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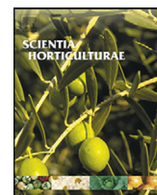

Effects of irrigation on fruit ripening behavior and metabolic changes in olive

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ABSTRACT

Olive (*Olea europaea*, cv Leccino) fruits grown under different water regimes were analyzed by metabolomics and specific transcript accumulation analyses. The fruit from non-irrigated (rain-fed) and irrigated trees cultivated under field conditions, with a seasonal water amount equivalent to the calculated crop evapotranspiration (ETc) was compared in the last developmental phase and, in particular, at commercial harvest. Metabolomics (GC–MS) analysis identified several hundred metabolites in ripe mesocarp, 46 of which showed significantly different contents in the rain-fed and irrigated samples. Some compounds involved in primary metabolism (carbohydrates, amino acids, organic acids) and secondary metabolism (squalene, simple phenols) appeared to be more abundant when irrigation was performed. Higher levels of total polyphenol were observed in the rain-fed fruit, which at ripening showed an increase in anthocyanin concentration. These data indicate that ripening in olives is affected by irrigation. In addition, expression analyses of three key polyphenol biosynthetic genes (*phenylalanine ammonia lyase* (*PAL*), *chalcone synthase* (*CHS*), *dihydroflavonol reductase* (*DFR*)) and two genes involved in triterpenoid metabolism (*β-amyrin synthase* and *cycloartenol synthase*) were also performed. The analyzed genes showed different expression patterns throughout ripening, and the resulting *PAL*, *DFR* and *β-amyrin synthase* transcript accumulation was found to be affected by the different water regimes at specific stages of fruit development.

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1. Introduction

In many of the world's agricultural areas including the Mediterranean region, water shortage and prolonged periods of drought are considered as the main production-limiting factors for a number of crops. Although it is well demonstrated that the olive, a typical Mediterranean crop, is a drought-tolerant species that can survive under prolonged periods of drought, irrigation has been introduced into modern intensive oil olive orchards (Lavee, 2011). In addition studies have assessed the physiological and yield responses of olive trees grown under different water regimes (d'Andria et al., 2004, 2009; Grattan et al., 2006; Tognetti et al., 2006; Iniesta et al., 2009). Different water regimes affect the general development and the composition of olive fruit (Alegre et al., 1999; Chaves et al., 2010) leading, however, to only slight changes in the taste of the resulting oil (Lavee, 2011). Particular attention has been given to the changes in phenolic compounds, present as a complex mixture in both olive fruits and oil (Patumi et al., 2002; Gomez-Rico et al., 2006; d'Andria et al., 2004, 2009) and characterized by antioxidant, anti-atherogenic, anti-cancerogenic properties (Hashim et al., 2008; Llorente-Cortes et al., 2010). Although this is not a general effect and responses may differ in relation to the genotype, generally olives harvested from irrigated trees show a lower accumulation of total phenols (Tovar et al., 2001; Romero et al., 2002; Marsilio et al., 2006). Contrasting evidence has not completely clarified the relation between water availability and the oleuropein content in the drupes (Patumi et al., 2002; Gomez-Rico et al., 2009). Besides phenol compounds, water availability may affect the concentration of other metabolites in olive fruit or oil. Interestingly, qualitative parameters, such as the unsaturated to saturated fatty acid ratio have been found to be higher in oils produced in rain-fed compared to irrigated conditions (Gomez-Rico et al., 2007). Although oil products have been characterized for important metabolites (Romero et al., 2002; Ben-Gal et al., 2011), information concerning the primary and secondary metabolic processes of olives cultivated under different water availability is still fragmentary. Compositional changes in mature fruit under different agronomic conditions (including water availability) are the result

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of an altered metabolism and physiology, which affect the developmental cycle including ripening. Metabolomics is often viewed as complementary to other functional genomic techniques, such as transcriptomics and proteomics, and is now increasingly used in plant sciences (Okazaki and Kazuki, 2012). Since metabolomes influence phenotypes more directly than transcripts or proteins, and changes are often amplified compared to those in the transcriptome or proteome, metabolomics analysis is extremely valuable in assessing the effects of environmental or agronomical factors on produce composition. Gas chromatography–mass spectrometry (GC–MS) is particularly suitable for the non-targeted metabolite profiling of volatile and thermally stable polar and non-polar metabolites (Parker et al., 2009).

The aim of this study was to evaluate the effects of irrigation on ripening and on the metabolic profiles of olives at commercial harvest. To complement this approach, and considering the lack of specific information, gene expression analyses were carried out for some genes in order to gain insight into the transcriptional regulation of important classes of secondary metabolism, such as phenol compounds and terpenoids, which are known to be affected by water availability in developing olives.

