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Oil content and composition in relation to leaf photosynthesis, leaf sugars and fruit sugars in maturing Koroneiki olives – The mannitol effect on oil

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Summary

In Koroneiki olive tree, leaf photosynthesis, and sucrose, glucose, fructose and mannitol concentrations in leaves and fruit were investigated at fruit maturity index (MI) 1.1, 3.8 and 6.9, along with oil accumulation and composition, total phenolics (TP) and total antioxidant capacity (TAC) in fruit during a fully productive season in experiment 1 (I). The effect of mannitol treatment at 50 and 100 mg L^{-1}, applied in mid-October, on oil content and composition, TP and TAC were investigated in fruit harvested 25 d after treatment, at an average MI of 3.4, in experiment 2 (II). In I, in leaves net photosynthesis, and sucrose, glucose and fructose concentrations decreased, but mannitol increased by advancing MI. In fruit, however, concentration of all sugars decreased apart from fructose, which increased. Oil content (% DW), already high initially at MI 1.1, increased slowly thereafter, exhibiting decreases in oleic acid (OL) and increases in linoleic (LL). TP and TAC decreased at MI 3.8, remaining stable afterwards. In II, increasing mannitol concentration promoted oil accumulation and OL in oil and reduced LL slightly, indicating an acceleration of olive metabolism. Practically, mannitol could be applied to hasten the harvest of olives, so as to avoid adverse winter conditions.

Introduction

Olive (*Olea europaea* L.) trees are cultivated in Mediterranean climates. They can grow under unfavourable conditions, such as on arid areas and hilly lands, where other fruit trees cannot grow. Modern olive cultivation has introduced dense plantations with irrigation and fertilization regimes, resulting in increased yield and better quality of the products, olives and olive oil (Loumou and Giourga, 2003). In the Mediterranean basin, olives remain among the most important species environmentally and economically (Conde et al., 2008). The European Union is the leading producer and consumer of olive oil, producing 73% and consuming 66% of the world's olive oil. The data indicate that the European Union has a problem with a surplus of 1 or 2 million olive trees (Economic Analysis of the Olive Sector, 2012). The growing popularity of olive oil is attributed to its high nutritional value, mainly due to unsaturated fatty acids (FA) and antioxidants (Visioli and Galli, 2002). Olive oil has a balanced composition of FAs, with a high content of oleic (OL) acid (80%) that has beneficial effects on human health and contributes to the resistance of olive oil to oxidation (Conde et al., 2008).

The olive belongs to the few plants that synthesize both polyols and oligosaccharides. In particular, it is capable of synthesizing the polyol mannitol as well as oligosaccharides of the raffinose family, raffinose and stachyose, all these being the end products of leaf photosynthesis, which along with sucrose are transported from the leaf to the fruit through the phloem (Flora and Madore, 1993; Sanchez and Harwood, 2002). Recently, research focusing on both yield and quality of olive oil has been increasing. Studies including photosynthesis in leaves, being the first step of oil biogenesis, have mainly referred to photosynthetic parameters in relation to fruit load (Proietti, 2000) or to leaf sugars and oil content in fruit (Proietti, 2003). Considering that the olive tree has an alternate fruit-bearing habit, fruit load in connection with fruit quality or oil content/quality is another area attracting much attention (Bustan et al., 2011; Lavee and Wodner, 2004). There is also a great number of studies on fruit harvested at different developmental stages in relation to oil composition (Anastasopoulos et al., 2011; Bustan et al., 2011), but the respective findings were not usually associated with photosynthesis and its products.

The objective of the present work was to study some successive steps throughout the oil biosynthesis chain, such as leaf photosynthesis rate, major sugar concentrations in leaves and fruit, oil content and FA composition in fruit during the late developmental stages, as a very first step to studying sugars and oil. It was desirable to monitor all these steps concomitantly during olive maturation in a fully fruit-bearing year, with emphasis on mannitol as playing an important role in oil biosynthesis. Mannitol concentration in fruit was indeed positively correlated with oil content (Issaoui et al., 2008; Marsilio et al., 2001). However, these results conflicted with observations by others, who claimed that the correlation of oil with fruit mannitol is rather negative (Nergiz and Engez, 2000; Nergiz and Ergonil, 2009). An early study (Wodner et al., 1988) suggested that fruit mannitol concentration might indicate the potential for oil biosynthesis and suggested a positive effect of mannitol application on oil content. In consequence, any relation between oil and fruit mannitol was also of interest to us, and mannitol being a transportable sugar in tissue, a preharvest treatment with mannitol was also considered. Koroneiki was the cultivar studied because it is the main olive cultivar grown in Greece and produces olive oil of exceptional quality, being fruity with an aroma of leaves and grass, enriched with notes of green apple and some astringency (Anastasopoulos et al., 2011).

Materials and methods

Source and handling of fruit

Self-rooted olive (*Olea europea* L. cv. Koroneiki) trees grown on the experimental orchard at the Agricultural University of Athens (latitude 37º 58′56′′, longitude 23º 42′47′′) were used during two consecutive years and corresponded to experiments 1 (I) and 2 (II) during the first and second year, respectively. Seven- and nine-year-old trees, spaced at 4 × 2.5 m and trained as vase, were used in I and II, respectively. All trees used were in fully productive seasons. In I, leaf photosynthesis was measured on six leaves per tree and on six trees on 5 November, 19 November and 22 December 2010. On the same dates, approximately 250 leaves and 350 fruit were also harvested from the same six trees. In II, trees were sprayed once with 50 mg L^{-1} or 100 mg L^{-1} mannitol (Fluka, Sigma-Aldrich Chemmie GmbH, Steinheim, Germany) or with water (controls) until run-off at the green olive maturation stage on 17 October 2011. Tween-20 at 0.05% (v/v) was added to all solutions. In II, approximately 350 olives from three trees per measurement of mannitol concentration were harvested on 10 November 2011. In both experiments, all

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harvested samples, macroscopically free of disorders and diseases, were transferred to the laboratory. On all harvest dates in I and II, a subsample of olives (three groups of 100 olives each) was used for the estimation of maturity index (MI) according to Hermoso et al. (1991). In I, mean MIs for the three replicates and the corresponding SEs of the means for the harvested olives were 1.1 (± 0.09), 3.8 (± 0.26) and 6.9 (± 0.08) on the three successive harvest dates, respectively, corresponding to green, cherry and black olives, respectively. In II, MIs for harvested controls and olives treated with 50 and 100 mg L^{-1} mannitol were 3.3 (± 0.185), 3.5 (± 0.23) and 3.5 (± 0.22), respectively. On each sampling day, sampling and sorting of leaves or fruit were all carried out according to a completely randomized design. Harvested leaves and fruit were stored at -80 ºC for a short time, freeze-dried and stored at -80 ºC again until analyses.

