Molecular and metabolic analyses in developing olive fruit in relation to different water regimes

F. Martinelli, L. Sebastiani and P. Tonutti
BioLabs, Scuola Superiore Sant'Anna
Piazza Martiri della Libertà 33, Pisa, Italy
p.tonutti@sssup.it

R. d'Andria
Istituto per i Sistemi Agricoli e Forestali del Mediterraneo, CNR, Via Patacca 85, Ercolano (NA), Italy

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Abstract

Despite the worldwide economic importance of olive (*Olea europaea* L.), little is known regarding the molecular and metabolic changes during fruit development and the modulation of quality-related metabolic pathways during drought stress. In this work we report the expression pattern of genes involved in important pathways of the secondary metabolism (polyphenols, terpenoids) during fruit development in rainfed or fully irrigated olive plants. Transcript levels of Phenylalanine ammonia lyase (PAL), Chalcone synthase (CHS), and Dihydroflavonol reductase (DFR) showed different expression pattern during fruit development and resulted (CHS and DFR) more expressed in the skin than in the pulp. PAL showed to be highly expressed before pit hardening and its expression decreased thereafter. CHS expression peaked at pit hardening and at veraison, while DFR resulted highly expressed only at fruit ripening indicating a possible crucial role played by these genes in the regulation of the flavonoid biosynthesis in the last developmental stages of olives. Water stress enhanced the expression of PAL and CHS at pit-hardening and of DFR in skin at ripening stage. Concerning terpenoids, Amyrin synthase (AS) showed a decreasing expression trend throughout development, while Lupeol synthase (LUS) resulted highly expressed in skin of ripe fruit. An induction of LUS gene expression was observed at the early developmental stages of fruit from water stressed plants. To gain insights into the metabolic changes of the fruit in relation to different water regimes in the field, we also start analyzing, using a high throughput approach, the mesocarp of ripe fruits by GC-TOF mass spectrometer and differences in metabolite composition were determined.

INTRODUCTION

Olive is a drought-tolerant species that can survive even under prolonged drought periods by developing resistance mechanisms to water stress (Chartzoulakis *et al*., 1999; Xiloyiannis *et al*., 1999). In Mediterranean agro-ecosystems water shortage is one of the main growth limiting factor and olive plants must finely regulate water balance for optimise their adaptation to the environment while reducing the intensity of water stress. In olive orchard the knowledge of these characters are relevant prerequisite for planning effective irrigation-scheduling protocols (Fernández *et al*., 2008).

Several studies have shown that irrigation enhances yield through increases in the size and number of fruits, and in the production of oil per surface unit. Less information is available on the composition and the molecular and metabolic processes of the olives of plants grown under different water regimes. Phenolic compounds are amongst the qualitative parameters most affected by irrigation, generally showing a negative trend with increasing water supply (d'Andria *et al*., 2009).

Fruit development is a genetically programmed process markedly influenced by environmental factors such as plant-water relationships. In this context and to better understand basic mechanisms regulating fruit development, molecular, genomics and post-genomics approaches are extremely useful and represent a novelty for *O. europaea*. Recently a large set of differentially expressed genes in developing olive fruits of cultivar Leccino have been identified (Galla *et al*., 2009), thus implementing the olive sequence database for further analyses.

In this work we report the expression pattern of some genes involved in important pathways of the secondary metabolism (polyphenols, terpenoids) and the results of a preliminary metabolomics approach in fruit from rainfed (control) and fully irrigated Leccino olive trees.

MATERIAL AND METHODS

The experimental site is 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 is sandy loam (1.76% organic matter, 1% $CaCO_3$, 0.15% N, pH 7.2), characterized by volumetric water content (m^3m^{-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 are planted 6 m apart with a plantation density of 555 plants ha^{-1}. The olive (cv. Leccino) trees used for the experiment were 15-year old. Plant were selected for uniformity in size and assigned to two treatments: a non-irrigated (water stressed) and an irrigated (control) with a seasonal water amount equivalent to 100% of maximum crop evapotranspiration (ETc). Irrigation water was delivered by a drip irrigation system from the beginning of pit hardening phenological phase (end of July), to early fruit veraison (beginning of October). Crop evapotranspiration 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, mm), a crop coefficient (kc) equal to 0.65 and a tree ground cover coefficient (kr) of 0.85. Irrigation volume of control was 181 mm while the ETc in the irrigation period was 191 mm.