2. Materials and methods

2.1. Experimental design, water management, water potential measurements and fruit sampling

A comparison between olive (*Olea europaea* (L.), cv Leccino) trees non-irrigated (rain-fed, RF) and irrigated (IR) with a seasonal water amount equivalent to the calculated crop evapotranspiration (ETc) was performed. The experimental site was located near Benevento (41°06′N, 14°43′E; 250 m above sea level), in a hilly olive-growing area of southern Italy. The soil was sandy loam (1.76% organic matter, 1% $CaCO_3$, 0.15% N, pH 7.2), characterized by a volumetric water content (m^3/m^3) of 35.6% at field capacity (soil matric potential of −0.03 MPa) and 21.2% at wilting point (soil matric potential −1.5 MPa), and an apparent bulk density of 1.25 t/m^3. Trees (15-year old) were planted 6 m between rows and 3 m within rows (density of 555 trees/ha). Water-relation measurements were carried out on six trees for each RF and IR conditions visually selected for homogenous tree size and crop load. Water was delivered by a drip irrigation system starting from the beginning of pit hardening (day of the year (DOY) 145) to the commercial harvest time (DOY 317) (Fig. 1). ETc was estimated from Class ‘A’ pan evaporation and data were corrected with a pan coefficient (kp) of 0.8 (to obtain reference crop evapotranspiration, ETo), a crop coefficient (kc) equal to 0.65 and a tree ground cover coefficient (kr) of 0.85. Irrigation volume was 181 mm while ETc during the irrigation period was 191 mm. Leaf water potentials were periodically measured at predawn (predawn Ψ_{leaf}) on three leaves per tree from three individual trees for each treatment. Stem water potential was measured at midday (midday Ψ_{stem}) on leaves previously enclosed in reflective envelopes for a period of 1–2 h to equilibrate Ψ_{leaf} to Ψ_{stem}. Predawn Ψ_{leaf} and midday Ψ_{stem} measurements were carried out detaching fully expanded leaves located in the middle part of the canopy and rapidly enclosing them in a Scholander pressure chamber (SKPM 1400, Sky Instruments, UK).

Fruits were separately sampled from three different trees for both IR and RF at DOY 229, 255, 272 and 302. The DOY 229 corresponded to post pit-hardening stage, DOY 255 to the second exponential growth stage, DOY 272 to the pre-veraison (“mature-green”) stage, whereas the last sampling date (DOY 302) to the ripe stage of the fruit (complete pigmentation of the epicarp). Fruit tissues were frozen in liquid nitrogen and stored at −80 °C. At commercial harvest (DOY 317), yield and average fruit weight for each of the three trees per treatment were recorded.

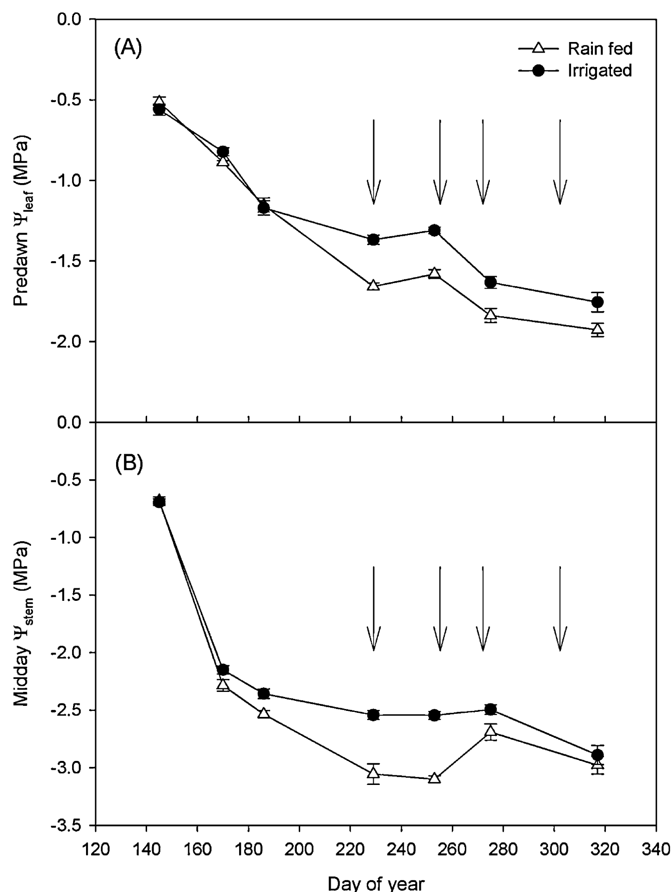

Fig. 1. Seasonal pattern of predawn leaf water potential (predawn Ψ_{leaf}) and midday stem water potential (midday Ψ_{stem}) measured in rain-fed and irrigated trees. DOY = day of the year. Arrows indicate the four dates of fruit sampling. Bars represent ±SD.