Leaf photosynthetic parameters

Photosynthetic parameters were measured on leaves near fruit on fruit-bearing shoots and selected randomly from the outer part of the canopy at a height of 1.5 m from the ground. Four measurements per leaf were carried out around 11.00 am – 1.00 pm on cloudless days with a portable photosynthesis system (Li-6400, LI-COR, Lincoln, NE, USA). The system operated at a steady light intensity (1200 μmol photons m^{-2} s^{-1}), provided by an LED light source, and CO_2 concentration (400 μL L^{-1}). The air flow in the chamber was 300 mL min^{-1}. Leaf net photosynthesis (Pn), stomatal conductance (g_s), substomatal CO_2 concentration (Ci) and transpiration rate (E rate) were expressed as the rate of moles of CO_2 assimilation per leaf surface unit (μmol m^{-2} s^{-1}), the rate of moles of H_2O exiting per leaf surface unit (mol m^{-2} s^{-1}), the moles of CO_2 per mole air (μmol mol air^{-1}) and the rate of moles of H_2O exiting per leaf surface unit (mmol m^{-2} s^{-1}), respectively.

Extraction and determination of soluble sugars

Sucrose, glucose, fructose and mannitol in both leaves and fruit were determined on powdered (with mortar and pestle) samples (Roussos et al., 2010). Briefly, 50 mg of powdered tissue were extracted with 2 mL water (HPLC grade) in a microwave apparatus at 400 W for 2 min, thrice. The extractions were centrifuged at 5000 × *g* for 6 min, thrice. The combined supernatants were filtered through a nylon syringe filter (0.2 μm pore size) before HPLC analyses. The separation of sugars was achieved with a Hamilton HC-75 cation exchange column, calcium form, (Bonaduz, Switzerland) at 80 ºC, operating with a Water 510 isocratic pump with a flow of 0.6 mL min^{-1} and connected to a Refractive Index detector in an HP 104 7A HPLC (Hewlett-Packard, Waldbronn, Germany) system. Peaks were identified and quantified with standards using a data processing system (Peak Simple 3.25), and expressed on a dry weight basis (μmol g^{-1} DW).

Moisture and oil content in fruit

Fruit slices from approximately 60 olives were dried at 60 ºC for 3 days and then at 105 ºC for 3 h. Weights before and after drying were used for the expression of results on a dry weight basis, minimizing the fluctuations due to different moisture content in samples. Oil percentage was estimated according to Tsantili (2014). The total oil content of fruit was determined from 5 g of dried sample extracted for 6 h with 50 mL petroleum ether (b.p. 40-60 ºC) using a Soxhlet apparatus.

Extraction of oil for FA composition

A cold-pressing method with a laboratory screw-press device was used for oil extraction (Christopoulos and Tsantili, 2015). Chilled (at 4 ºC) fruit slices (50 g) were compressed between two parallel stainless steel plates of 10 × 5 cm pre-cooled at 4 ºC and each at a torque of 30 N m^{-1}. The recovered oil was used for analyses after clarification by centrifuging at 5000 × *g* for 3 min.

Fatty acid composition

The FA composition of the oil samples was determined by gas chromatography of fatty acid methyl esters (FAMEs). Esterification was conducted in tubes (12 × 100 mm) with Teflon-lined screw cap. One hundred milligrams of oil and 1 mL 0.5 N methanolic potassium hydroxide solution were added to the tube, the headspace was flushed with N_2 and the tube was heated in a water-bath at 90 °C for 10 min. After cooling the tube smoothly, 1 mL 14% (w/v) methanolic boron trifluoride was added. The heating step was then repeated, followed by cooling at room temperature. One millilitre of deionized water and 0.5 mL hexane were added and FAMEs were extracted by vigorous shaking for 1 min. The tubes were then centrifuged at 2,500 × *g* for 5 min and the top hexane layer was transferred to a vial for GC analysis (Christopoulos and Tsantili, 2015). FAs were analyzed by injecting 1 μL FAME into a GLC (HP 5890 Series II, HP, USA) equipped with a split/splittless injector (ratio 50:1), a flame ionization detector and a capillary column (DB-23, J &W Scientific, UK; 60 m length × 0.25 mm i.d., 0.25 μm film thickness), according to Christopoulos and Tsantili (2015) after some modifications. The carrier gas was helium. The injector and detector temperatures were 270 ºC and 280 ºC, respectively. The oven temperature program was as follows: isotherm at 190 ºC for 30 min, increase to 230 ºC at a rate of 10 ºC min^{-1} and isotherm at 230 ºC for 5 min. The identification and quantification of peaks were carried out with FAME standards (GLC-20, Supelco, UK; Me93, Larodan Fine Chemicals, Sweden), and the results were expressed as % (w/w) in oil.

Extraction for total phenolics (TP) and total antioxidant capacity (TAC)

Frozen olive slices were powdered with a mortar and pestle in liquid nitrogen before phenolic extraction. A quantity of 500 mg of powdered tissue was added to 80% v/v acetone in deionized water (1 mL 100 mg^{-1} tissue) and the mixture was placed in an ultrasonic ice-bath for 15 min. The samples were then centrifuged at 4000 × *g* for 5 min. The extraction was repeated thrice and the combined supernatants were used for TP and TAC (Tsantili, 2014).

Determination of TP and TAC

A modified Folin-Ciocalteu method was used for the TP determination (Tsantili et al., 2010). Specifically, 0.2 mL of diluted olive fruit extract was added to a tube containing 2.6 mL of water and 0.2 mL of Folin-Ciocalteu reagent. The tube was stirred and allowed to stand at room temperature for 6 min. Then, 2 mL (7% w/v) of sodium carbonate was added to the mixture. After 90 min, absorbance was measured at 750 nm *versus* a blank. The results were expressed as gallic acid equivalents on a dry weight basis (μmol g^{-1} DW).

TAC was estimated according to Brand-Williams et al. (1995). A volume of 0.1 mL of extract diluted with deionized water was added to a tube containing 3.9 mL 2,2-diphenyl-1-picryhydrazyl solution (0.06 mM in methanol). After 30 min incubation at room temperature, the decrease in absorbance was measured at 515 nm *versus* a blank. The results were expressed as trolox acid (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid) equivalents on a dry weight basis (μmol g^{-1} DW).

Statistical analyses

The significance of the effect of sampling date or MI in I and of mannitol concentration treatment in II on the measured variables were

estimated by one-way analyses of variance (ANOVA). The presented SEs in tables and figures were calculated from the residual variances. Photosynthetic parameters were estimated on three replicates of 12 leaves each, whereas the remaining determinations were on three replicates of 60 leaves or fruit each. Principal Component Analyses and pair wise correlations were applied to get an overview of the main variation in the data and interpret variable relationships among the variables determined in I. Data analyses were conducted using JMP 7.0.1 (SAS Institute, Cary, NC, USA).