In correspondence of four fruit developmental stages (from pit-hardening to ripening) (Fig. 1), fruits have been sampled from water stressed and control plants, RNA extracted according to Galla *et al*. (2009) and semi-quantitative RT-PCR performed using specific primers for phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), dihydroflavonol reductase (DFR)**,** amyrin synthase (AS) and lupeol synthase (LUS) according to the manufacturer's instructions (Reverse Transcription System, Promega, USA). Quantum 18S RNA Universal kit was used as housekeeping. Cycle conditions were adapted for each gene following the instructions Quantum 18S RNA Universal kit (Ambion Inc.). The PCR products were separated by electrophoresis on 1.5% agarose gels. Amplification mix was prepared using goTaq protocol (Promega). The amplification products were scanned and quantified using the Quantity One software (BioRad).

Metabolomics analysis was performed by GC-TOF mass spectrometer. Ripe fruits (3 replicates for control and water-stressed samples) were frozen in liquid nitrogen, and stored at -80˚C until analysis. For each sample, 20 mg pulp was ground and 2 ml pre-chilled extraction solvent (MetOH:$CHCl_3$ 1:1). After vortex and centrifugation, the supernatant was analyzed with a Pegasus III TOF GC-Mass spectrometer that has a resolution power of profiling 400 compounds. The relative concentrations were determined by peak area (mm^2). All peak detections were manually checked for false positive and false negative assignments.

RESULTS AND DISCUSSION

Water stress induced a slight decrease in PAL gene expression in at the first sampling date (early pit-hardening) but an up-regulation was observed at the second sampling date (late pit-hardening) when the highest level of transcript accumulation throughout fruit development was detected (Fig. 2) and when the highest rates of polyphenol accumulation occur (Martinelli and Tonutti, unpublished). Similarly, CHS showed an increased expression in control fruit at late pit-hardening stage when water stress conditions induced an enhanced CHS transcription. Differently from PAL, the highest CHS expression level was observed in correspondence of ripening in skin tissues (Fig. 2). DFR transcripts were detected only in the last stages of fruit development in both pulp and skin, but only in skin water stress induced an increased expression of this gene (Fig. 2). The increased expression of PAL and CHS might account for the higher accumulation of phenols observed in olives of water stressed trees (Tovar *et al*., 2002). The increased expression of both CHS and DFR observed in the last developmental stage suggests that the flavonoid biosynthetic pathway, leading to the production of compounds as flavonols, flavones and anthocyanins (Fig 2 -

simplified scheme), is regulated by these two genes. Both genes showed high expression level in skin of ripening fruit indicating that, as observed in other fruit species, peel is highly active in terms of flavonoid metabolism in olives.

The expression analyses of two oxidosqualene cyclase genes Amyrin synthase (AS) and Lupeol synthase (LUS) that are responsible for specific steps of the triterpenoid biosynthetic pathway (Fig 3 - simplified scheme) indicate that AS is actively transcribed during the early growth stages and water stress induced a higher expression throughout development (Fig. 3). A marked up-regulation of LUS expression was detected in fruit of water stressed trees in correspondence of two sampling dates (early development and before the onset of ripening). Differently from AS, LUS appeared markedly expressed in the skin of ripe fruit.

To gain insight into overall changes in the fruit metabolism under water stress, we also start analyzing pulp of mature fruits sampled from water stressed and control plants by a metabolomics approach by means of GC-TOF mass spectrometer. Chromatograms revealed the presence of differences between the two samples (Fig. 4) and deep data analysis is now in progress in order to identify specific metabolites.