2.2. Total polyphenol and anthocyanin quantification

A pool (10–15 fruits) of frozen fruit tissue (mesocarp + epicarp) was separately ground in a pre-chilled mortar for each of the three biological replicates per treatment. Aliquots (0.5 g) were used for extractions with 25 mL methanol (80%, v/v), agitated in darkness at 4 °C for 15 min. After centrifugation at 5000 rcf for 10 min, supernatant was recovered and the entire procedure was repeated for three times. Supernatants were combined, filtered and used for analysis. Total polyphenol concentration was determined using the Folin–Ciocalteau method and absorbance was measured at 750 nm. Concentrations were expressed as gallic acid equivalents/g FW. Concentration of anthocyanins was expressed as cyanidin-3-glucoside equivalents/g FW determined using Beer’s law of spectrometer readings at 535 nm and an extinction coefficient of 29,600.

2.3. Gene expression analysis

RNA was extracted from mesocarp + epicarp (at DOY 229, 255, and 272), and separately from mesocarp and epicarp (at DOY 302) according to Galla et al. (2009). Three biological replicates (fruit sampled from different trees) were considered as a pool of homogeneous 5–6 fruits each. Semi-quantitative RT-PCR was performed using specific primers (Table 1) for *phenylalanine ammonia lyase* (*PAL*), *chalcone synthase* (*CHS*), *dihydroflavonol reductase*

Table 1
Genes and primers used in semi-quantitative PCR analyses.

Gene	Primers
PAL	F 5′-CGCCGTGCTTACCCCTCCGTGG-3′ R 5′-TGAAGCCAAGCCAGAACCAACAGCC-3′
CHS	F 5′-TCATGATGTACCAACAGGGCTGCTTCG-3′ R 5′-GGCCGCTCCACCCCAATCACC-3′
DFR	F 5′-GCT TCT GGC TTC ATC GGC TCA TGG-3′ R 5′-CTC CTT CAC ATC CGT GGA TTG CTT CGT-3′
β-amyrin synthase	F 5′-CGG AAA TTG AAG GGA GTT CAC CCC TG-3′ R 5′-CGG CGT TTT CAG CTG GCC AAT GG-3′
cycloartenol synthase	F 5′-AGA AGT GGA TTC TGG ATC ATG GTG GTG C-3′ R 5′-AAT TGG CCC CAC AAA CCT CTT CCC-3′

(*DFR*), *cycloartenol synthase* and *β-amyrin synthase* genes according to the manufacturer's instructions (Reverse Transcription System, Promega). 18S RNA was used as housekeeping gene following the instructions of the Quantum 18S RNA Universal kit (Ambion Inc.). Amplification mix was prepared using the goTaq protocol (Promega) and amplification products were scanned and quantified using Quantity One software (BioRad).

2.4. Metabolite extraction and derivatization

For the metabolomics analysis, six biological replicates (two replicates for each of the three individual sampling), represented by a pool of 10 fruits (mesocarp + epicarp) collected at DOY 302 (mature stage) were used. For each sample, 2 mL of pre-chilled extraction solvent (MetOH:$CHCl_3$:1:1) (v/v) was added to 20 mg of ground tissue and maintained in agitation at 4 °C per 5 min. After vortexing and centrifugation (6000 rpm for 2 min), aliquots of 20 μl of supernatant whereas completely dried in a SpeedVac concentrator. After derivatization using methoxyamine and N-methyl-N-(trimethylsilyl)trifluoroacetamide, samples were analyzed using the Agilent GC–quadrupole MS. The following GC/MS conditions were used. An Agilent 6890 GC oven was ramped by 10 °C/min from 60 °C (1 min initial time) to 325 °C (10 min final time), resulting in a 37.5 min run time with cooling down to 60 °C. 1 μL was injected into the Agilent split/splitless injector at 250 °C by a 10 μL syringe with 4 sample pumps, 1 pre-injection wash and 2 post-injection washes. No viscosity delay or dwell time was applied using a fast plunger speed. Samples were introduced in both splitless and split conditions. For splitless conditions, a helium purge flow of 10.5 mL/min was applied for 1 min (8.2 psi). A constant flow rate of 1 mL/min helium was used as carrier gas. The quadrupole mass spectrometer was switched on after a 5.90 min solvent delay time, scanning from 50 to 600 u. The source temperature was set to 230 °C and the quadrupole temperature was 150 °C. Prior to acquisition, the MSD was autotuned using FC43 according to the instrument manual. When using split injections, parameters used were identical as given above but with a split ratio of 1:10 and a split flow rate of 10.3 mL/min.

2.5. Data acquisition, peak identification and statistical analysis

Missing values in the data matrix were replaced with "XX". Compounds that were not detected in at least 10% of the sample within a class were discarded.

Internal standards were spiked at the moment of the analysis for each samples. Relative concentrations were determined by peak area (mm^2). All peak detections were manually checked for false positive and false negative assignments. Retention time locking reduces run-to-run retention time variation. The Agilent Fiehn GC/MS Metabolomics RTL Library was employed for metabolite identifications. This library is one of the most comprehensive library of metabolite GC/MS spectra that is commercially available. It contains searchable GC/MS EI spectra and retention time indexes from approximately 700 common metabolites.