Results and discussion

Photosynthetic parameters

The Pn was at 17.1 μmol CO_2 m^{-2} s^{-1} (Fig. 1A) on the first measuring date, but decreased gradually to 14.8 μmol CO_2 m^{-2} s^{-1} on the third date, with the seasonal change being significant ($P < 0.05$) and affected by decreases in temperature, while the light intensity was stable (1200 μmol photons m^{-2} s^{-1}), as described earlier in Materials and methods. During measurements, the temperature was 22.3 ºC, 20.8 ºC and 16.3 ºC on the first, second and third date, respectively, while the corresponding RH was 32.4%, 37.2% and 46.2%. Pn on the last date (22 December) was rather high and might be attributed to the relatively high temperature for the winter season. In an earlier study, the leaves of the Koroneiki olive cultivar were found to have the highest Pn among olive cultivars, while Pn in all studied cultivars was lower during summer and late autumn (end of November) than during spring and early autumn (Hagidimitriou and Pontikis, 2005). However, in Arbequina and Maurino, Pn, measured monthly from July to November, was highest in mid November (Proietti et al., 2012). In this study, the values of g_s ranged between 0.186 and 0.173 mol m^{-2} s^{-1}, while those of Ci between 246 and 205 μmol mol air^{-1} (Fig. 1B and 1C, respectively), but the changes in both parameters were not significant ($P > 0.05$). The E rate also did not significantly decrease during the season (Fig. 1D), although the effect of date was of borderline significance ($P = 0.0503$). In another olive study (Proietti et al., 2012), g_s decreased and Ci and E rate increased, but determinations were carried out for many months, allowing the opportunity to exhibit large changes, in contrast to the present results determined only within 1.5 months.

Stomatal density was lowest in Koroneiki among five cultivars studied (Hagidimitriou and Pontikis, 2005), providing an adequate justification of the high resistance of Koroneiki to drought. In the present results, this could be associated with almost stable g_s when Pn decreased, indicating non-stomatal limitation for leaf photosynthesis. The pairwise correlation among photosynthetic parameters showed non-significant relations (Tab. S1). In contrast to this, a positive linear relation between Pn and g_s was found in control Coratina olive leaves measured on young trees early in summer (Angelopoulos et al., 1996).

Major soluble sugars in leaves and fruit

In leaves, the initial concentrations of sugars were 31.93, 218.1, 33.9 and 140.4 μmol g^{-1} DW for sucrose, glucose, fructose and mannitol, respectively (Fig. 2A, 2C, 2E, 2G). Sucrose, glucose and fructose decreased gradually, resulting in final concentrations reduced by 1.64-, 1.22- and 1.17-fold, respectively, in comparison to the initial ones. In contrast, mannitol concentration in leaves gradually rose, reaching a final level of 1.34-fold higher than the initial value, while the Pn decreased by 1.2-fold within the same time period. The seasonal effect was significant for all sugar changes in leaves (Fig. 2). In another study on Koroneiki, leaf glucose and fructose were at higher and sucrose at lower levels than here, while the leaf mannitol level was similar to the present results (Roussos et al., 2010). However, the experimental conditions were different from this study since the trees were very young and growing in pots. A detailed study on carbohydrate allocation to vegetative tissues and roots in Barnea olive tree in a fully production year showed that in leaves, mannitol and

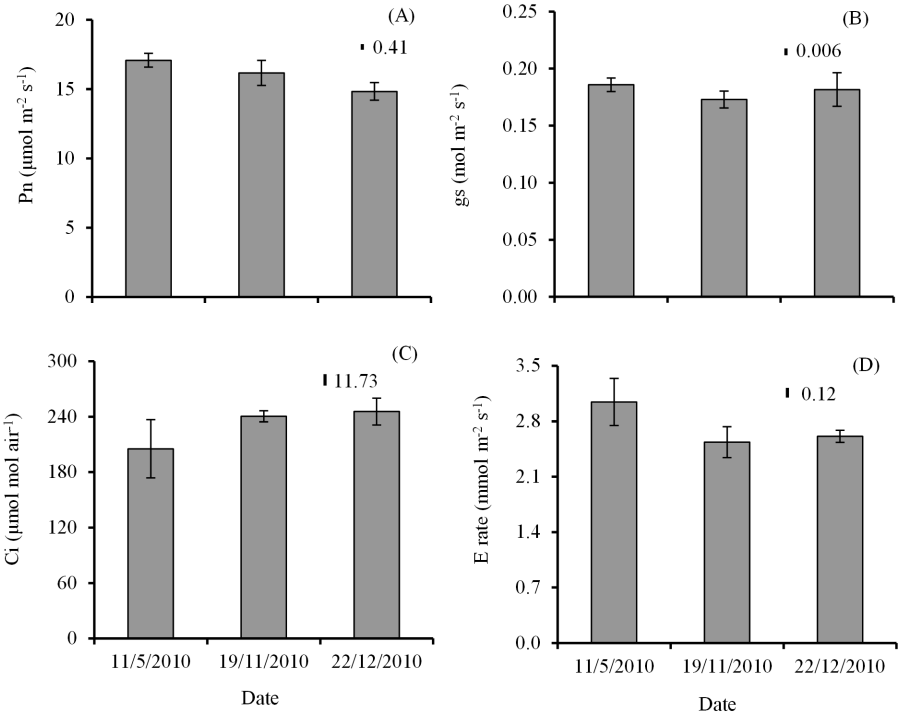

Fig. 1: Net photosynthesis (Pn), stomatal conductance (g_s), intracellular CO_2 concentration (Ci) and transpiration rate (E rate) in response to seasonal development in Koroneiki leaves, in 2010. A, Pn; B, g_s; C, Ci; D, E rate. Bars without numbers correspond to standard deviations; bars with numbers to Standard Errors from the residual variance (ANOVA) of three replicates, d.f. = 6. In A, $P < 0.05$, whereas in B, C and D, $P > 0.05$ in all of them.

