Received: 16 July 2021 | Revised: 14 February 2022 | Accepted: 26 April 2022

DOI: 10.1111/pce.14340

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

Carbon supply and water status regulate fatty acid and triacylglycerol biosynthesis at transcriptional level in the olive mesocarp

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Funding information
Ministerio de Ciencia, Innovación y Universidades, Grant/Award Numbers: AGL2015-71585-R, AGL2017-87871-R (AEI/FEDER, UE)

Abstract

The relative contribution of carbon sources generated from leaves and fruits photosynthesis for triacylglycerol biosynthesis in the olive mesocarp and their interaction with water stress was investigated. With this aim, altered carbon source treatments were combined with different irrigation conditions. A higher decrease in mesocarp oil content was observed in fruits under girdled and defoliated shoot treatment compared to darkened fruit conditions, indicating that both leaf and fruit photosynthesis participate in carbon supply for oil biosynthesis being leaves the main source. The carbon supply and water status affected oil synthesis in the mesocarp, regulating the expression of *DGAT* and *PDAT* genes and implicating *DGAT1-1*, *DGAT2*, *PDAT1-1*, and *PDAT1-2* as the principal genes responsible for triacylglycerol biosynthesis. A major role was indicated for *DGAT2* and *PDAT1-2* in well-watered conditions. Moreover, polyunsaturated fatty acid content together with *FAD2-1*, *FAD2-2* and *FAD7-1* expression levels were augmented in response to modified carbon supply in the olive mesocarp. Furthermore, water stress caused an increase in *DGAT1-1*, *DGAT1-2*, *PDAT1-1*, and *FAD2-5* gene transcript levels. Overall, these data indicate that oil content and fatty acid composition in olive fruit mesocarp are regulated by carbon supply and water status, affecting the transcription of key genes in both metabolic pathways.

KEYWORDS
DGAT, fatty acid composition, fatty acid desaturase, fruit photosynthesis, *Olea europaea*, olive oil, PDAT, triacylglycerol synthesis, water stress

1 | INTRODUCTION

Olive oil is a major edible oil mainly composed (95%–98%) of triacylglycerols (TAG), which consist of a glycerol backbone esterified by three fatty acids. Its exceptional nutritional, organoleptic, and technological properties are due to its well-balanced fatty acid composition, as well as the presence of minor components, such as antioxidants and vitamins (Aparicio & Harwood, 2013). According to the European Commission Regulation (2003), oleic acid is the major fatty acid in olive oil (55%–83%), while linoleic acid accounts for 4%–21% and linolenic acid for less than 1%. In contrast, although the cultivar is the main determinant of olive oil fatty acid composition,

environmental factors and culture conditions have also been linked to variations in the fatty acid profile (Beltrán et al., 2004). Many studies have evaluated the effect of different water regimes on olive oil yield and composition (Fernández, 2014; Gonçalves et al., 2020; Sánchez-Rodríguez et al., 2020), demonstrating that even if water stress decreases fruit yield (Greven et al., 2009; Moriana et al., 2003), fruit volume (Gómez-del-Campo et al., 2014), fresh weight, cell size (Rapoport et al., 2004) and the mesocarp/endocarp ratio (Gucci et al., 2009), olive oil content in the mesocarp is not affected (Costagli et al., 2003); however, the fatty acid composition can be slightly changed (Ahumada-Orellana et al., 2018; Gómez-Rico et al., 2007; Hernández et al., 2018; Tovar et al., 2002). In the processes of fruit growth and oil synthesis, photosynthesis and its limitation by water deficit play a key role since it is the main supplier of reduced carbon (Lawlor & Cornic, 2002).

The olive fruit is a drupe consisting of an exocarp, a mesocarp, and a woody endocarp, which consists of a woody shell enclosing one or, rarely, two seeds (Sanchez, 1994). Total fruit weight comprises 70%–90% mesocarp, 9%–27% endocarp and 2%–3% seed. Accumulation of oil in the fruit begins in the mesocarp and the seed after the lignification of the endocarp when both tissues are properly differentiated (Sanchez, 1994). At the usual harvest time for oil production, the mesocarp contains about 30% oil, while the seed has 27%, and no oil is deposited in the endocarp (Connor & Fereres, 2005). Because the mesocarp weight is very much higher than the seed weight, much more of a fruit's oil is in the mesocarp than in the seed. In fact, the fatty acid composition of the olive oil is similar to that of the mesocarp but different from that of the seed (Hernández et al., 2016).

The ultimate precursor of carbon for TAG synthesis is CO_2, which is fixed during photosynthesis (Sanchez, 1994; Sánchez & Harwood, 2002). Notably, the olive mesocarp possesses the remarkable characteristic of having a high TAG content together with active chloroplasts, which enables it to fix CO_2 under photosynthetic conditions (Sanchez, 1994). Therefore, unlike oilseeds, there are two sources of carbohydrates for fruit growth and lipid biosynthesis in the olive mesocarp: (i) sugars imported from the phloem, coming in turn from the leaves, and (ii) sugars formed by photosynthesis in the fruit (Sánchez & Harwood, 2002). In both cases, sugars are catabolized in the fruit mesocarp via glycolysis to form pyruvate, which is converted into acetyl-CoA, the precursor of de novo fatty acid biosynthesis. Because photosynthesis in fruit, measured by CO_2 exchange, rarely reaches the compensation point, it has been suggested that the organ contribution to the carbon economy is rather modest (Blanke & Lenz, 1989). More recently, pioneering studies using olive fruit from the cultivar 'Picual' with an altered carbon supply strongly suggest that fruit photosynthesis contributes significantly to oil biosynthesis (Sánchez, 1995; Sánchez & Harwood, 2002).

Fatty acid biosynthesis in higher plants begins in the plastids, with oleoyl-ACP being the main product of plastidial fatty acid biosynthesis (Harwood, 2005). The synthesized acyl-ACPs can either be utilized within the plastid for glycerolipid assembly, for glycerolipid assembly and further desaturation, or cleaved by specific thioesterases to free fatty acids, activated to acyl-CoAs, and exported to the cytosol. In this way, they are available in the endoplasmic reticulum for incorporation into membrane glycerolipids and to allow de novo TAG formation via the Kennedy pathway, where diacylglycerol acyltransferase (DGAT) is the enzyme that catalyses the final acylation of diacylglycerol (DAG) to yield TAG. *DGAT1* enzymes have been mainly related to the accumulation of TAG in oilseeds, while *DGAT2* is responsible for the incorporation of unusual fatty acids into TAG (Bates, 2016). Additionally, an alternative acyl-CoA independent reaction catalysed by the phospholipid:diacylglycerol acyltransferase (PDAT) has been described for TAG synthesis (Dahlqvist et al., 2000), which transfers an acyl group from phosphatidylcholine (PC) to DAG, producing TAG. Moreover, oleic acid can be further desaturated to linoleic and linolenic acids by the activity of membrane-bound fatty acid desaturases (FAD). FAD2 and FAD3 are located in the endoplasmic reticulum, and FAD6 and FAD7/8 are in the chloroplast. These enzymes differ not only in their cellular localizations but also in their lipid substrates and electron donor systems (Shanklin & Cahoon, 1998).

In olive, two *DGAT* genes (*OeDGAT1-1* and *OeDGAT2*) have been isolated and characterized, showing overlapping but distinct expression patterns during olive mesocarp growth (Banilas et al., 2011). Recently, we have cloned and characterized three olive *PDAT* genes (*OePDAT1-1*, *OePDAT1-2*, and *OePDAT2*) and their contribution to oil synthesis in olive fruit has been investigated (Hernández et al., 2021a). Furthermore, we have also identified two new *DGAT1* genes (*OeDGAT1-2* and *OeDGAT1-3*) in the olive genome (Unver et al., 2017). Concerning FAD, five genes encoding microsomal oleate desaturases (*OeFAD2-1* to *OeFAD2-5*) have been reported (Hernández et al., 2005, 2020), whereas only one *OeFAD6* gene has been identified to date (Banilas et al., 2005; Hernández et al., 2011). It has been suggested that *OeFAD2-2* and *OeFAD2-5* are the main genes determining the linoleic acid content in the olive mesocarp and therefore in the virgin olive oil (Hernández et al., 2009, 2020). Four members of the olive linoleate desaturase gene family have been isolated and characterized, two microsomal (*OeFAD3A*, Banilas et al., 2007; *OeFAD3B*, Hernández et al., 2016) and two plastidial (*OeFAD7-1*, Poghosyan et al., 1999; *OeFAD7-2*, Hernández et al., 2016), with *OeFAD7-1* and *OeFAD7-2* as the main genes that contribute to the linolenic acid present in the olive oil (Hernández et al., 2016).