Data were statistically analyzed using Agilent Mass Profiler Professional Software with default parameters for noise reduction, normalization, mass spectral and compound identification. The significance of effects of Irrigation (I), Time (T), and $I \times T$ treatments on predawn Ψ_{leaf}, midday Ψ_{stem}, and flesh concentrations of polyphenols and anthocyanins were calculated with repeated measures analysis of variance (repeated measures ANOVA). The significance of the effect of irrigation treatments on the concentration of metabolites and on gene expression was calculated with one-way ANOVAs ($P \leq 0.05$) with the Duncan post hoc test.

Table 2
Probability of the significance of the effects of Irrigation, Time, and Irrigation × Time treatments on predawn Ψ_{leaf}, midday Ψ_{stem}, and fruit concentrations of polyphenols and anthocyanins calculated with repeated measures analysis of variance.

Source of variation	Probability			
	Predawn Ψ_{leaf}	Midday Ψ_{stem}	Polyphenols	Anthocyanins
Irrigation (I)	<0.001	<0.001	<0.002	0.140
Time (T)	<0.001	<0.001	<0.001	0.001
$I \times T$	0.005	0.002	0.290	<0.001

3. Results and discussion

The aim of this work was to study the metabolic changes of olive fruit in response to irrigation by a metabolomics approach and expression analysis of specific genes involved in important pathways of secondary metabolism (phenol compounds and terpenoids).

In terms of environmental parameters, a total of 156.3 mm of rainfall was registered from the beginning of May to harvest (November 13th). Rainfall events occurred in May (20.5% of the total), June (13.3%), September (20.6%), October (41%), and November (4.6%). No rainfall was registered in July and August.

Irrigation, time, and $I \times T$ significantly affected both pre-dawn Y_{leaf} and midday Y_{stem} (Table 2). Significant differences between irrigation treatments in pre-dawn Y_{leaf} occurred for the first time on DOY 229 and were maintained until the end of the experiment (Fig. 1A). Maximum significant differences between irrigation treatments in midday Y_{stem} were measured between DOYs 229 and 253 when the first two fruit samples were collected (Fig. 1B).

Fruit yield of IR trees was significantly higher than RF trees (10.2 ± 1.13 and 7.0 ± 0.55 kg/tree ± SD, respectively). Similarly, irrigation increased fresh fruit weight (2.2 ± 0.07 and 1.6 ± 0.06 g/fruit ± SD in IR and RF trees, respectively).

3.1. Polyphenol metabolism and anthocyanin accumulation

Several studies have focused on how different water regimes affect olive oil yield and quality (fatty acid composition and ratio, acidity, peroxide number, phenolic compound concentration), and organoleptic properties (fruiting, bitterness, pungency) (Patumi et al., 2002; Berenguer et al., 2006; Gomez-Rico et al., 2007; Tognetti et al., 2007). Particular attention has been paid to the changes in phenolic compounds, present as a complex mixture in both olive fruit and oil (Patumi et al., 2002; d'Andria et al., 2004, 2009), and recent studies have also focused on other healthy metabolites such as α-tocopherol and squalene (Palese and Nuzzo, 2010; Ben-Gal et al., 2011).

Irrespectively of the treatment, polyphenols decreased significantly during fruit ripening, whereas anthocyanins accumulated significantly only after DOY 272 (Fig. 2 and Table 2). For all the

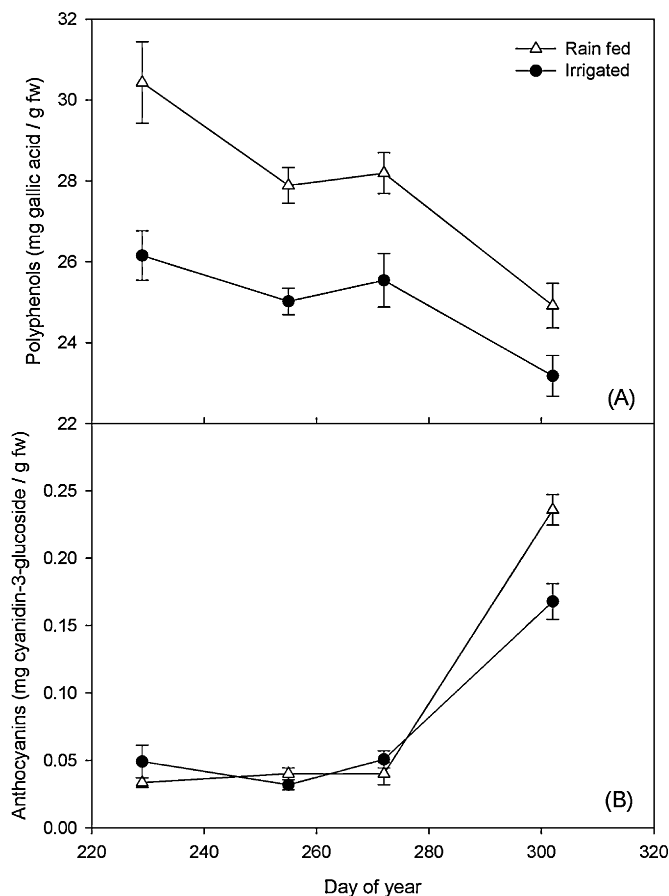

Fig. 2. Total polyphenol (A) and anthocyanin (B) concentration in fruit (mesocarp + epicarp) sampled from rain-fed and irrigated olive trees in correspondence of four developmental stages. Bars represent ±SD.