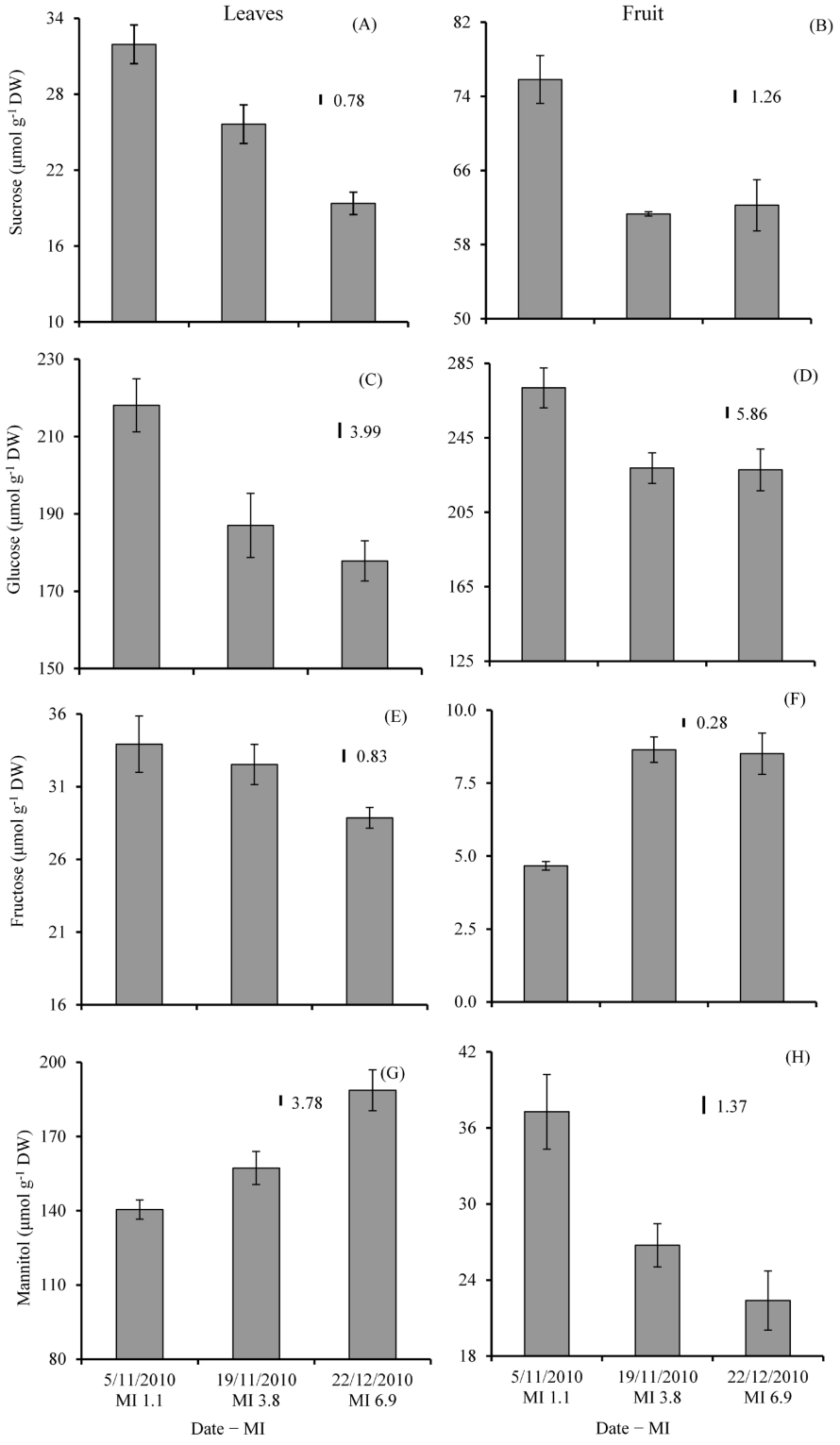

Fig. 2: Sucrose, glucose, fructose and mannitol, in leaves and fruit, in response to seasonal development and fruit maturity indices (MI) in Koroneiki olive fruit, in 2010. A, C, E and G, leaves; B, D, F and H, fruit. Bars without numbers correspond to standard deviations; bars with numbers to Standard Errors from the residual variance (ANOVA) of three replicates, d.f. = 6. In E, $P < 0.05$, in D, $P < 0.01$, whereas in A, B, C, F, G and H, $P < 0.001$ in all of them.

sucrose concentrations were highest in February, but declined thereafter, exhibiting fluctuations (Bustan et al., 2011). In November, leaf mannitol and sucrose were approximately 236 and 117 µmol g^{-1} DW, respectively, with both sugars being much higher than found here. According to the findings in Barnea, mannitol is the predominant carbohydrate in leaves, and this agrees with the present results for December, but disagrees with the sugars determined earlier here, when glucose was found at higher levels than mannitol. Indicatively, in other olive studies, the molar ratio of glucose/mannitol was 1.9 (Flora and Madore, 1993) or 1.2 (Cataldi et al., 2000) in olive

plant extracts. Indeed, the present concentration ratio of leaf glucose/mannitol ranged from 1.55 to 0.94 at MIs from 1.1 to 6.9, respectively. Glucose and mannitol in Koroneiki leaves did remain the major sugars during the whole experiment, although exhibiting an opposite pattern of changes. Significant relationships were observed among all leaf sugars, being negative between mannitol and sucrose, and glucose and fructose, but positive for the remaining relationships (Tab. S1). In leaves, mannitol showed the strongest, but negative correlation with sucrose (r = -0.923). Additionally, significant correlations were found among photosynthetic parameters and sugars, with that between Pn and leaf mannitol being the strongest and negative (r = -0.821), followed by positive correlation between Pn and leaf sucrose (r = 0.804) (Tab. S1). In other words, leaf mannitol increased at reduced photosynthesis late in the season and in contrast to decreasing leaf sucrose.

It is well known that mannitol and sucrose or raffinose saccharides are direct photosynthetic products and comprise the transportable forms of carbon. In particular, mannitol is synthesized in the cytosol and transported to vacuoles rapidly, and indeed, in the mesophyll close to the CO_2 fixation site (Flora and Madore, 1993). It is synthesized in mature leaves from mannose-6-phosphate, translocated *via* the phloem to sink tissues, stored or oxidized to mannose, and used for energy supply or a carbon source (Conde et al., 2008). Additionally, it has a protective role in plants, being a compatible solute and an oxygen radical scavenger (Shen et al., 1997; Lo Bianco et al., 2011), coping with abiotic or biotic stress. In general, soluble sugars might protect plant tissues from stress, either directly when they exist at high concentrations (Van den Ende and Valluru, 2009) or due to their signalling role, resulting indirectly in production of reactive oxygen species scavengers (Bolouri-Moghaddam et al., 2010). Indeed, mannitol was found to increase the capacity of chloroplasts to scavenge radicals and in contrast to sucrose, glucose and fructose, it does not repress photosynthesis or result in any harmful effect even at high concentrations (Bolouri-Moghaddam et al., 2010; Shen et al., 1997). Consequently, the mannitol increase in Koroneiki leaves in December might be attributed to a protective role against subsequent winter conditions.

In this study, the seasonal effect was considered as an integrated factor influencing fruit, reflecting mainly the interaction between fruit state and weather conditions. Fruit load does not seem to affect sugar allocation between leaves and fruit, apart from some extreme cases, such as severe fruit thinning, when soluble sugar accumulation in leaves and fruit was observed (Bustan et al., 2011; Haouari et al., 2013). In fruit in our study, the concentrations of sucrose, glucose, fructose and mannitol at MI 1.1 were 75.81, 271.81, 4.67 and 37.28 μmol g^{-1} DW, respectively (Fig. 2B, 2D, 2F, 2H). During the growing season, sucrose, glucose and mannitol decreased, whereas fructose increased. Decreases by 1.21-, 1.19- and 1.66-fold in sucrose, glucose and mannitol, respectively, and an increase by 1.79-fold in fructose were observed between the first and third date. However, the sugar changes, albeit significant, occurred mainly at MI 3.8, and the levels remained almost stable thereafter. The sum of these sugars, expressed per DW, decreased (by 1.21-fold) during fruit development, and glucose remained the predominant sugar in fruit during the entire experiment. These decreases in sugar and glucose predominance in fruit both agreed with other work on three olive cultivars studied from August to December (Wodner et al., 1988). The main difference between the two studies lies in the absolute glucose concentrations, being high in Koroneiki and low in the other cultivars. In contrast to these results, mannitol concentration increased during maturation in Hojiblanca olives (Marsilio et al., 2001), reaching levels close to those in green Koroneiki fruit, whereas the minimum sucrose level in Koroneiki fruit was higher than the minimum in Hojiblanca. The differences could be ascribed to compensation between mannitol and sucrose in fruit, depending on maturity stage, cultivar or environment. Moreover, in black olives, mannitol varied between 21 in Duro and 99 μmol g^{-1} DW in Thasos (Marsilio et al., 2001), with mannitol in Koroneiki being within this range. It should be noted that Koroneiki, being a late ripening cultivar with long oil-filling period (Parvini et al., 2015), possibly utilizes mannitol for longer periods than other cultivars. Indicatively, Koroneiki fruit exhibited significant and positive correlations between mannitol and sucrose or glucose, but negative between fructose and mannitol, and sucrose and glucose (Tab. S1). Relations between sucrose and fructose (r = -0.975) and between mannitol and glucose (r = 0.935) were among the strongest observed. Among the relations of fruit sugars with leaf sugars, fruit mannitol with leaf sucrose exhibited a highly significant positive relation, with the highest correlation coefficient observed (r = 0.907; Tab. S1). Taking into consideration the systems of sugar loading into the phloem and unloading and the complexity of sugar interconversions and metabolism, it would not be possible to gain detailed information about sugar transport. Moreover, fruit photosynthesis occurs at the green stage (Blanke and Lenz, 1989) and contributes to fruit carbon economy. In the fruit gas phase, CO_2 concentration rises not only due to fruit mitochondrial respiration of the photoassimilates imported from the leaves *via* the phloem, but also due to the prevention of CO_2 diffusion by cuticle impermeability. In green olives, particularly, it was shown that the photosynthetic products contribute to some extent to oil synthesis (Sanchez, 1995).