Summary statement

Triacylglycerol biosynthesis and fatty acid composition depend on the carbon supply and the water status, which control the transcription of *PDAT* and *DGAT* genes involved in oil accumulation and *FAD2* and *FAD7* genes related with unsaturated fatty acid composition.

Water availability represents one of the main limitations in agriculture; therefore, the application of deficit irrigation strategies, and consequently water stress, on olive crops is unavoidable. Empirical evidence suggests that oil synthesis is less sensitive to water stress than other growth processes in the plant (Iniesta et al., 2009), including fruit growth (Hernandez-Santana et al., 2018), although the physiological basis for this is poorly known, and a better understanding of the regulation of oil synthesis by the water supply is needed. To understand the molecular mechanisms that regulate oil biosynthesis in the photosynthetic olive mesocarp under different water conditions, the main objectives of this study were: (i) to evaluate the relative contribution of the different carbon sources, generated by leaf and fruit photosynthesis, for TAG biosynthesis in the olive mesocarp, and their interaction with water stress and (ii) to study the regulatory mechanisms involved in these metabolic processes. To achieve these objectives, different olive fruit carbon source treatments (control, darkened fruit, and girdling and defoliated shoot) were employed and combined with two irrigation conditions (well-watered [WW] and water-stressed [WS]) to assess their effects on the mesocarp oil content and fatty acid composition. In addition, the effect of these treatments on the expression levels of genes encoding TAG synthesizing enzymes (DGAT and PDAT) and membrane-bound fatty acid desaturases (FAD2 and FAD7) was investigated in this tissue.

2 | MATERIALS AND METHODS

2.1 | Experimental orchard and climate conditions

This study was conducted in a commercial super-high-density olive orchard (*Olea europaea* L. cv. Arbequina), located in Utrera (Seville, southwest Spain) (37°15′ N, −5°48′ W), during the 2018 irrigation season from July to October. 'Arbequina' olive trees were 12 years old, planted in a 4 × 1.5 m formation (1667 trees ha^{-1}) and in rows oriented north-northeast to south-southwest. The soil in the orchard had a sandy top layer and a bottom clay layer (Arenic Albaquaf; USDA 2010, https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_050915.pdf). Further details on the orchard characteristics can be found in Fernández et al. (2013).

The Mediterranean climate is dominant in the region with mild rainy winters and hot, dry summers. Most of the annual rainfall occurs from late September to May. In the area, average values of potential evapotranspiration (ET_O) and precipitation (P) were 1482 and 500 mm, respectively, for the 2002–2018 period (data recorded at the Los Molares station, Regional Government of Andalusia, near the study area). For that period, the average maximum ($T_{a,\ max}$) and minimum ($T_{a,\ min}$) air temperatures were 24.8°C and 10.6°C, respectively. The hottest months are July and August, whose $T_{a,\ max}$ values over 40°C are recorded nearly every year, with peak values rarely over 45°C. Vapour pressure deficit (VPD) values over 7 KPa are reached once per year between July and August.

2.2 | Irrigation and altered carbon source treatments

Two irrigation treatments were applied: a WW treatment, whose trees were irrigated daily at full irrigation (FI) to replace irrigation needs (IN), and a WS treatment, whose trees were under a regulated deficit irrigation (RDI) regime and received 45% of IN during a 5-week period after pit hardening finished, following the strategy described by Fernández et al. (2013). Each irrigation treatment was carried out in three 12 × 16 m plots in a randomized design ($n = 3$). There were 24 trees per plot with the measurements made in two central trees to avoid border effects. Dripper lines were close to the trunk, and each one had five 2 L h^{-1} drippers separated by 0.5 m. IN was calculated daily based on a simplified version of the stomatal conductance model evaluated in the same olive orchard and described in greater detail in Fernandes et al. (2018) and Hernandez-Santana et al. (2021).

In addition to the irrigation treatment, an altered carbon source treatment with three levels was applied to the fruit of two central trees of each irrigation treatment (WW and WS), starting immediately after the lignification of the stone at 12 weeks after flowering (WAF). Four similar shoots of those trees were selected to establish three different altered carbon source treatments to determine the carbon source: a control fruit treatment (C), in which the shoot and fruit were not altered; a darkened fruit treatment (D), in which the fruit of the shoot was covered from sunlight with black fabric to avoid photosynthesis in the fruit but allow ventilation, and thus, the carbon source was only that via phloem from other parts of the plant; and a girdled and defoliated shoot treatment (G), in which the leaves of the shoot were detached and the bark of the shoot was girdled to the phloem, so the fruit was deprived of the external supply of photosynthates for their growth. Fruit temperature was measured with wired thermocouples in fruits bagged and sun-exposed, and no significant differences were found between both conditions. In all treatments, selected shoots had the same number of fruits, and the trees had the same number of fruit-bearing shoots.

2.3 | Sample collection

The olive fruit was harvested at three different developmental stages: immediately after pit hardening (12 WAF) when oil accumulation began, fully green (20 WAF) when the rate of TAG synthesis and oil accumulation was at its maximum and turning (24 WAF) when the fruit changed colour from green to purple. For each biological replicate, 2 g of olive mesocarp tissue was collected from at least 10 different olives harvested from the two central trees contained in each of the three plots and three treatments. The skin of the olive fruit was peeled off, and the mesocarp samples were quickly frozen in liquid nitrogen and stored at −80°C.

2.4 | Plant-based sensors

2.4.1 | Fruit dendrometers (fruit DW)

For each of three plots of an irrigation treatment, the diameter of one fruit in two trees was measured by dendrometer, giving a total of six monitored fruit per irrigation treatment. The fruit dendrometers were adapted using a linear potentiometer (model MM(R)10-11) with an internal spring return (Megatron Elektronik GmbH & Co.) coupled to a sensor holder. The sensors were connected to a data logger (CR1000; Campbell Scientific Ltd.), which saved data on fruit equatorial diameter every 5 min. In addition, the equatorial diameter and fruit dry weight (DW) of six olives were measured in each plot where the trees were monitored every 15 days from May to October. With those data, a correlation of both variables was performed to obtain non-linear regression ($y = ax^b$) for WW and WS treatments considering phenological stages as indicated in Supporting Information: Figure S1. Then, both equations were used to simulate fruit DW continuously from fruit dendrometer measurements (Supporting Information: Figure S1).

2.4.2 | Sap flow sensors (accumulated A_N)

One central tree for each plot was monitored with sap flow sensors (Tranzflo NZ Ltd.) using the compensation heat pulse (CHP) method (Green et al., 2003) to derive sap flux density (J_s, mm h^{-1}) values. In both irrigation treatments, one extra tree was monitored in one plot. Measurements were made every 30 min for the whole study period and controlled by a CR1000 datalogger connected to an AM25T multiplexer (Campbell; Campbell Scientific Ltd.).

J_s was measured at a depth of 5 mm below the cambium for continuous gas exchange measurements. The process to obtain g_s from J_s measurements in olive is described in Hernandez-Santana et al. (2016), and the A_N modelling was performed as in Hernandez-Santana et al. (2018). Briefly, a regression was established between g_s measured in new, sun-exposed leaves and J_s measured in the trunk of the same tree, divided by VPD values calculated from the weather station at the orchard. The J_s/VPD versus g_s calibration equations were established using 10–23 data points from each instrumented tree. The stomatal conductance values used for the equations were obtained from measurements of g_s and A_N, which were conducted on four clear days from May to August, every 30–60 min from dawn to noon, in three sun-exposed current-year leaves per instrumented tree. The data from measuring maximum g_s and A_N in two leaves per tree, conducted every other week in every tree instrumented with sap flow probes, from mid-July to the beginning of October, at 8:00–9:00 GMT in the same plots were also used. For the gas exchange measurements, two portable photosynthesis systems (Li-Cor 6400-XT; LI-COR, Inc.), with a 2 × 3 cm standard chamber, at ambient light and CO_2 conditions were used. g_s was simulated every 30 min to model A_N using the model by Farquhar et al. (1980), using the simulated g_s as the input. Details on the modelling and measurements needed to apply the model in this olive orchard can be found in Hernandez-Santana et al. (2018). Accumulated A_N was calculated by summing the quantity of simulated A_N every 30 min until the moment the value was shown.