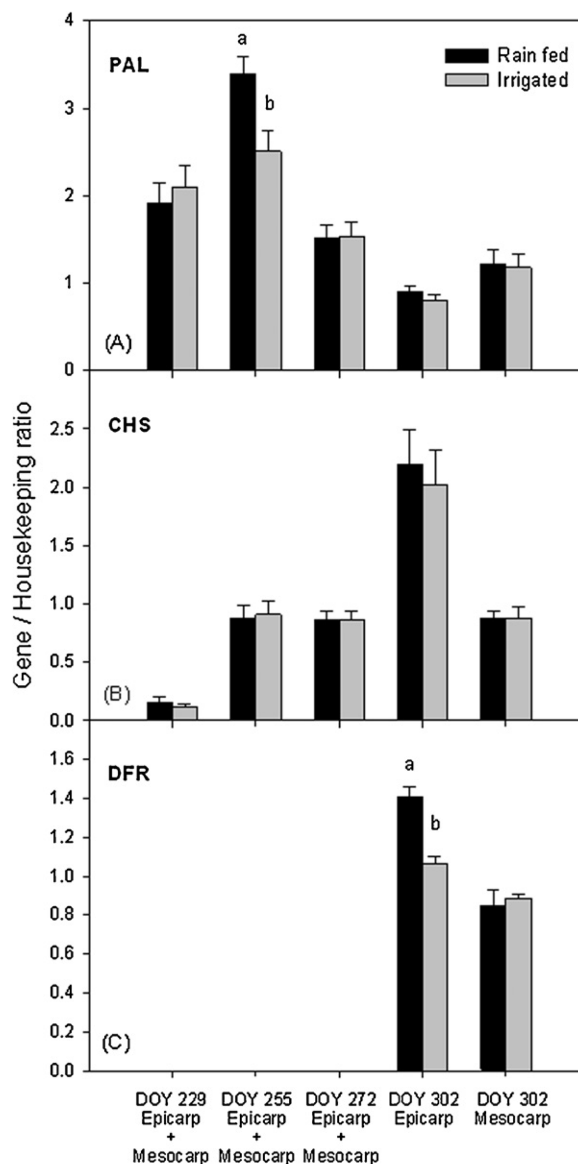

Fig. 3. Expression analysis of genes involved in phenol compound biosynthesis during olive fruit development from pit-hardening to ripe stage. (A) *phenylalanine ammonia lyase* (*PAL*), (B) *chalcone synthase* (*CHS*), and (C) *dihydroflavonol reductase* (*DFR*). Different letters indicate significant difference between RF and IR samples ($P \leq 0.05$).

sampling dates, polyphenol concentration was significantly higher in RF than in IR fruit (Fig. 2A and Table 2). Differences between treatments regarding polyphenol concentration remained constant throughout the experiment as suggested by the non-significant effect of the $I \times T$ interaction (Table 2). It cannot be ruled out that a dilution effect may have been present in olives undergoing the irrigation treatment. At ripening, anthocyanin accumulation was higher in RF than in IR fruit, which caused significant differences between treatments regarding this parameter in relation to the last sampling date (complete epicarp pigmentation with pulp still not accumulating anthocyanins) (Fig. 2B and Table 2). Reduced water availability is known to induce a higher accumulation of total phenols in olives and the resulting oil (Tovar et al., 2001; Romero et al., 2002; Marsilio et al., 2006). However, the effect of irrigation seems to affect the pattern of specific polyphenol compounds in different ways: for example, Patumi et al. (2002) reported an increase in tyrosol, oleuropein aglycones, and oleuropein under water stress conditions, while hydroxytyrosol appeared to be positively affected by irrigation. On the other hand, Tovar et al. (2001) found that the three most important compounds of the olive phenolic fraction (4-(acetoxyethyl)-1,2-dihydrobenzene, the dialdehydic form of elenoic acid linked to tyrosol and to hydrotyrosol, and the oleuropein aglycon) decreased with irrigation. Hydroxytyrosol, tyrosol, and vannilic acid were apparently unaffected, whereas vanillin content increased following the application of a linear irrigation strategy. These contrasting data concerning, in particular, minor components of the phenolic fraction may be due to the different experimental and environmental conditions. The analyses conducted in our study are in agreement with the general decrease in total polyphenol content detected in olives from well-watered trees, which was observed from the earliest sampling date (229 DOY). At this early stage, PAL activity is high, as observed by Morello et al. (2005) in Arbequina and Farga cultivars, thus indicating an active participation of the phenylpropanoid pathway in the metabolic processes of the fruit. The activation of this pathway is probably crucial for setting up part of the total phenol compound pool, which reaches its highest values at these intermediate stages of fruit growth and development (Morello et al., 2005; Ortega-Garcia and Peragon, 2010). This hypothesis is supported by analyzing the expression trend of polyphenol-related genes, and in particular of *PAL*. In fact, *PAL* transcript accumulation appeared to be higher, as it relates to the two initial sampling dates with a peak at DOY 255 (Fig. 3A). At this developmental stage, a significant lower accumulation of *PAL* transcripts was observed in the IR samples. In terms of *CHS*, the first gene in the flavonoid biosynthetic pathway, the expression pattern showed an increase from the first to the second sampling date, and later, in the epicarp only,