Oil content

In this study, oil content was 54.2, 60 and 64.71% (w/w DW) at MI 1.1, 3.8 and 6.9, respectively, and this gradual increase was affected significantly by advancing MI (Tab. 1). However, the majority of oil accumulation seemed to take place at MI <1.1. This observation was in general agreement with other studies on olive cultivars (Wodner et al., 1988), where approximately 80% of the oil content was accumulated until fruit reached 50% black stage. Additionally, Koroneiki exhibited approximately 8.3% mannitol in fruit and an already high oil accumulation at MI 1.1, in accordance with the proposal that fruit mannitol indicates the potential for oil accumulation (Wodner et al., 1988). In other studies, the maximal oil content was observed at different MI, with differences depending on cultivar (Bodoira et al., 2015; Dag et al., 2014).

The main oil constituents, triacylglycerols, are formed from the FAs synthesized in the plastids with glycerol-3-phosphate *via* the Kennedy pathway. FA biosynthesis, concisely, requires acetyl-CoA as a precursor, which can be formed from carbohydrates *via* glycolysis in the plastid, as well as, from pyruvate in the mitochondria that hydrolyzed to acetate, transported to plastid and forms acetyl-CoA. (Sanchez and Harwood, 2002). In this work, the oil percentage exhibited significant and relatively strong correlations with all determined sugars in both leaves and fruit. Oil accumulation exhibited particularly strong and negative correlations with leaf sucrose (r = -0.979) and fruit mannitol (r = -0.953), while being positively correlated with leaf glucose (r = 0.937) and leaf mannitol (r = 0.915) (Tab. S1). The negative correlation of fruit mannitol and oil accumulation during the late stages of fruit development here agrees with the findings of two studies (Nergiz and Engez, 2000; Nergiz and Ergonil, 2009), while not necessarily disagreeing with others (Marsilio et al., 2001; Wodner et al., 1988). Wodner et al. (1988) reported that the high levels of fruit glucose in Uovo di Piccione and of fruit fructose in Manzanillo at the beginning of August, followed by their sharp decrease, might be responsible for the rapid oil accumulation in those cultivars. The discrepancy of results between studies might be attributed to different experimental conditions, cultivars and season.

The significant and negative correlation between oil percentage and

Pn (r = -0.757; Tab. S1) was of interest, and might be associated with the late season that the experiment took place. CHERBIY-HOFFMANN et al. (2015) showed that shading applied at the beginning of phase III of fruit development reduced oil accumulation, directly confirming the necessity for sunlight and photosynthesis during olive maturation. In this study, the increase in leaf mannitol might reflect either the increased requirement of leaves for mannitol to cope with seasonal change and stress, or 'reduced transport necessity/ability' of mannitol to fruit after the completion of oil accumulation. Indeed, it was postulated that leaves have an important role as carbohydrate storage organs, while exhibiting prolonged photosynthetic activity to store adequate levels of reserves for tree survival under the unpredictable Mediterranean climatic conditions (BUSTAN et al., 2011).

Oil composition

The oil composition is very important for human nutrition. At MI 1.1, the values of palmitic (PA), palmitoleic (PO), stearic (ST), OL, vaccenic (VA), LL, linolenic (LN), arachidic (AR) and gondoic (GO) were 11.4, 1.2, 2.32, 73.43, 2.53, 6.70, 1.1, 0.49 and 0.4% (w/w in oil), respectively (Tab. 1). PA decreased at MI 6.9, while OL decreased at MI 3.8 and remained almost stable thereafter. PO showed no consistent changes, but LL exhibited a continuous increase that was highly significant. The remaining acids ST, VA, LN, AR and GO were fairly constant. All these observations were confirmed statistically (Tab. 1). At the end of the experiment, PA and OL were reduced by 1.1- and 1.03-fold, respectively, while LL increased by 0.64-fold in comparison to initial levels. At MI 1.1, the values of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA), unsaturated fatty acids/saturated fatty acids (UFA/SFA) and linolenic acid/linoleic acid (ω-6/ω-3) were 14.22, 77.55, 7.8, 6 and 6.09, respectively, but significant changes occurred, resulting in SFA and MUFA values reduced by 1.08- and 1.04-fold, respectively, whereas PUFA, UFA/SFA and ω-6/ω-3 increased by 1.48-, 1.09- and 1.43-fold, respectively, by the end of the experiment. These changes during fruit maturation were in general agreement with another study on Koroneiki (ANASTASOPOULOS et al., 2011).

Here, OL was already at 73% of total FAs early in November. OL then decreased to approximately 70%, indicating that the most active oil biosynthesis occurred early during fruit development, in agreement with other studies (BODOIRA et al., 2015). The decrease in OL at the two last sampling dates resulted in an apparently negative correlation of OL with oil percentage (Tab. S1). OL was also significantly positively correlated with fruit glucose, sucrose and mannitol, and leaf glucose and sucrose, with the strongest correlation being with fruit glucose (r = 0.955) (Tab. S1).

The main FAs in Koroneiki oil were found to be OL and LL (Tab. 1), as expected. In general, stearoyl-ACP Δ9-desaturase in the plastids and oleoyl-ACP Δ9-desaturase in the endoplasmic reticulum are

Tab. 1: Content of individual fatty acids (FA), total saturated (SFA), mono-unsaturated (MUFA), poly-unsaturated FA (PUFA) and total unsaturated FA (UFA), on the unsaturation degree (UFA/SFA) and ω-6/ω-3 ratios in oil, as well as, on oil content, total phenolics concentration (TP) and total antioxidant capacity (TAC) in the flesh in Koroneiki olive fruit, in response to seasonal development and fruit maturity indices (MI) in 2010.