2.5 | Oil content and fatty acid composition

Two different methodologies were used to determine the oil content of olive mesocarp tissue. With respect to the complete period of olive fruit development and ripening, six fruit per tree from the two irrigation treatments were harvested every 2 weeks. Lipids were extracted from the mesocarp tissue by the method of Hara and Radin (1978). Mesocarp oil content (%) was determined by gravimetric quantification of total lipid weight after solvent evaporation in a centrifugal vacuum concentrator Basic Model *5301* (Eppendorf).

In the case of irrigation and altered carbon source treatments, olive mesocarp samples (1.5 g) corresponding to each biological replicate were lyophilized (VirTis BenchTop 2K Freeze Dryer; SP Industries Inc.), and the dry weight (DW) was determined. The lyophilized samples were ground in a mortar, and three aliquots of 100 mg DW were sampled for fatty acid analysis. Lipids were extracted as described by Hara and Radin (1978). Fatty acid methyl esters were produced by acid-catalysed transmethylation (Garcés & Mancha, 1993) and analysed by gas-liquid chromatography (Román et al., 2015). Heptadecanoic acid was used as an internal standard to calculate the fatty acid content in the samples. The mesocarp oil content (% DW) was calculated as the sum of the different fatty acids in 100 mg DW. The fatty acid composition was expressed in mol % of the different fatty acids. Both types of data are presented as the means ± standard error (SE) of three biological replicates, each having three technical replicates.

2.6 | Total RNA extraction and cDNA synthesis

Total RNA isolation was performed using 100 mg FW of frozen olive mesocarp tissue and the Spectrum™ Plant Total RNA kit (Sigma-Aldrich). Contaminating DNA was removed from RNA samples using a TURBO DNA-free kit (Ambion). RNA quality was verified using a QIAxcel Advanced System (Qiagen), scoring RNA integrity using indicators such as RIS (RNA Integrity Score), and values between 6.5 and 7 were obtained. cDNA synthesis was carried out with the SuperScript™ III First-Strand Synthesis System (Invitrogen) according to Hernández et al. (2009).

2.7 | Quantitative real-time PCR

Gene expression analysis was performed by quantitative real-time PCR (qRT-PCR) using a CFX Connect real-time PCR System and iTaq

Universal SYBR Green Supermix (BioRad), as previously described by Hernández et al. (2019). Primers for gene-specific amplification were designed using the Primer3 program (http://bioinfo.ut.ee/primer3/) and the Gene Runner program (Supporting Information: Table S1). The housekeeping olive ubiquitin2 gene (*OeUBQ2*, AF429430) was used as an endogenous reference for normalization (Hernández et al., 2009). The relative expression level of each gene was calculated using the equation $2^{-\Delta C_t}$, where $\Delta C_t = (C_{tGOI} - C_{tUBQ2})$ (Livak & Schmittgen, 2001; Pfaffl, 2004). This method has the advantage of making comparisons at the level of gene expression across developmental stages, treatments, and genes. The data are presented as means ± SE of three biological replicates, each having two technical replicates per 96-well plate.

2.8 | Statistical analyses

The average of accumulated A_N, fruit DW, oil content, and relative gene expression values for each plot was calculated. Data for accumulated A_N and fruit DW were analysed by one-way analysis of variance (ANOVA), and oil content and relative gene expression were analysed by two-way ANOVA with $p < 0.05$ as a significance level. Statistical analyses were carried out using SigmaPlot® software (Systat Sotfware). In the graphs, the vertical bars represent the SE of the mean of the plot of each treatment.

3 | RESULTS

3.1 | Effect of different altered carbon source and irrigation treatments on the oil content and fatty acid composition of the olive fruit mesocarp

The dynamics of accumulated A_N and fruit DW during the development and ripening of olive fruit (cv. Arbequina) followed similar trends (Figure 1a, b), with lower values for both variables in the WS treatment than for WW. While the increment rate of accumulated A_N (0.29 mol m^{-2} day^{-1}) and fruit DW (0.0018 g day^{-1}) was constant in WW for most of the studied period, the rate decreased (0.19 mol m^{-2} day^{-1} and 0.0002 g day^{-1} for accumulated A_N and fruit DW, respectively) when deficit irrigation started (DOY 196) in the WS treatment. Increment rates recovered after resuming irrigation (DOY 243) to nearly as high as those for the WW treatment (Figure 1a, b). A similar pattern was observed for oil accumulation rates in the olive fruit mesocarp (Figure 1c). The mesocarp oil accumulation rate for WS treatment was lower during the deficit irrigation period than for WW treatment, but the rate increment was higher in WS than in WW during irrigation recovery (4.52% day^{-1} in WS compared to 3.69% day^{-1} in WW), yielding a similar oil content in the mesocarp for both treatments at harvest.

When the different altered carbon source treatments were applied to olive fruit, a decrease in the oil content of the fruit

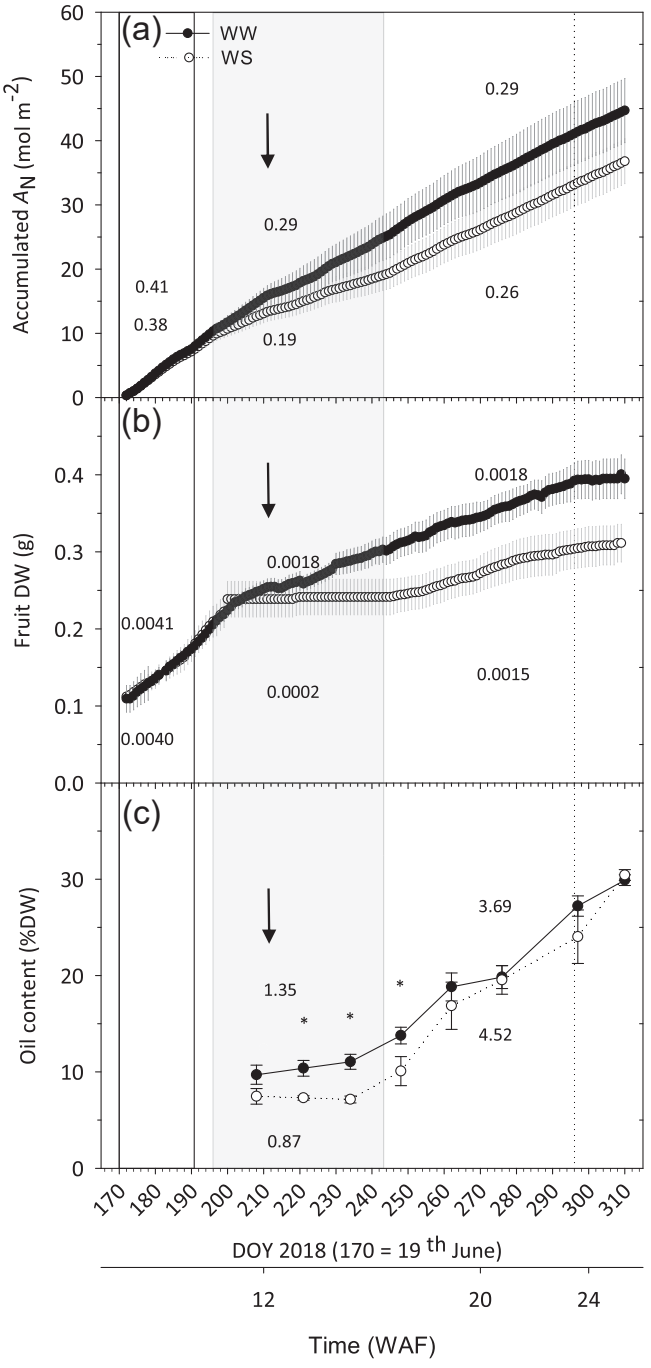

FIGURE 1 Estimated daily accumulated photosynthesis (accumulated A_N) (a), simulated daily fruit DW (b) and fruit oil content (% DW) in mesocarp tissue (c). Numbers represent slope growth of the curve during different periods (accumulated A_N/day, fruit DW g/day and % oil content/day respectively). Well-watered trees (WW) and water-stressed trees (WS) are represented. Grey area is the deficit irrigation period (DOY 196–243), which occurred before the sampling dates 20 and 24 WAF. Vertical solid lines indicate the period of pit hardening (DOY 170–191), and vertical dotted line is the date where fruit ripening starts. The three times of sampling are indicated in WAF. Data are mean ± SE from three different plots. Asterisks means differences ($p \leq 0.05$) according to one-way ANOVA. The arrow indicates the beginning of the carbon source treatments.