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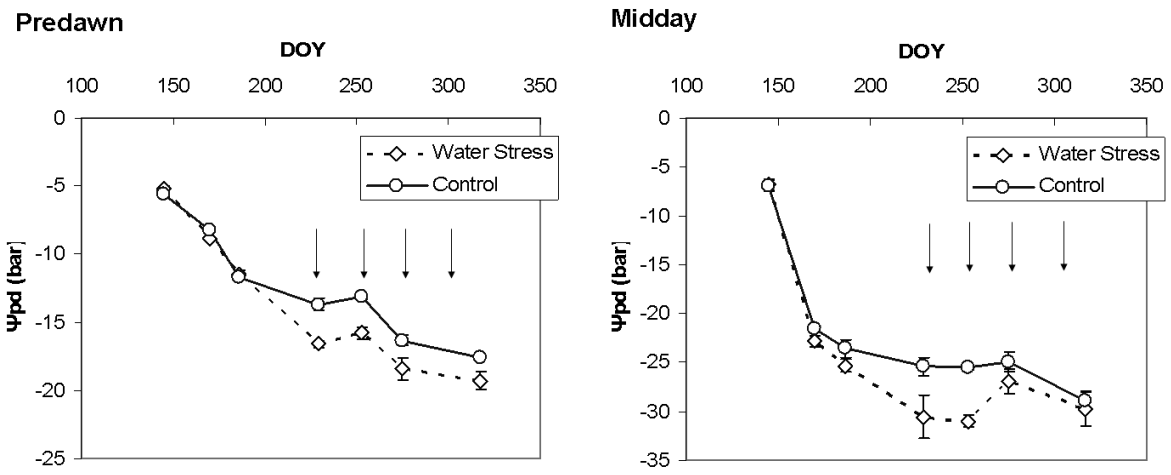

Fig. 1. Predawn and midday (covered leaves) leaf water potential dynamics in water stress and control treatments. Arrows = olive sampling dates. DOY = day of the year.