in line with ripening (Fig. 3B). No significant effects of the different water regimes were observed on the expression of this gene. *DFR* transcripts were not detected at DOY 229, 255, and 272, while in ripe fruit, both in the mesocarp and epicarp, *DFR* was actively transcribed. A lower accumulation of *DFR* transcripts was observed in the epicarp of IR samples at DOY 302 (Fig. 3C). Our molecular data indicate that the different water regimes had no effect on *PAL* gene expression during ripening (DOY 272 and 302), while a general reduction in PAL activity has been observed by Tovar et al. (2002) throughout ripening when irrigation treatments were applied to cv Arbequina in field conditions. In addition to the fact that transcript accumulation and enzyme activity patterns do not always correlate due to post-transcriptional and post-translational regulatory mechanisms, discrepancies in the results may be due to different genotypes and experimental conditions (including the water supply management). *CHS* did not show any change in transcript accumulation when comparing RF and IR samples. Such behavior appears to be different from that in other fruit species such as, for example, grapes, where *PAL* and *CHS* genes (together with other flavonoid genes) are up-regulated in ripening red-skinned berries (Castellarin et al., 2007; Deluc et al., 2009) under limited water availability.

As reported in a previous paper (Martinelli and Tonutti, in press), DFR seems to play a key role in the transcription regulation of the anthocyanin pathway, as also observed in onions (Kim et al., 2005). Although a coordinated up-regulation of several genes leading to anthocyanin synthesis has been observed in terms of color development and the progress of ripening (Galla et al., 2009), *DFR* transcripts, not detectable in the early stages of olive fruit growth and development, start accumulating specifically when pigmentation changes take place (Martinelli and Tonutti, in press), as observed in this work concerning epicarp tissues.

The higher expression level observed for *DFR* in the RF epicarp samples correlates with the increase in anthocyanin concentration. This gene is not considered as a key point in the transcriptional regulation of the anthocyanin pathway in *Vitis vinifera* (Boss et al., 1996). Our data reinforce the hypothesis of the presence of some variable regulatory steps and mechanisms in this pathway in different plant species. The lower anthocyanin concentration detected at the complete pigmentation of the epicarp (DOY 302) of the IR samples could indicate that ripening is affected by the specific treatment and, as observed in grapes (Castellarin et al., 2007), an acceleration in the olive fruit's development to maturity occurs when the water supply is limited (Alegre et al., 1999). Tovar et al. (2002) found that, in irrigated olive trees, fruit slowed down the ripening process, and reached the same maturity index later than the fruit of the deficit irrigation treatment.

3.2. Metabolomics analysis

Fruits collected at DOY 302 (complete pigmentation of the epicarp) were analyzed to obtain quantitative analyses of more than 250 metabolites by Agilent/Frontier pyrolysis GC–quadrupole MS. The content of 46 metabolites appeared to significantly differ (P-value <0.1) in the two samples considered. Of these 46 compounds, 13 (unidentified) accumulated at a higher level in the RF samples, and the other way round for the 33 remaining metabolites. In terms of the group of metabolites accumulating at a higher level in the IR samples, 19 were identified based on spectra similarities to known compounds present in the Agilent Fiehn GC/MS Metabolomics RTL Library. Of these, specific compounds belonging to the three most important primary metabolism categories of fruit (carbohydrates, organic acids and amino acids) were detected (Fig. 4). L-Asparagine, galacturonic acid, shikimic acid, and allose showed the highest differences between the two samples. Less pronounced but still significant changes were observed for other carbohydrate compounds such as palatinitol and organic acids, such as quinic and glyceric acids, as well as for vanillic acid and squalene (Fig. 4). A slight increase was detected in the IR samples in terms of the content of two organic acids (citraconic and alpha-ketoglutaric acid), three carbohydrates (isopropyl β-D1-thiogalactopyranoside, myo-inositol and maltose), two amino acids