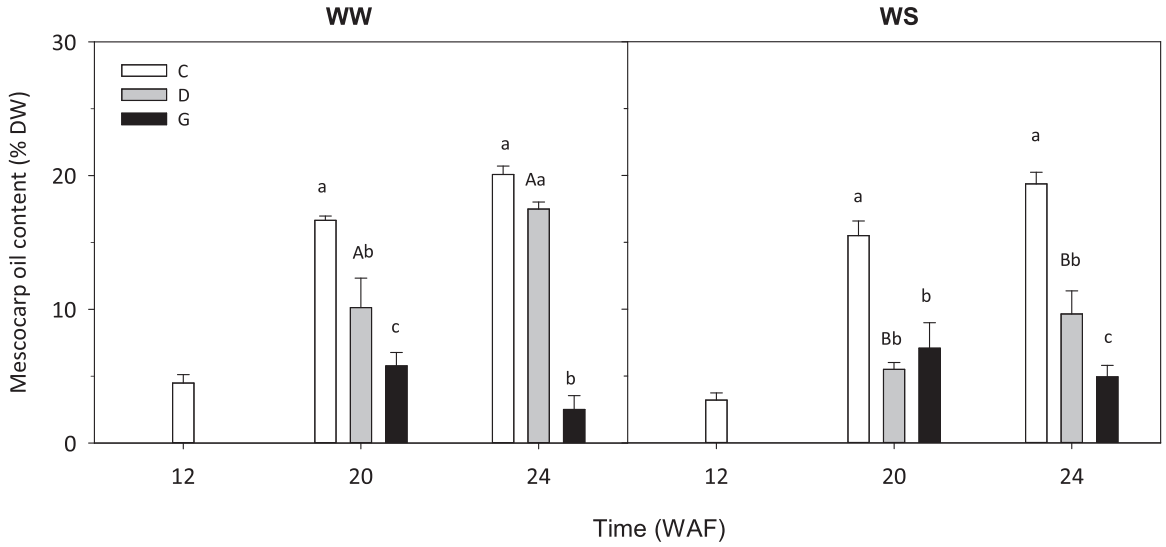

FIGURE 2 Oil content (% DW) in mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, mesocarp oil content was analysed as described in Section 2. Data are mean ± SE from three biological replicates. Capital letters determine significant differences ($p \leq 0.05$) between both irrigation treatments within each fruit altered carbon source treatment and fruit development period according to two-way ANOVA. Lowercase letters determine significant differences ($p \leq 0.05$) between fruit treatments according to two-way ANOVA.

mesocarp compared to C was observed, although to a different extent (Figure 2). In WW conditions, G fruit showed the highest reduction of mesocarp oil content at both times, whereas D fruit exhibited a diminution that was statistically significant at 20 WAF but not at 24 WAF. Regarding WS treatment, a stronger decrease for D fruit and milder reduction in G fruit for both 20 and 24 WAF was found compared to WW treatment. In contrast, the mesocarp oil content of C and G fruit was not significantly different between WW and WS treatments at 20 and 24 WAF (Figure 2). D fruit in WS conditions exhibited a lower oil content in the mesocarp compared to WW treatment. Interestingly, D and G treatments accelerated fruit ripening, especially in the case of G fruit (Supporting Information: Figure S2). However, this effect was slowed under water stress.

Furthermore, different altered carbon source conditions and levels of water stress were studied to determine their effect on the unsaturated fatty acid composition of the olive fruit mesocarp (Figure 3). The altered carbon source treatment substantially affected the fatty acid profile. In WW at 20 WAF, a significant reduction of oleic acid together with an increase in the linoleic and linolenic acid content was detected in the mesocarp of G fruit relative to C and D fruit. Similarly, this occurred in the WS treatment at 20 WAF, except for the linolenic acid content. In this case, its content was not only increased in G fruit mesocarp but also in the mesocarp of fruit from the D treatment. Overall, similar trends were observed at 24 WAF in the different altered carbon source and irrigation treatments, except for linolenic acid in the WS treatment, where the D and G treatments showed no significant differences. However, no differences were observed in the fatty acid composition of olive fruit mesocarp when comparing the two irrigation treatments.

3.2 | Expression levels of genes encoding TAG synthesizing enzymes in the olive fruit mesocarp under different altered carbon source and irrigation conditions

To examine the effect of the different altered carbon source and irrigation treatments on the expression of the genes involved in the last step of TAG synthesis, the transcript levels of *DGAT* and *PDAT* genes in the olive fruit mesocarp were determined by qRT-PCR. Since no significant differences were found in oil content and fatty acid composition between 20 and 24 WAF stages, we decided to analyse gene expression only at 20 WAF.

With respect to the different altered carbon source treatments and concerning the analysed *DGAT* genes (Figure 4), only *DGAT2* transcript levels decreased significantly in the G treatment compared to C and D in WW conditions at 20 WAF. In the case of WS treatment, D fruit mesocarp showed a significant decrease of *DGAT1-1* expression levels compared to C, while G conditions produced a strong reduction in the mesocarp transcript levels not only for *DGAT1-1* but also for *DGAT2* compared to C. Interestingly, when comparing WS conditions with WW treatment, a significant increase in the expression levels of *DGAT1-1* and *DGAT1-2* genes was observed at 20 WAF in C fruit mesocarp, and the *DGAT1-2* gene also had significantly increased mesocarp transcript levels in the G treatment. Expression of the *DGAT1-3* gene was not detected in any condition.