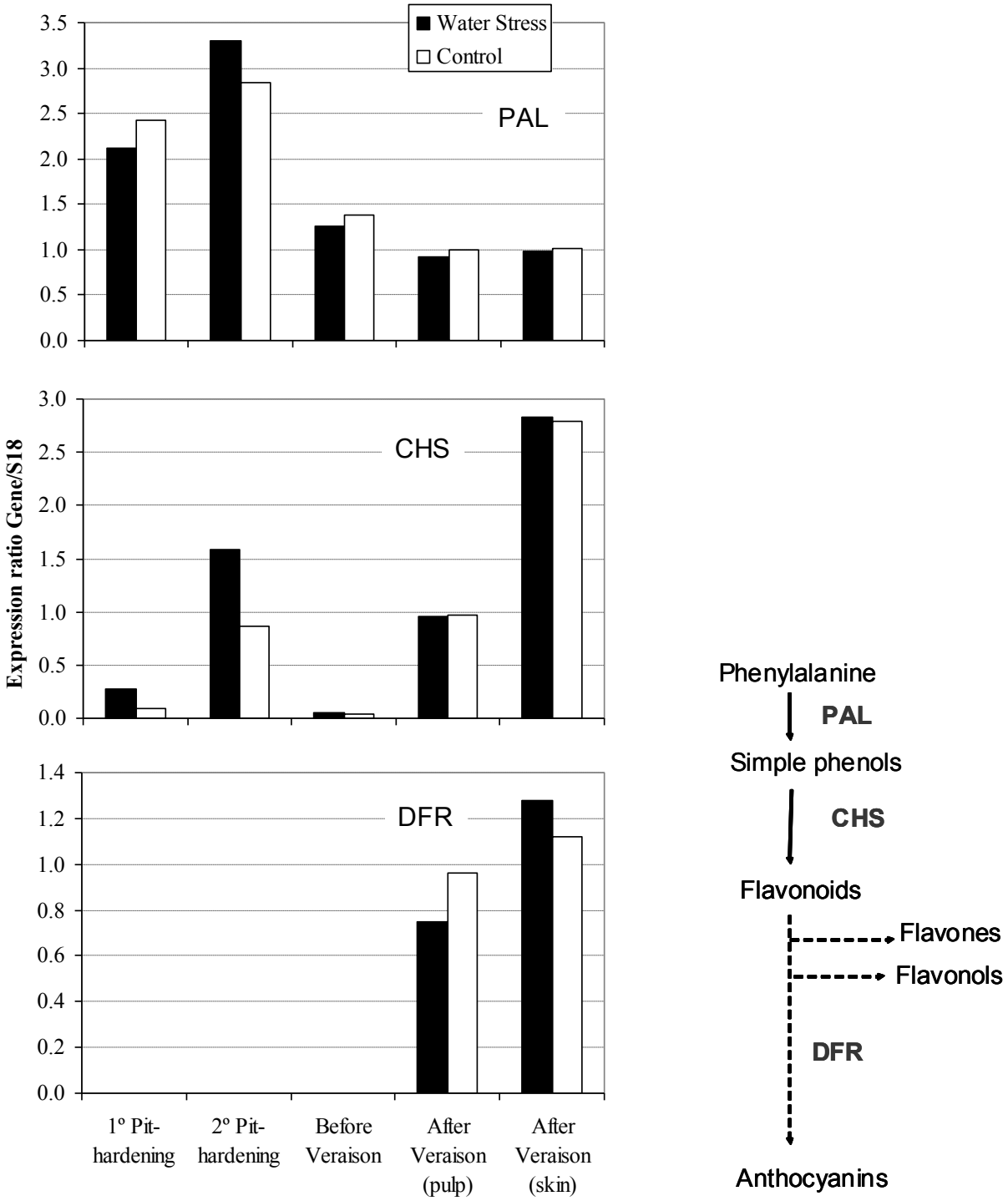

Fig. 2. Transcript accumulation of PAL, CHS and DFR in olives sampled at different developmental stages from water stressed and control plants (left). Simplified flavonoid biosynthetic pathway (right).

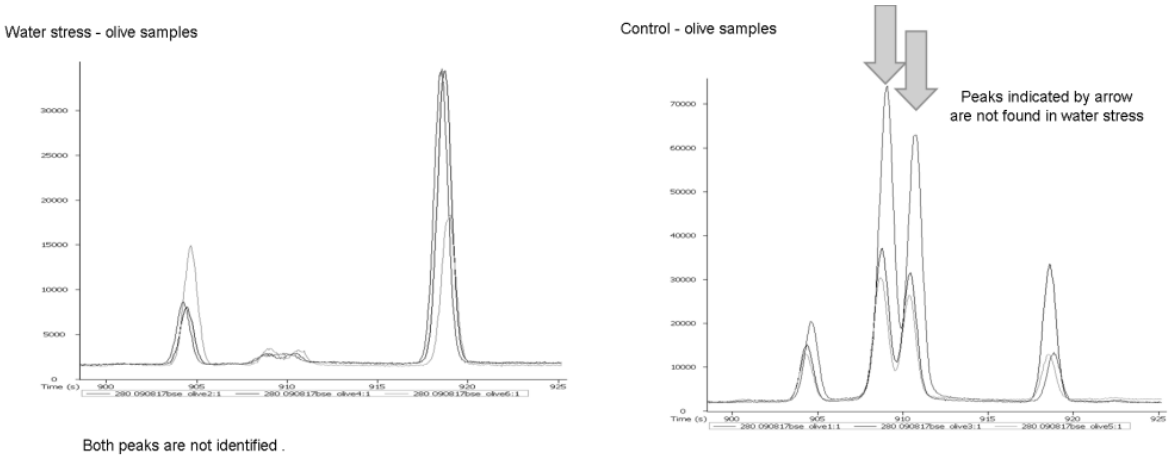

Fig 4. GC-TOF mass spectrometer and metabolite samples spectra from water stressed (left) and control plants (right).