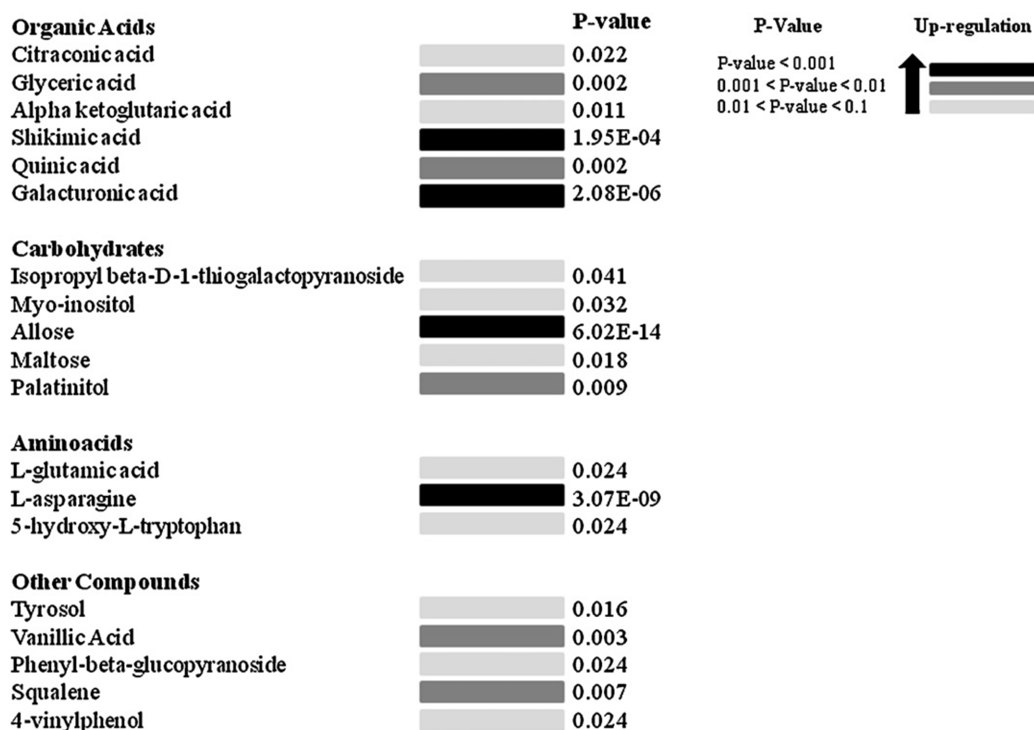

Fig. 4. List of metabolites showing significant higher concentrations in IR compared to RF ripe fruit samples. Statistical analysis was performed using Agilent Mass Profiler Professional Software (P-value ≤0.1 without any corrections for multiple comparisons). The darker is the colour, the more pronounced is the difference between the samples.

(L-glutamic acid and 5-hydroxy-L-tryptophan), and three phenolic compounds (4-vinylphenol, phenyl-beta-glucopyranoside, and tyrosol). The two carbohydrates with the highest difference in IR and RF were allose and the sugar alcohol palatinitol. As far as we know, these two molecules have never been detected before in olive fruit. The fact that five carbohydrate molecules appeared to be more abundant in IR samples reinforces the hypothesis that irrigation affects the ripening physiology of olives. Cherubini et al. (2009) and Migliorini et al. (2011) reported that sugar concentrations decrease throughout olive fruit ripening, following a sygmoidal model tending to an asymptote. Olives remain green for a long period, with their chloroplasts are active even when changes in pigmentation occur and fruit photosynthesis is a secondary source of sugars for the fruit itself (Conde et al., 2008). A delay in the onset and evolution of ripening would therefore sustain the carbon economy, thus reducing the sugar decrease, which is mainly due to the transformation of these molecules into oil.

Even though organic acids are minor components of olive fruit (about 1.5% of the fleshy part), they may play an important role in the fruit's skin color and processing by affecting the buffering activity of olive tissues (Marsilio et al., 1978; Arslan and Ozcan, 2011). Malic and citric acids are the two main organic acids in olives: few other organic acids, namely lactic, oxalic, galacturonic, succinic, and tartaric have been identified and studied in this species (Arslan and Ozcan, 2011). Although there are marked differences in relation to the specific organic acid, variety and growing location, the amount of organic acids generally decreases throughout ripening (Arslan and Ozcan, 2011). In a previous paper, malic and citric acids were shown not to be affected by different water regimes (Patumi et al., 2002). In the present work, the marked increase in galacturonic acid and, to a lesser extent, glyceric and quinic acids (the latter has never been described in olive fruit, as far as we know) in the IR samples, again reinforces that there is likely a delay in fruit maturation and ripening.