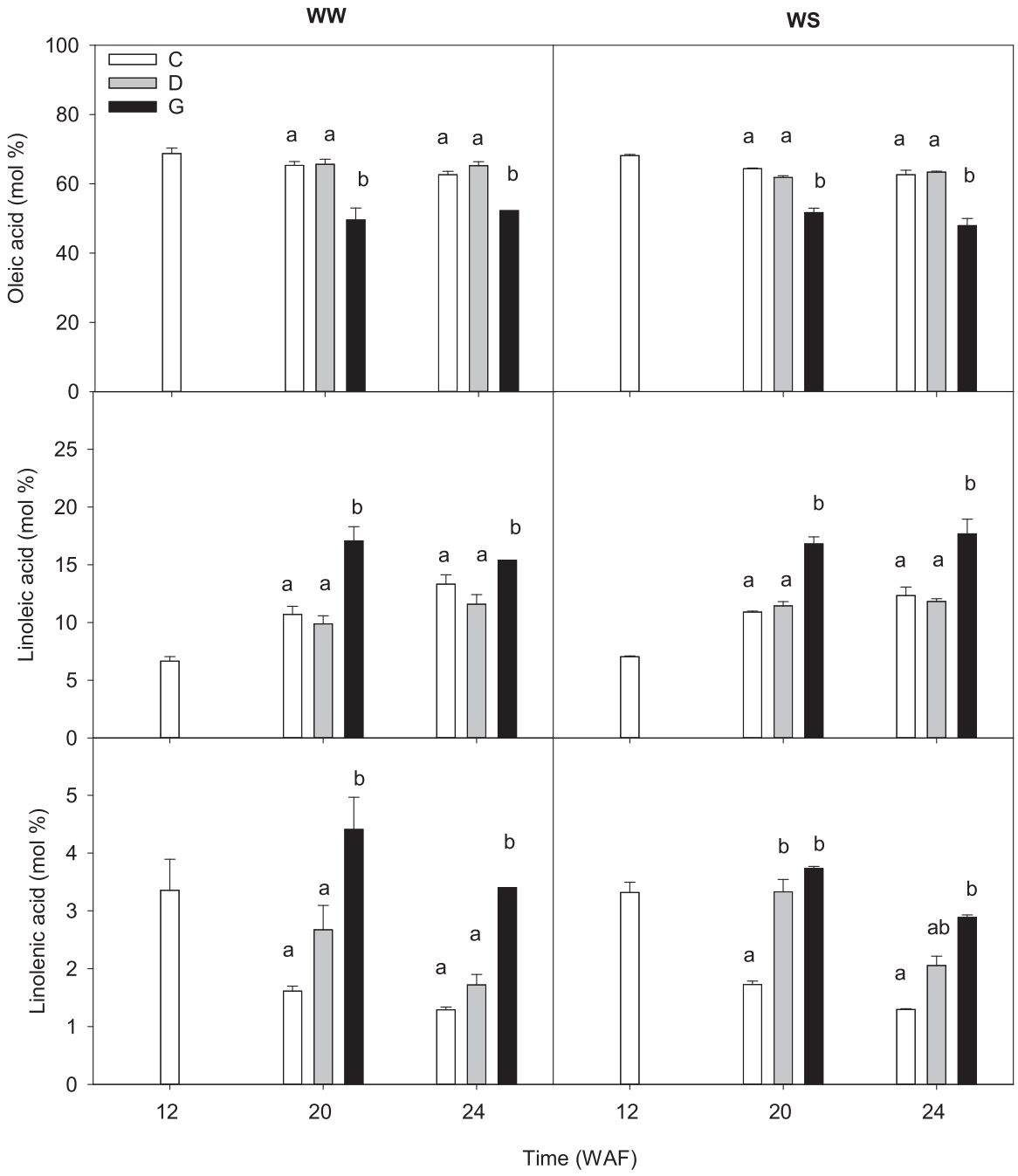

FIGURE 3 Oleic, linoleic and linolenic acids percentage in the mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, fatty acid composition was analysed as described in Section 2. Data are mean ± SE from three biological replicates. Letters determine significant differences ($p \leq 0.05$) between fruit altered carbon sources according to two-way ANOVA.

In relation to *PDAT* genes (Figure 5), only significant changes were detected in the transcript levels of the *PDAT1-1* gene. Regarding altered carbon source conditions, in the WS treatment, a strong decrease in its expression levels was observed in D and G fruit mesocarp with respect to C. When WW and WS conditions were compared, a strong increase in *PDAT1-1* transcript levels was detected in the mesocarp of C fruit. Expression of the *PDAT2* gene was not observed in any case.

3.3 | Transcript levels of membrane-bound fatty acid desaturase genes in the olive fruit mesocarp under different altered carbon source and irrigation treatments

The effect of different altered carbon source and irrigation conditions on the expression levels of oleate and linoleate desaturase genes in

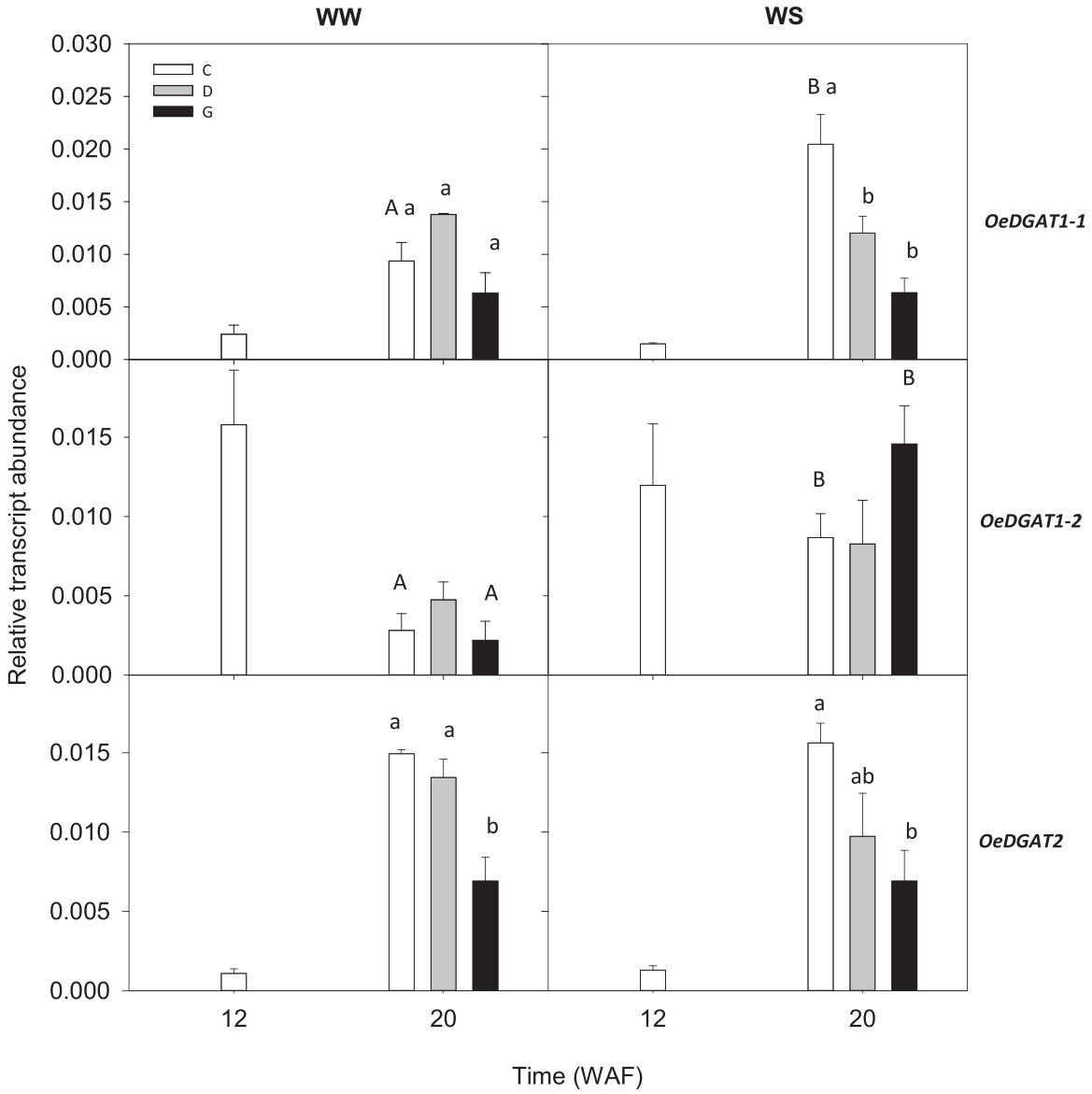

FIGURE 4 Relative transcript abundance of olive *DGAT* genes in the mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, relative transcript abundance was determined by qRT-PCR as described in Section 2. Data are mean ± SE from three biological replicates. Capital letters determine significant differences ($p \leq 0.05$) between both irrigation treatments within each fruit carbon source according to two-way ANOVA. Lowercase letters determine significant differences ($p \leq 0.05$) between fruit altered carbon sources according to two-way ANOVA. Letters are not shown when significant differences were not found.

the olive fruit mesocarp was also analysed. In particular, the transcript levels of *FAD2* and *FAD7* genes were determined, since they have been demonstrated as the main genes responsible for the linoleic and linolenic acid synthesis, respectively, in this tissue (Hernández et al., 2009, 2016, 2020, 2021b).

In the case of *FAD2* genes (Figure 6) and altered carbon source treatments, under the WW treatment, D and G fruit mesocarp showed a significant increase in *FAD2-2* gene expression levels compared to C, with that increase higher for G than for D fruit mesocarp. In WW conditions, the mesocarp of G fruit also showed a significant increment in *FAD2-1* transcript levels compared to that of C and D fruit. Concerning the WS regime, *FAD2-2* showed higher expression in G fruit mesocarp than in C and D, while *FAD2-5* exhibited a significant reduction of its expression levels in the mesocarp from D and G treatments compared to C. When comparing the same altered carbon source treatment but under WW or WS conditions, *FAD2-1* had increased transcript levels in D fruit mesocarp but decreased in G, D and G fruit mesocarp had decreased expression of *FAD2-2*, and C had increased expression of *FAD2-5* in the mesocarp. Expression of *FAD2-3* and *FAD2-4* genes was not detected in any condition.

