration (Bosabalidis & Kofidis, 2002) and thus, lead to more efficient water use.

The increased leaf thickness and the higher number of palisade cell layers with decreased leaf area in the juvenile phase seem to be due to palisade cell division and elongation (Pemberton *et al.,* 1989). The reduced thickness of the leaves of the mature phase was mainly due to the reduced spongy mesophyll parenchyma as compared to the other treatments (Cantos *et al.,* 2002). Similar results have been also reported by Lavee *et al*. (1996) and Garcia *et al.* (2000) who stated that plants with juvenile growth differ morphologically from those with mature growth.

The reduction of stomatal density in the leaves of juvenile phase might be due to the greater enlargement of epidermal cells (Dengler, 1979). The increase of stomatal number in the leaves of MP does not reflect an efficient mechanism regulating water loss (Klich, 2000), but it might be considered as a possible mechanism for stomatal acclimation (Woodward, 1987).

The significantly higher density of non-glandular hairs in the leaves of mature phase might be interpreted not only as a mechanical protection against biotic factors (Johnson, 1975), but also as an additional retardation element in gas diffusion pathway (Nobel, 1983). The latter affects water loss by creating a zone of still air which reduces diffusion of water vapor from leaf interior to atmosphere (Klich, 2000). Hairs also reflect radiation and reduce its absorption resulting thus in the reduction of leaf heating and consequently of leaf transpiration (Premachandra *et al.,* 1991; McWhorter, 1993; Karabourniotis *et al,*. 1995; Klich *et al.,* 1997).

These arrangements of the leaf structural elements coupled with the lower densities of stomata and non-glandular hairs (scales) in the leaves of juvenile phase may act as an efficient mechanism to

improve the photosynthetic rate and may explain the higher Pn (Fox, 1996). However, the obtained results support the argument that, the anatomical differences contribute to differences in water transport characteristics coming out with direct effects on stomatal conductance and photosynthetic rate (Kaufmann & Fiscus, 1984). Moreover, the higher Pn values exhibited in JP leaves compared with those of the other treatments could presumably explain the higher rates of water use efficiency. This result agrees with Darrow's *et al.,* (2002) hypothesis that in juvenile phase the higher values of stomatal conductance seem to favour photosynthetic rate than transpiration.

CONCLUSIONS

It seems that the juvenile phase in olive trees is, when compared to the other phases, physiologically characterized by a higher photosynthetic rate and stomatal conductance, and morphologically by a smaller leaf size, smaller number of hairs and stomata, higher leaf thickness, and thicker palisade and spongy parenchymas. The mature phase has a lower photosynthetic rate and stomatal conductance, and a decreased cell size in all leaf histological components. Moreover, these changes in leaf morphology, anatomy and allocation of water might contribute to explain the significantly higher Pn in the leaves of juvenile phase. Nevertheless, further investigations are required to determine which ones of the leaf architectural characteristics are genetically or physiologically related to the high photosynthetic rate.

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