		Units	Date – MI			SE[b]	P [c]
			5/11 – MI 1.1	19/11 – MI 3.8	22/12 – MI 6.9		
Oil	Palmitic $C_{16:0}$	%	11.41 ± 0.10 [a]	11.73 ± 0.28	10.20 ± 0.21	0.12	***
	Palmitoleic $C_{16:1\ n-9}$	%	1.20 ± 0.06	1.39 ± 0.05	1.19 ± 0.07	0.03	*
	Stearic $C_{18:0}$	%	2.32 ± 0.04	2.42 ± 0.08	2.41 ± 0.16	0.06	NS
	Oleic $C_{18:1\ n-9}$	%	73.43 ± 0.46	70.14 ± 0.66	70.75 ± 0.50	0.31	***
	Vaccenic $C_{18:1\ n-11}$	%	2.53 ± 0.06	2.67 ± 0.09	2.47 ± 0.10	0.05	NS
	Linoleic $C_{18:2\ n-9,12}$	%	6.70 ± 0.39	9.09 ± 0.34	10.38 ± 0.13	0.18	***
	Linolenic $C_{18:3\ n-9,12,15}$	%	1.10 ± 0.05	1.16 ± 0.04	1.19 ± 0.05	0.03	NS
	Arachidic $C_{20:0}$	%	0.49 ± 0.01	0.53 ± 0.04	0.54 ± 0.04	0.02	NS
	Gondoic $C_{20:1\ n-11}$	%	0.40 ± 0.02	0.41 ± 0.01	0.41 ± 0.01	0.01	NS
	SFA $C_{v:0}$	%	14.22 ± 0.07	14.67 ± 0.35	13.15 ± 0.39	0.18	**
	MUFA $C_{v:1}$	%	77.55 ± 0.45	74.61 ± 0.70	74.83 ± 0.41	0.31	***
	PUFA $C_{v:n\ (n\geq1)}$	%	7.80 ± 0.41	10.25 ± 0.38	11.57 ± 0.13	0.19	***
	UFA	%	85.35 ± 0.04	84.85 ± 0.33	86.40 ± 0.39	0.17	**
	UFA/SFA		6.00 ± 0.03	5.78 ± 0.16	6.57 ± 0.22	0.09	
	ω-6/ω-3		6.09 ± 0.29	7.85 ± 0.09	8.75 ± 0.40	0.17	***
Flesh DW	Oil	%	54.28 ± 0.59	60.03 ± 1.32	64.71 ± 0.97	0.58	***
	TP	μmol g^{-1}	208.00 ± 10.24	136.63 ± 6.00	140.63 ± 4.68	4.25	***
	TAC	μmol g^{-1}	161.66 ± 2.29	104.00 ± 8.26	107.66 ± 4.25	3.19	***

[a] Numbers are means ± standard deviation
[b] Standard Error from the residual variance (ANOVA) of three replicates, d.f. = 6.
[c] Probabilities.
NS, not significant.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

responsible for OL and LL synthesis, respectively (SANCHEZ and HARWOOD, 2002). During the late developmental stages, LL increases at expense of OL were possibly due to oleoyl-Δ9-desaturase, while stearoyl-Δ9-desaturase seemed to be still active for OL synthesis (BANILAS et al., 2005). Indeed, in this work, LL was positively related to oil percentage (r = 0.979), but negatively to OL. As mentioned earlier, LL contributes to oil oxidation, which is undesirable and thus, these increases should be avoided.

Changes in SFA, MUFA, PUFA, UFA/SFA could be largely attributed to OL and LL changes. The UFA/SFA ratio along with the OL percentage value belong to the criteria for the characterization of oil quality. Koroneiki exhibits a high proportion of OL, being higher than in other cultivars (PARVINI et al., 2015). Additionally, decreases in the ratio of MUFA/PUFA were connected with oil deterioration during advanced fruit development and/or in relation to field temperature increases (DAG et al., 2014). Here, MUFA/PUFA decreased gradually, being 9.94, 7.27 and 6.46 at MI 1.1, 3.8 and 6.9, respectively, indicating a deterioration at advanced maturation. The ratio of ω-6/ω-3 was included into the present results since it is considered important for diseases prevention or therapy (SIMOPOULOS, 2008).

Total phenolics (TP) and total antioxidant capacity (TAC)

At MI 1.1, determinations of TP and TAC were 208 μmol g^{-1} DW and 161.66 μmol g^{-1} DW, respectively (Tab. 1). Both variables reduced considerably at MI 3.8 and remained almost stable thereafter. At MI 6.9, TP and TAC were reduced by 1.47- and 1.5-fold, respectively, against the initial values. These reductions during ripening complied with other studies (MORELLO et al., 2005). Here, TP was positively and strongly related to TAC, as expected (TSANTILI, 2014). Both TP and TAC showed highly significant and positive correlations with fruit sucrose, glucose and mannitol, but negative with fruit fructose (Tab. S1). These correlations could be explained by the antioxidant character of sugars, as mentioned earlier. It is also noted that TAC exhibited a highly significant, positive and strong relationship with OL (r = 0.973), but a negative one with LL (r = -0.903), suggesting the prevention of LL increases.

Principal component analysis overview

The PCA was performed for all 24 variables determined and already presented in Fig. 1, Fig. 2 and Tab. 1. It showed two interpretable components, explaining together 76.05% (eigenvalue 3.55) of the total variation (Fig. 3). In score plot, all fruit were separated according to their MI, in green, red and black olives. Correlations among the variables presented in Tab. S1 are summarized in the load plot, in two main groups, G1 and G2, the left-side and the right-side groups, respectively. Particularly, G1 includes Ci, leaf mannitol, fruit fructose, oil %, LL, LN, AR and GO, whereas G2 includes Pn, leaf fructose, leaf sucrose, leaf glucose, fruit mannitol, fruit sucrose, fruit glucose, OL, TP, TAC and E rate. Positive correlations are shown among the variables in each separate group, whereas negative ones among variables between groups.

The mannitol effect

The previous experiment confirmed the close relationship among sucrose and glucose in both leaf and fruit, and oil content. However, the reduction of fruit mannitol in parallel with oil accumulation was not a definitely expected observation. It is known that the higher mannitol in fruit, the more energy is released from mannitol degradation that could be available for oil synthesis *via* acetyl-CoA (MARSILIO et al., 2001). Considering the transportable character of mannitol (CONDE et al., 2008) in conjunction with the above observations, the authors proceeded to preharvest treatments with mannitol. Indeed, in II, the oil percentage increased progressively by advanced mannitol concentration, being higher by 1.043- and 1.076-fold in fruit treated with the lower and higher mannitol concentration, respectively, in comparison with controls (Tab. 2). Additionally, OL, ST and MUFA increased, whereas LL, PUFA and ω-6/ω-3 were lowered by increasing mannitol concentration. Also, the ratio of MUFA/PUFA increased from 7.43 in controls to 8.41 and 8.97 by mannitol at 50 and 100 mg L^{-1}, respectively. These changes seemed to conform to the retention of oil quality in mannitol treated olives. Values of PA, PO, VA, LN, AR, GO, SFA and UFA/SFA were not affected significantly by the treatment. However, in II, MI of controls and