Regarding *FAD7* genes (Figure 7), no differences in their mesocarp transcript levels were found in fruit under WW or WS

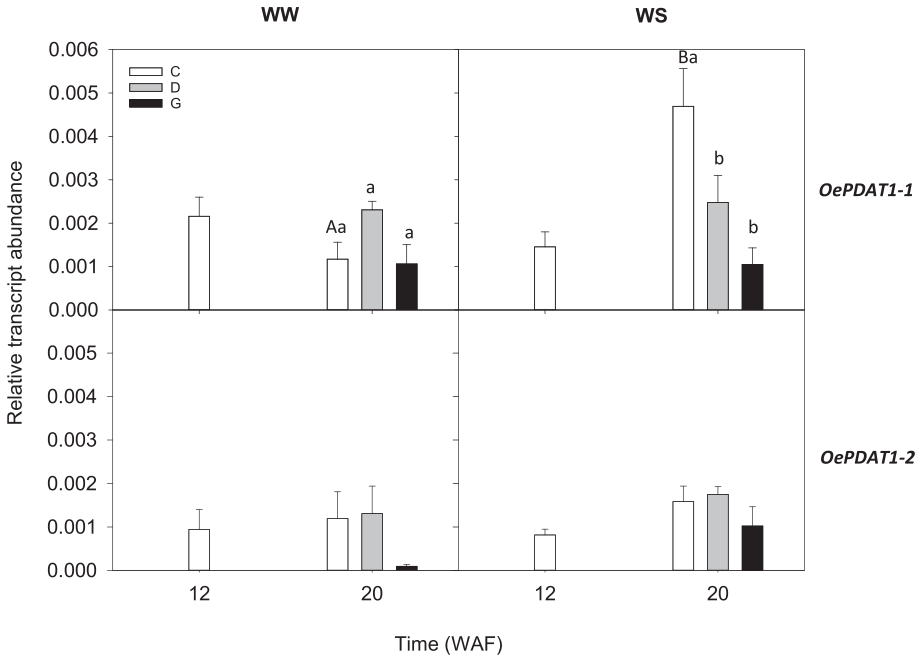

FIGURE 5 Relative transcript abundance of olive *PDAT* genes in the mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, relative transcript abundance was determined by qRT-PCR as described in Section 2. Data are mean ± SE from three biological replicates. Capital letters determine significant differences ($p \leq 0.05$) between both irrigation treatments within each fruit carbon source according to two-way ANOVA. Lowercase letters determine significant differences ($p \leq 0.05$) between fruit altered carbon sources according to two-way ANOVA. Letters are not shown when significant differences were not found.

conditions at 20 WAF for any of the altered carbon source treatments. However, when the WS regime was compared to WW conditions, a significant increase in *FAD7-1* expression was observed in G fruit mesocarp.

4 | DISCUSSION

Our work provides novel insights into the regulation of TAG accumulation and fatty acid composition in response to carbon source availability and water stress in a photosynthetic oil fruit mesocarp. The two treatments (altered carbon source and irrigation) produced a range of carbon availability for the synthesis of TAG, which together with the regulation of key gene transcript levels allowed us to progress our understanding of olive oil synthesis and its response to water stress.

As a methodological consideration, we cannot discard that the removal of phloem in the girdled and defoliated shoot treatment affected other processes than just the transport of sugars to the fruit. Phloem has an important signalling role facilitating electrical signals (Sukhov et al., 2019), as well as the transport of some chemical molecules, including phytohormones (Jorgensen et al., 1998). However, most of the processes of fruit development are regulated and performed from the fruit itself (Giovannoni, 2004; Pesaresi et al., 2014).

4.1 | Leaf and fruit photosynthesis participate in the carbon supply for oil biosynthesis in olive fruit mesocarp, with a major contribution of photoassimilates imported from the leaves

To examine the origin of the carbon source, generated from leaf and fruit photosynthesis, for TAG synthesis and oil content in the olive fruit mesocarp, different altered carbon source conditions were applied. As shown in Figure 2, a reduction in the oil content was found in the mesocarp of olive fruit from D and G altered carbon source treatments compared to the control. These results showed that not only the import of photoassimilates from leaves but also fruit photosynthesis contribute to oil biosynthesis in olive mesocarp since oil accumulation was observed in both carbon source treatments. However, although fruit photosynthesis is important, it is not an alternative to carbon import. In accordance with our results, transcriptomic studies performed in the avocado mesocarp showed high transcript levels for Rubisco and PEPc, suggesting a role for fruit photosynthesis in the carbon supply for oil biosynthesis in this oil fruit (Kilaru et al., 2015).

In addition, the higher decrease in the mesocarp oil content detected in G fruit related to D fruit in WW conditions indicated a major contribution of leaf photosynthesis with respect to fruit photosynthesis. This is true even in the case that the reduction observed in the mesocarp of D fruit could be partially due to the

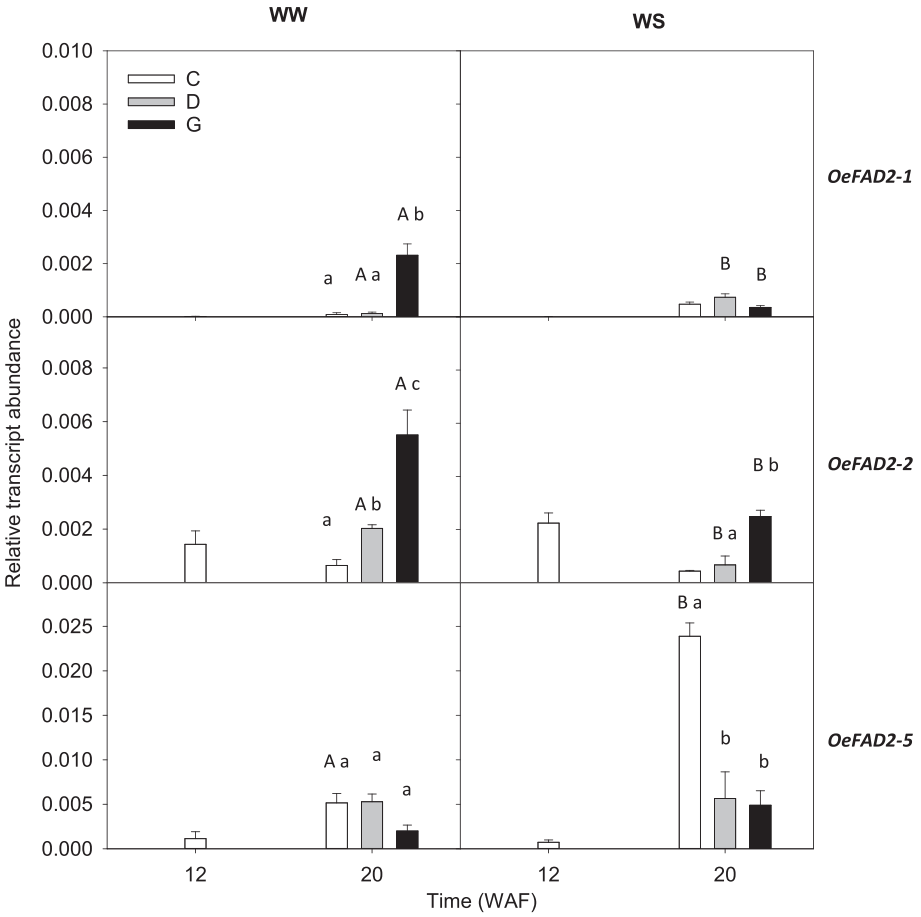

FIGURE 6 Relative transcript abundance of olive *FAD2* genes in the mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, relative transcript abundance was determined by qRT-PCR as described in Section 2. Data are mean ± SE from three biological replicates. Capital letters determine significant differences ($p \leq 0.05$) between both irrigation treatments within each fruit carbon source according to two-way ANOVA. Lowercase letters determine significant differences ($p \leq 0.05$) between fruit altered carbon sources according to two-way ANOVA. Letters are not shown when significant differences were not found.

inactivation of acetyl-CoA carboxylase, which catalysed the first committed and the only light-regulated step of de novo fatty acid synthesis in plants (Ye et al., 2020). In contrast to our results, a similar contribution to oil synthesis was reported for heterotrophic and autotrophic olives from the Picual cultivar (Sánchez, 1995; Sánchez & Harwood, 2002). This discrepancy could be explained because in those studies, the autotrophic treatment of defoliating leaves from the same branch as the fruit was not accompanied by phloem girdling. This is essential to prevent the import of photoassimilates from leaves from other branches of the olive tree, as it is shown in Supporting Information: Figure S3 where no significant differences in oil content were found between control and defoliated shoot treatment. In addition, the distinct magnitude of the decrease in oil content caused by the altered carbon source treatments could also be due to the use of a different cultivar. In this sense, it has been recently demonstrated that the effect of light availability on olive fruit development is cultivar-dependent (Reale et al., 2019).