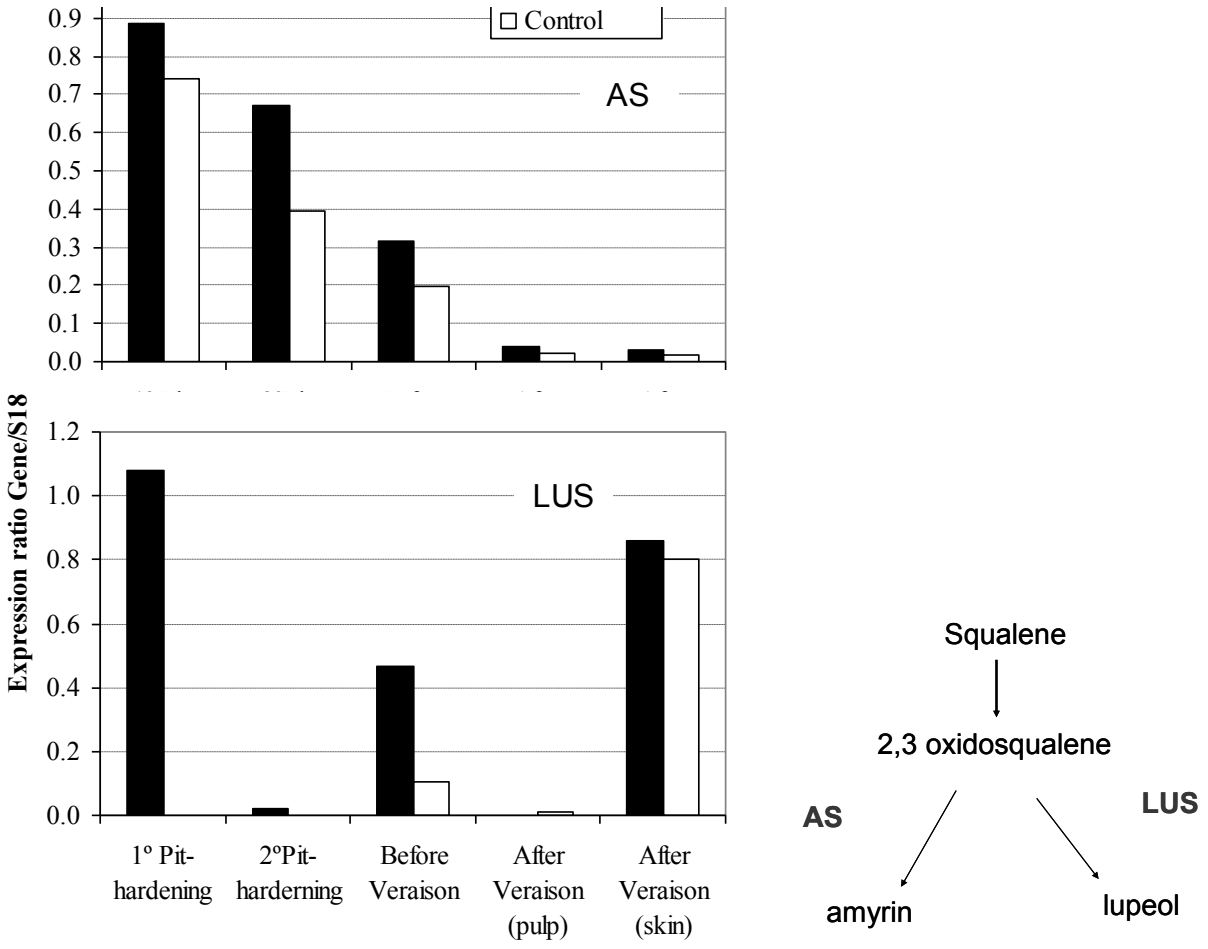

Fig. 3. Transcript accumulation of AS and LUS in olives sampled at different developmental stages from water stressed and control plants (left). Simplified triterpenoid biosynthetic pathway (right).