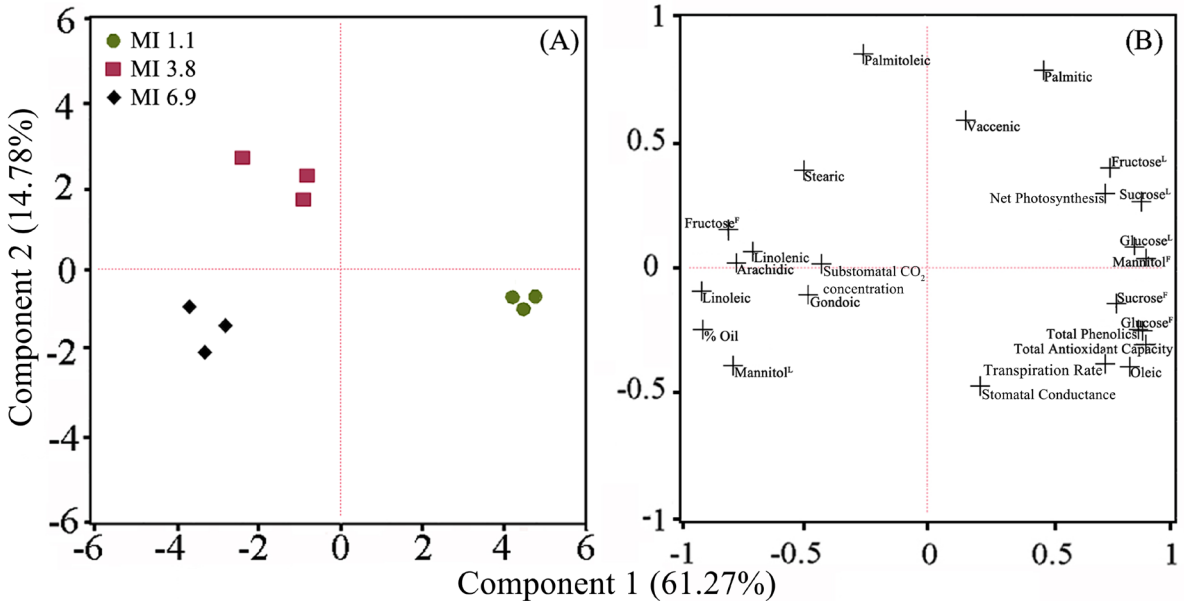

Fig. 3: Principal Component Analysis (PCA) in Koroneiki olive tree according to 24 variables (related to leaf photosynthetic parameters and sugars, and fruit sugars, oil content, fatty acids and total antioxidants) affected by seasonal growth - maturity index, in 2010 (A and B). In PCA: A, Score plot; B, Load plot. In A: MI, maturity index; circle, green olives at MI 1.1; squares, red olives at MI 3.8; rhombus, black olives at MI 6.9. In B: dagger indicates the position of each variable in load plot. Numbers in parentheses correspond to the percentage of the total variance explained by each component. ^{L}, leaf; ^{F}, fruit.

Tab. 2: Effect of preharvest mannitol treatment on content of individual fatty acids (FA), total saturated (SFA), mono-unsaturated (MUFA), poly-unsaturated (PUFA), and total unsaturated FA (UFA), on the unsaturation degree (UFA/SFA) and ω-6/ω-3 ratios in oil, as well as, on oil content, total phenolics concentration (TP) and total antioxidant capacity (TAC) in the flesh in Koroneiki olive fruit, in 2011.

		Units	Mannitol concentration (mg L^{-1})			SE[b]	*P* [c]
			Control	50	100		
Oil	Palmitic $C_{16:0}$	%	13.86 ± 0.67[a]	13.45 ± 0.75	12.92 ± 0.34	0.35	NS
	Palmitoleic $C_{16:1\ n-9}$	%	1.10 ± 0.06	1.02 ± 0.08	0.97 ± 0.06	0.04	NS
	Stearic $C_{18:0}$	%	3.42 ± 0.02	3.18 ± 0.10	3.60 ± 0.08	0.04	**
	Oleic $C_{18:1\ n-9}$	%	68.78 ± 0.60	70.44 ± 0.84	71.22 ± 0.77	0.43	*
	Vaccenic $C_{18:1\ n-11}$	%	2.01 ± 0.01	1.74 ± 0.19	1.92 ± 0.04	0.06	NS
	Linoleic $C_{18:2\ n-9,12}$	%	8.79 ± 0.14	7.89 ± 0.47	7.29 ± 0.55	0.25	*
	Linolenic $C_{18:3\ n-9,12,15}$	%	0.92 ± 0.07	0.86 ± 0.05	1.00 ± 0.06	0.04	NS
	Arachidic $C_{20:0}$	%	0.52 ± 0.04	0.62 ± 0.03	0.58 ± 0.04	0.02	NS
	Gondoic $C_{20:1\ n-11}$	%	0.30 ± 0.04	0.42 ± 0.10	0.33 ± 0.01	0.04	NS
	SFA $C_{v:0}$	%	17.80 ± 0.62	17.24 ± 0.82	17.10 ± 0.27	0.31	NS
	MUFA $C_{v:1}$	%	72.19 ± 0.63	73.62 ± 0.96	74.44 ± 0.74	0.46	*
	PUFA $C_{v:n\ (n\geq1)}$	%	9.71 ± 0.16	8.75 ± 0.49	8.29 ± 0.53	0.25	*
	UFA	%	81.89 ± 0.47	22.37 ± 0.53	82.73 ± 0.29	0.26	NS
	UFA/SFA		4.61 ± 0.19	4.79 ± 0.25	4.84 ± 0.09	0.10	NS
	ω-6/ω-3		9.58 ± 0.70	9.20 ± 0.78	7.29 ± 0.85	0.45	*
Flesh DW	Oil	%	52.30 ± 1.18	54.55 ± 1.35	58.73 ± 0.85	0.66	***
	TP	μmol g^{-1}	230.44 ± 10.48	172.22 ± 4.63	184.12 ± 9.65	4.99	***
	TAC	μmol g^{-1}	146.75 ± 3.09	99.44 ± 8.37	128.31 ± 4.93	3.40	***

[a] Numbers are means ± standard deviation.
[b] Standard Error from the residual variance (ANOVA) of three replicates, d.f. = 6.
[c] Probabilities.
NS, not significant.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

treated olives with the lower and higher mannitol concentration were 3.3, 3.5 and 3.5, respectively, whereas the treatment effect was not significant ($P > 0.05$; SE = 0.212). Besides, mannitol concentration reduced TP and TAC in fruit, and this could be attributed rather to an effect of mannitol on promoting the olive metabolism. The current results seemed to be promising for increasing the oil accumulation, while no quality oil deterioration seemed to occur.

Conclusions

This study was a first attempt to investigate different steps in the chain of oil accumulation during fruit maturation in Koroneiki olives. Being aware of the long chain and the complexity of various steps, the study was focused on photosynthesis, as the initial step, on changes in concentrations of the major sugars in leaves and fruit, and on oil accumulation and composition in green, green-cherry and black olives from early November up to late December. According to results, leaf sucrose, glucose and fructose decreased during fruit maturation, while leaf mannitol increased, with glucose and mannitol remained the major sugars in leaves on all sampling dates. In fruit, glucose and sucrose were the major sugars, even after their decreases during fruit maturation. However, fruit fructose increased, whereas fruit mannitol decreased. Preharvest treatment with mannitol resulted in increased oil accumulation without decreasing the oil quality, as estimated by FA analysis. The mannitol effect seemed to be related to an accelerated metabolism. Koroneiki exhibits a long oil accumulation period. Thus, mannitol treatment seemed to be promising for increasing the oil accumulation earlier than usual and thus, harvest would be completed before the unfavourable winter conditions that make the harvest hard and deteriorate the oil quality. However, the positive mannitol treatments raised questions concerning the physiology of exogenous mannitol uptake, transport and utilization. Consequently, further studies are needed to investigate the mannitol effects from the aspect of basic research and of applied, as well.