In contrast, the WS treatment relative to the WW regime enhanced the reduction in oil content observed in the mesocarp of D olives, whereas in G olive mesocarp the detected decrease was attenuated (Figure 2). These data show that water stress affects the carbon supply for oil synthesis in olive mesocarp more severely in the case of the photoassimilates from leaves than in that involving fruit photosynthesis. This result can be explained by the lower water status of water-stressed plants, which reduced their capacity to transport sugars via the phloem from leaves to fruit (Martre et al., 2011), illustrating the importance of imported carbon in the fruit for oil synthesis. Consistently, no differences existed in the mesocarp oil content between WW and WS in G fruit since they do not rely on the carbon imported from leaves. It is also remarkable how the sum of both D and G mesocarp oil content equals C in all situations (Figure 2), confirming that the source of carbon for oil biosynthesis in the olive mesocarp was from both fruit and leaf photosynthesis.

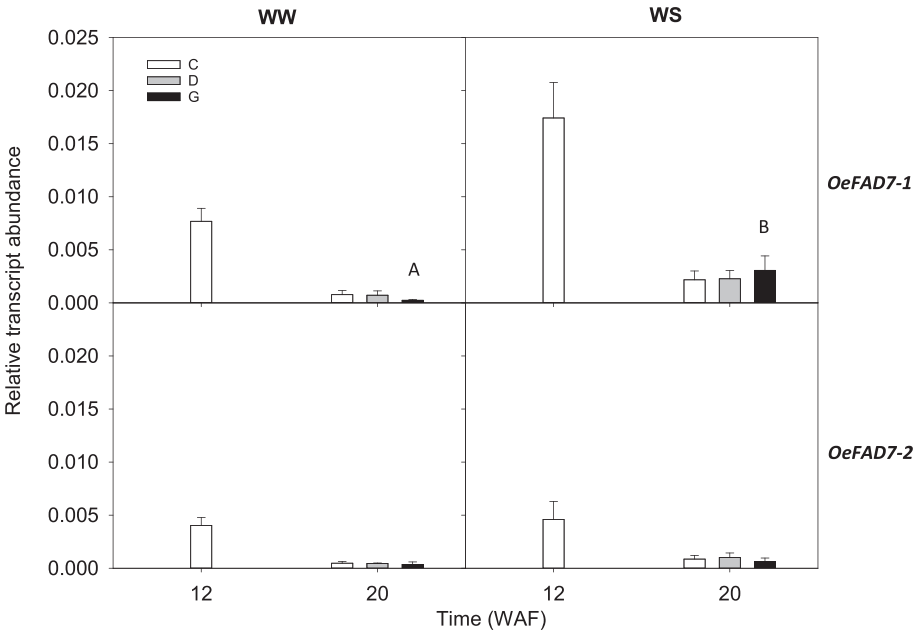

FIGURE 7 Relative transcript abundance of olive *FAD7* genes in the mesocarp tissue of olives (cv. Arbequina) grown under different altered carbon source conditions: control (C), darkened fruits (D) and girdled and defoliated shoots (G); and two different irrigation treatments: well-watered (WW) and water-stressed (WS). The deficit irrigation period occurred before the sampling dates 20 and 24 WAF. At the indicated times, relative transcript abundance was determined by qRT-PCR as described in Section 2. Data are mean ± SE from three biological replicates. Capital letters determine significant differences ($p \leq 0.05$) between both irrigation treatments within each fruit carbon source according to one-way ANOVA. Lowercase letters determine significant differences ($p \leq 0.05$) between fruit altered carbon sources according to two-way ANOVA. Letters are not shown when significant differences were not found.

4.2 | The oil content of olive mesocarp is not affected by water stress without altered carbon source treatments

The imposed water stress without altered carbon source treatments reduced the carbon assimilation rate estimated at the leaf level (Figure 1a) and decreased the fruit size (Figure 1B), but the mesocarp oil content was not affected (Figures 1c and 2). It is important to note that the water stress treatment was imposed for 5 weeks starting at 10 WAF. Afterwards, FI was resumed some 2–3 weeks before the sampling at 20 WAF. One of the most intriguing aspects when applying RDI strategies in olive is that a significant reduction of irrigation is not usually proportionally reflected in the reduction of oil yield (Hernandez-Santana et al., 2017, 2018; Iniesta et al., 2009). The tolerance of olive fruit to water stress has been explained by the lower sensitivity of oil synthesis processes to water deficit than other processes, such as vegetative growth (Hernandez-Santana et al., 2017). A complementary explanation for the maintenance of the mesocarp oil content in WS compared to WW is that oil synthesis could have used the spare carbon that was not used for fruit growth. Growth and oil synthesis are the major carbon sinks of the fruit. However, the factor impairing fruit growth may have been the water status of the fruit (Dell'Amico et al., 2012; Fernandes et al., 2018; Girón et al., 2015), as an increasing number of works have demonstrated that the water status is more growth limiting than carbon availability (Fatichi et al., 2014; Körner, 2015; Steppe et al., 2015). The fruit growth inhibition produced by water factors, in turn, would have resulted in extra carbon available for other biosynthetic routes in the mesocarp, such as fatty acid biosynthesis and TAG accumulation.

4.3 | Carbon supply and water status affect oil synthesis in the olive mesocarp, regulating *DGAT* and *PDAT* transcript levels

The specific contribution of *DGAT* and *PDAT* enzymes to the synthesis of TAG is a key point to elucidate the mechanisms that regulate oil accumulation in oil crops, and their specific involvement is dependent on the species (Chapman & Ohlrogge, 2012). In olive, since there are no mutants available, a different approach was employed by altering the carbon supply to the fruit, together with two different irrigation regimes, to examine if the detected changes in mesocarp oil content correlated with alterations in the transcript levels of the corresponding *DGAT* and *PDAT* genes in this tissue.

In WW plants, the decrease in the mesocarp oil content noted in G fruit (Figure 2) was parallel to the reduction in the expression levels of *DGAT2* and *PDAT1-2* genes detected in this tissue (Figure 4). In the case of WS olives, the diminution in oil content measured in D fruit mesocarp (Figure 2) coincided with the observed decrease of the *DGAT1-1*, *DGAT2*, *PDAT1-1*, and *PDAT1-2* transcript levels in the mesocarp (Figures 4 and 5), while the reduction in oil content noted

in G fruit mesocarp correlated with the diminution of *DGAT1-1*, *DGAT2*, and *PDAT1-1* expression levels observed in this tissue.