As reported above, our metabolomics data showed that irrigation leads to an increase in squalene in olive fruit. These results agree with previous findings that demonstrated an increased squalene content in the fruit of Leccino cultivars following irrigation (Tognetti et al., 2007), Barnea and Souri (Ben-Gal et al., 2011). The increased concentration in squalene in the IR samples may be the result of an increased synthesis and/or a reduced production of sterols and non-steroidal triterpenoids that share the common precursor represented by (3S)-2,3-oxidosqualene (OS) formed from squalene through the activity of squalene monooxygenase (Stiti et al., 2007). The amount of squalene is high in growing and immature olive fruit, and then decreases at the later stages of development (Sakouhi et al., 2011). In the triterpenoid biosynthetic pathway, OS is cyclised into two different molecules such as cycloartenol (precursor of sterols) and β-amyrin, which is the precursor of triterpenes. The triperpenoid content in olive fruit considerably changes throughout development: non-steroidal triterpenoids are produced at the early stages of fruit growth when sterol biosynthesis is not activated and the squalene content is high. Later on in the fruit's development and during ripening, a dramatic change is observed with a marked decrease in the content of α- and β-amyrin associated with a progressive increase in sterol end-products (Stiti et al., 2007). The expression of two genes responsible for the cyclization of squalene in both cycloartenol (*cycloartenol synthase*, sterol pathway) and β-amyrin (*β-amyrin synthase*, triterpenoid pathway) was determined. The time-course expression data of *β-amyrin synthase* throughout fruit development appear to be in agreement with the above described pattern. Only in the samples collected at DOYs 229 and 255 did irrigation induce a down-regulation of this gene (Fig. 5A). No significant differences throughout fruit development and no

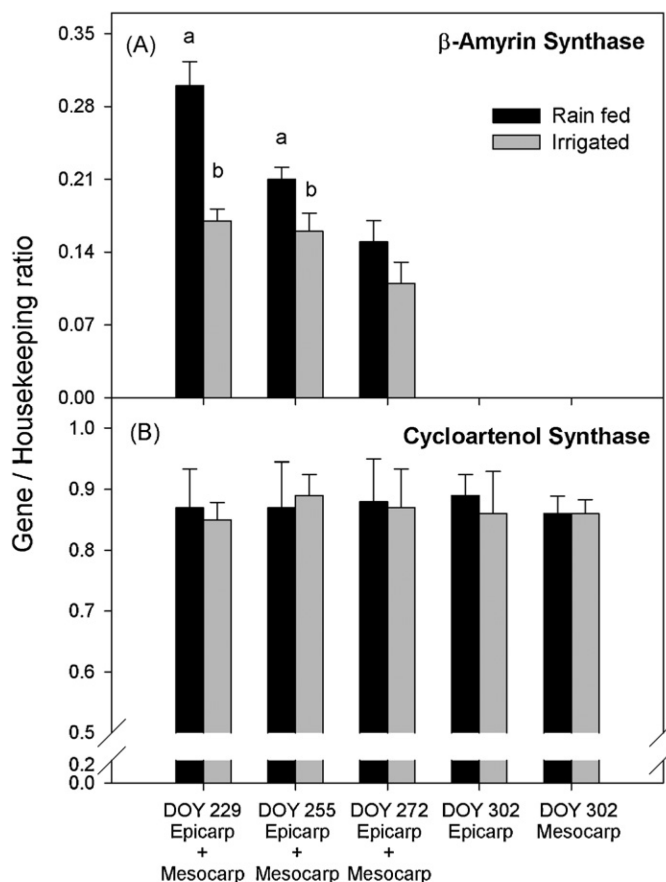

Fig. 5. Expression analysis of genes involved in terpenoid metabolism during olive fruit development from pit-hardening to mature stage. (A) *β-amyrin synthase* and (B) *cycloartenol synthase*. Different letters indicate significant difference between RF and IR samples ($P \leq 0.05$).

effects of different water regimes were observed in *cycloartenol synthase* expression (Fig. 5B). Thus, the increase in squalene content in the IR ripe fruit samples may be the result of an enhanced (or prolonged) synthesis of squalene, rather than a reduced catabolism to produce sterols. This hypothesis needs to be validated through detailed and specific metabolic and molecular analyses concerning specific biosynthetic steps of squalene.

Considering that also shikimic acid was found to be more abundant in the IR than the RF samples (Fig. 4), it is likely that the whole shikimate pathway, which is upstream of the synthesis of vanillic acid and 4-vinylphenol (via coumaric acid) and tyrosol (via 4-hydroxyphenylacetaldehyde), is a specific metabolic target in olives grown under different water conditions. Analyses of phenylalanine and tyrosine levels, precursors of vanillic acid and 4-vinylphenol (phenylpropanoid pathway) and tyrosol (phenylethanoid pathway), respectively, would be useful to better elucidate these aspects. The increase in the identified phenol compound content in the IR sample confirms the complexity of the olive fruit response to different water availability conditions in terms of a modulation of specific secondary metabolic pathways.

In conclusion, this work shows that the water supply in olive grove management is a key agronomic factor affecting fruit growth and development. Our data suggest that by providing irrigation from the second half of the fruit growing cycle (i.e. after pit hardening), the ripening process is delayed and specific primary and secondary metabolisms are affected with a marked effect on the fruit composition at harvest.

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