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Tab. S1: Pairwise correlations among photosynthetic parameters, sugars in leaves and fruit, oil content, individual fatty acids (FA), total phenolics concentration (TP) and total antioxidant capacity (TAC) in olive fruit of different sampling dates – maturity index(MI), in 2010[#].

	Pn	g_s	Ci	E rate	SUC^L	GLU^L	FRU^L	MAN^L	SUC^F	GLU^F	FRU^F	MAN^F	% Oil	PA	PO	ST	OL	VA	LL	LN	AR	GO	TP
g_s	0.298^{NS}																						
Ci	-0.490^{NS}	-0.410^{NS}																					
E rate	0.478^{NS}	0.644^{NS}	-0.369^{NS}																				
SUC^L	0.804**	0.087^{NS}	-0.692*	0.533^{NS}																			
GLU^L	0.581^{NS}	0.331^{NS}	-0.694*	0.780*	0.876**																		
FRU^L	0.725*	0.159^{NS}	-0.305^{NS}	0.668*	0.813**	0.819**																	
MAN^L	-0.821**	0.051^{NS}	0.511^{NS}	-0.495^{NS}	-0.923***	-0.762*	-0.813**																
SUC^F	0.625^{NS}	0.574^{NS}	-0.772*	0.844**	0.775*	0.877**	0.598^{NS}	-0.653^{NS}															
GLU^F	0.662^{NS}	0.266^{NS}	-0.494^{NS}	0.750*	0.786*	0.773*	0.641^{NS}	-0.721*	0.817**														
FRU^F	-0.662^{NS}	0.451^{NS}	0.768*	-0.755*	-0.868**	-0.857**	-0.596^{NS}	0.690*	-0.975***	-0.844**													
MAN^F	0.750*	0.185^{NS}	-0.525^{NS}	0.756*	0.907***	0.872**	0.825**	-0.875**	0.836**	0.935***	-0.863**												
% Oil	-0.757*	-0.144^{NS}	0.648^{NS}	-0.663^{NS}	-0.979***	0.937***	-0.869**	0.915***	-0.819**	-0.834**	0.852**	-0.953***											
PA	0.655^{NS}	-0.252^{NS}	-0.329^{NS}	0.121^{NS}	0.734*	0.533^{NS}	0.750*	-0.819**	0.271^{NS}	0.244^{NS}	-0.316^{NS}	0.522^{NS}	-0.687*										
PO	0.122^{NS}	-0.235^{NS}	0.104^{NS}	-0.381^{NS}	-0.019^{NS}	-0.143^{NS}	0.173^{NS}	-0.126^{NS}	-0.367^{NS}	-0.556^{NS}	0.388^{NS}	-0.291^{NS}	0.083^{NS}	0.631^{NS}									
ST	-0.209^{NS}	-0.160^{NS}	0.051^{NS}	-0.515^{NS}	-0.267^{NS}	-0.406^{NS}	-0.317^{NS}	0.326^{NS}	-0.333^{NS}	-0.618^{NS}	0.277^{NS}	-0.439^{NS}	0.354^{NS}	0.044^{NS}	0.422^{NS}								
OL	0.522^{NS}	0.385^{NS}	-0.634^{NS}	0.741*	0.716*	0.778*	0.490^{NS}	-0.577^{NS}	0.866**	0.955***	-0.876**	0.847**	-0.765*	0.077^{NS}	-0.667*	-0.583^{NS}							
VA	0.311^{NS}	-0.020^{NS}	-0.110^{NS}	-0.157^{NS}	0.231^{NS}	0.162^{NS}	0.408^{NS}	-0.208^{NS}	-0.166^{NS}	-0.107^{NS}	0.187^{NS}	0.007^{NS}	-0.195^{NS}	0.541^{NS}	0.615^{NS}	-0.052^{NS}	-0.173^{NS}						
LL	-0.779*	-0.221^{NS}	0.717*	-0.662^{NS}	-0.967***	-0.913***	-0.790*	0.880**	-0.855**	-0.893**	0.886**	-0.955***	0.979***	-0.582^{NS}	0.204^{NS}	0.406^{NS}	-0.852**	-0.170^{NS}					
LN	-0.438^{NS}	-0.119^{NS}	0.584^{NS}	-0.396^{NS}	-0.619^{NS}	-0.638^{NS}	-0.426^{NS}	0.579^{NS}	-0.523^{NS}	-0.712*	0.496^{NS}	-0.624^{NS}	0.645^{NS}	-0.269^{NS}	0.290^{NS}	0.703*	-0.740*	-0.332^{NS}	0.735*				
AR	-0.507^{NS}	-0.332^{NS}	0.514^{NS}	-0.541^{NS}	-0.744*	-0.597^{NS}	0.492^{NS}	-0.623^{NS}	-0.699*	0.650^{NS}	-0.626^{NS}	0.695*	-0.281^{NS}	0.261^{NS}	0.638^{NS}	-0.717*	-0.326^{NS}	0.728*	0.677*	-0.520^{NS}			
GO	-0.528^{NS}	-0.023^{NS}	0.109^{NS}	-0.238^{NS}	-0.520^{NS}	-0.367^{NS}	-0.465^{NS}	0.403^{NS}	-0.262^{NS}	-0.612^{NS}	0.364^{NS}	-0.493^{NS}	0.482^{NS}	-0.226^{NS}	0.260^{NS}	0.587^{NS}	-0.516^{NS}	-0.323^{NS}	0.526^{NS}	0.503^{NS}	0.813**		
TP	0.664^{NS}	0.488^{NS}	-0.701*	0.835**	0.778*	0.836**	0.583^{NS}	-0.711*	0.968***	0.906***	-0.949***	0.880**	-0.823**	0.251^{NS}	-0.446^{NS}	-0.498^{NS}	0.922***	-0.196^{NS}	-0.877**	-0.630^{NS}	-0.638^{NS}	-0.363^{NS}	
TAC	0.657^{NS}	0.464^{NS}	-0.622^{NS}	0.828**	0.790*	0.845**	0.615^{NS}	-0.683^{NS}	0.939***	0.959***	-0.936***	0.909***	-0.839**	0.215^{NS}	-0.528^{NS}	-0.539^{NS}	0.973***	-0.131^{NS}	-0.903***	-0.686*	-0.712*	-0.484^{NS}	0.977***

[#] Pn, net photosynthesis; g_s, stomatal conductance; Ci, substomatal carbon dioxide concentration; E rate, transpiration rate; SUC, sucrose; GLU, glucose; FRU, fructose; MAN, mannitol; ^{L}, leaf; ^{F}, fruit; PA, palmitic; PO, palmitoleic; ST, stearic; OL, oleic; VA, vaccenic; LL, linoleic; LN, linolenic; AR, arachidic; GO, gondoic.

NS, not significant.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.