These results implicate *DGAT1-1*, *DGAT2*, *PDAT1-1* and *PDAT1-2* as the genes that could be involved in TAG biosynthesis and oil accumulation in the olive mesocarp, with a major role for *DGAT2* and *PDAT1-2* in conditions of no water stress. The participation of *DGAT1-1* and *DGAT2* genes in the synthesis of TAG in this tissue has been previously reported for cultivar Koroneiki (Banilas et al., 2011). In addition, in the 'Picual' and 'Arbequina' mesocarp, it has been proposed that the incorporation of linoleic acid into TAG may occur preferentially via the Kennedy pathway, with a minor contribution of *PDAT* activity (Hernández et al., 2020). However, according to our data, not only *DGAT* but also *PDAT* genes could participate in oil synthesis and accumulation in the mesocarp of olive fruit in conditions of altered carbon supply and water stress, with *DGAT* and *PDAT* enzymes cooperating to guarantee the synthesis of TAG. In accordance with these results, the contribution of *PDAT1-2* to the synthesis of TAG in olive fruit mesocarp has been recently described (Hernández et al., 2021a). In addition, previous studies indicated that *PDAT* may also contribute to TAG biosynthesis in olive callus culture (Hernández et al., 2008). In oilseeds, an overlapping role for *DGAT* and *PDAT* in oil accumulation has been proposed (Zhang et al., 2009). Remarkably, the *DGAT* and *PDAT* genes involved in this regulation of oil synthesis encode enzymes located at the final step of the TAG biosynthetic pathway without affecting membrane lipid biosynthesis. Furthermore, our results suggest that TAG synthesis is a priority for oil fruit mesocarp even under water stress conditions. Fruit plays a role in disseminating the seeds to accumulate storage substances to attract animals and enhance the success of disseminating the matured seeds. Therefore, mesocarp oil accumulation rather than fruit size might be a better option for the plant.

Our data also indicate that both carbon supply and water status affect oil synthesis in the olive mesocarp, regulating the transcription of *DGAT* and *PDAT* genes. In this sense, the MYB96 transcription factor has been reported to activate *DGAT1* and *PDAT1* expression in Arabidopsis seeds (Lee et al., 2018). Interestingly, Arabidopsis MYB96 triggers not only drought-related traits, such as stomatal closure (Seo et al., 2009) but also regulates ABA-dependent TAG biosynthesis in vegetative tissues as carbon and energy storage, to further ensure plant growth and development under long-term drought stress conditions (Lee et al., 2019).

Conversely, the substantial increase detected for *DGAT1-1*, *DGAT1-2*, and *PDAT1-1* gene expression levels in C fruit mesocarp at 20 WAF under WS treatment compared to WW conditions (Figures 4 and 5) indicates that these genes were transcriptionally upregulated by water stress. In opposition to the effect of water stress in C fruit mesocarp, no significant changes were found between WW and WS in the transcript level of *DGAT* and *PDAT* genes in D fruit mesocarp, and only one gene, *DGAT1-2*, was significantly upregulated in the mesocarp of G fruit. The upregulation of these genes seemed to be related to water stress and not to the imposition of altered carbon source conditions, as suggested by their expression under WW conditions. However, it is worth noting that both water stress and altered carbon source treatments have the reduction of available carbon in common.

4.4 | Modifying the carbon supply alters *FAD2* and *FAD7* expression levels and unsaturated fatty acid composition in the olive mesocarp

The carbon availability not only affected the oil content of the mesocarp but also its fatty acid composition. The alterations observed in the unsaturated fatty acid content were accompanied by changes in the transcript levels of the membrane-bound fatty acid desaturase genes responsible for their desaturation, indicating that the source of carbon supply for fatty acid biosynthesis regulates FADs at the transcriptional level in olive mesocarp.

In the case of WW fruit, the diminution in the oleic acid percentage and the increase in linoleic and linolenic acid seen in the mesocarp of G olives (Figure 3) correlated with the observed augmentation in the mesocarp *FAD2-1* and *FAD2-2* transcript levels (Figure 6). Regarding WS olives, the decrease in oleic acid together with the augmentation in linoleic and linolenic acid in G fruit mesocarp was parallel to the detected increase in the expression levels of *FAD2-2* and *FAD7-1* genes detected in this tissue (Figures 3, 6, and 7).

Therefore, *FAD2-1*, *FAD2-2*, and *FAD7-1* were the fatty acid desaturase genes that could be responsible for the changes in the unsaturated fatty acid composition observed in response to the altered carbon supply in the olive mesocarp. The expression of these genes could be increased in response to the new carbon supply conditions to ensure the correct redistribution of available carbon. In oilseeds, two different transcription factors were reported to regulate the expression of *FAD2* genes, such as bHLH in sesame (Kim et al., 2007) and Dof11 in rapeseed (Sun et al., 2018). However, no similar information has been reported in oil fruit.

Additionally, in C fruit mesocarp at 20 WAF under WS conditions, a high increase of *FAD2-5* transcript levels was detected in comparison with the WW treatment (Figure 6). In a previous study, a reduction in *FAD2-5* gene expression levels during olive mesocarp development was observed under water stress (30 RDI) (Hernández et al., 2020). Together, these results suggest that *FAD2-5* is the olive *FAD2* gene regulated by water supply. However, the dissimilar responses detected could be due to the different water treatments and the distinct climatic conditions in the years of the two studies. In addition, it has been reported that drought stress increased *FAD2* expression in mandarin seedlings (Gimeno et al., 2009) and purslane leaves (D'Andrea et al., 2015), whereas osmotic stress enhanced the transcription of *FAD2* in lima bean leaves (Zhang et al., 2011) and Arabidopsis seedlings (Zhang et al., 2012). Furthermore, *FAD7* has also been reported to be involved in drought resistance, since the antisense expression of an Arabidopsis *FAD7* gene in transgenic tobacco plants reduced drought tolerance (Im et al., 2002).

Although changes in the percentage of fatty acid composition in the olive fruit mesocarp were small, their role might be crucial for the

plant since changes in the degree of fatty acid desaturation relate to the activation of intracellular signalling in response to abiotic stress. Polyunsaturated fatty acids, such as linoleic and linolenic acids, are precursors of oxylipins, including stress-related phytohormones, such as jasmonic acid (JA), which are involved in the mechanisms of the stress response (Wasternack & Feussner 2018).

5 | CONCLUSIONS

In the present study, a stronger reduction in the oil content of mesocarp tissue was detected in olive fruit under girdled and defoliated shoot treatment compared to darkened fruit conditions. This indicates that even though both leaf and fruit photosynthesis participate in the carbon supply for oil biosynthesis in olive fruit mesocarp, the major contribution of photoassimilates is imported from the leaves. Our results also demonstrated that carbon supply and water status affect oil synthesis and fatty acid composition in the olive mesocarp, regulating the transcript levels of *DGAT*, *PDAT*, and *FAD* genes. In particular, *DGAT1-1*, *DGAT2*, *PDAT1-1*, and *PDAT1-2* seemed to be the genes involved in TAG biosynthesis and oil accumulation in this tissue, with a major role for *DGAT2* and *PDAT1-2* in WW conditions. In addition, *FAD2-1*, *FAD2-2*, and *FAD7-1* transcript levels and polyunsaturated fatty acid content increased in olive fruit mesocarp in response to the altered carbon supply. Moreover, *DGAT1-1*, *DGAT1-2*, *PDAT1-1*, and *FAD2-5* gene expression levels in olive mesocarp were transcriptionally upregulated by water stress. This study represents a significant advance in the understanding of the molecular mechanisms regulating TAG synthesis and composition in the olive mesocarp. In the future, this information will allow the development of molecular markers for the marker-assisted selection of new olive cultivars with increased oil content in olive fruit. Furthermore, these results provide for a better understanding of how olive mesocarp TAG content and composition are affected by water deficit, permitting us to choose a better irrigation strategy and to decide how much and when water stress can be imposed, which is critical to obtain olive oil with the highest yield and quality with minimum irrigation.

ACKNOWLEDGEMENTS

This study was supported by Spanish Ministry of Science, Innovation and Universities through Research Grants AGL2015-71585-R and AGL2017-87871-R (AEI/FEDER, UE). A. Montero and A. Perez-Martin helped us with field measurements. A.P.-A. was the recipient of a contract from the FPI-CSIC program (Spain). We thank *Internacional Olivarera, S.A.U. (Interoliva)*, for allowing us to make the experiments at Sanabria orchard.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Perez-Arcoiza, A., Luisa Hernández, M., Dolores Sicardo, M., Hernandez-Santana, V., Diaz-Espejo, A. & Martinez-Rivas, J. M. (2022) Carbon supply and water status regulate fatty acid and triacylglycerol biosynthesis at transcriptional level in the olive mesocarp. *Plant, Cell & Environment*, 1–15. https://doi.org/10.1111/pce.14340