**Effect of a Regulated Deficit Irrigation Strategy in a Hedgerow
‘Arbequina’ Olive Orchard on the Mesocarp Fatty Acid
Composition and Desaturase Gene Expression with Respect to
Olive Oil Quality**

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ABSTRACT

The effect of three irrigation strategies on the olive oil quality in a hedgerow ‘Arbequina’ olive orchard with 1667 trees ha⁻¹ was assessed. Olive trees were fully irrigated, with the crop water demand replaced through daily irrigation (FI), or irrigated according to two regulated deficit irrigation (RDI) treatments, in which the total irrigation supplies amounted to 60% and 30% of the water supplied to the FI treatment. Oil content, fatty acid composition and desaturase genes expression in fruits from the three treatments were analysed. Although the total oil content and the amounts of the diverse lipid classes were not affected by the different irrigation treatments, changes in the fatty acid composition during the mesocarp development and ripening in the three irrigation treatments were detected. The 30RDI strategy caused a decrease in the linoleic acid content in olive mesocarp and, therefore, in the olive oil. The lack of correlation with the expression of known oleate desaturase genes (*FAD2-1*, *FAD2-2* and *FAD6*) points out to an un-identified oleate desaturase gene that could be repressed by water stress. Linolenic acid content and expression levels of plastidial linoleate desaturase genes (*OeFAD7-1* and *OeFAD7-2*) increased under 30RDI conditions at the beginning of fruit development. However, this effect was not noticeable in the olive oil because it was not observed during fruit ripening. All these data enlighten the molecular mechanisms related to the fatty acid desaturation in the oil of olive trees under water stress and point out to the 30RDI as suitable strategy when olive quality is the main production target.

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26 **KEYWORDS**

27 *Olea europaea*, olive fruit, fatty acid desaturase, gene expression, olive oil, water deficit.

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29 **ABBREVIATIONS USED**

30 ACP, acyl carrier protein; ER, endoplasmic reticulum; ETc, potencial crop evotranspiration;
31 FAD2, microsomal ω 6 desaturase; FAD3, microsomal ω 3 desaturase; FAD6, plastidial ω 6
32 desaturase; FAD7/8, plastidial ω 3 desaturase; FI, fully irrigated; IN, irrigation needs; *P*,
33 precipitation; PC, phosphatidylcholine; RDI, regulated deficit irrigation; SAD, stearyl-ACP
34 desaturase; SD, standard deviation; SHD, super high density; TAG, triacylglycerol; WAF,
35 weeks after flowering; qRT-PCR, quantitative real-time PCR.

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38 **1. Introduction**

39 Olive (*Olea europaea* L.) is one of the most economically relevant tree crops in the
40 Mediterranean basin. Historically, olives for oil production have been rain-fed cultivated, with
41 widely spaced trees, to take full advantage of the water stored in the soil during winter rains for
42 the next spring and summer growth. More recently, new orchards are planted at higher plant
43 densities and have localised irrigation system, resulting in higher yields and improvement in
44 the alternate-bearing behaviour (Beede and Goldhamer, 1994). Within the last two decades,
45 hedgerow orchards with plant densities over 1500 trees ha⁻¹, also called super high density

(SHD) orchards, facilitate the use of over-the-row mechanical harvesters and bring orchards into production sooner after planting. Tree spacing, pruning, weed control, pest management, nutrition and irrigation are being managed intensively in this type of orchards. However, there is still inconsistent information about the specific effect of irrigation on the production and quality of the oil. Current evidence suggests that a deficit irrigation strategy could be the best option for SHD olive orchards, since problems derived from excessive tree vigour can be minimized by reduced irrigation (Connor, 2006). Regulated deficit irrigation (RDI) consists on replacing crop evotranspiration (ET_c) in the phases of the growing cycle when the crop is most sensitive to water stress, and reducing or even withholding irrigation for the rest of the cycle (Chalmers et al., 1981). The quantity of water applied to trees in SHD orchards has a deep impact on olive oil quality (Berenguer et al., 2006; Dag et al., 2015; García et al., 2013, 2017; Gómez-del-campo and García, 2013; Rosecrance et al., 2015). However, the effect of different irrigation strategies on the olive oil fatty acid composition remains unclear.

The olive oil is obtained by mechanical extraction from the olive fruit, which is a drupe consisting of a fleshy pericarp and a woody endocarp that encloses a single seed. The pericarp, in turn, consists of an outer epicarp and an inner mesocarp. The olive mesocarp forms and stores TAG and, possessing active chloroplasts, it has the capability of fixing atmospheric CO_2 and incorporating it into storage material (Sánchez, 1994). TAG fatty acid composition determines the technological and nutritional properties of the virgin oil. Oleic acid is the major fatty acid in the olive oil (55-83%), whereas linoleic acid accounts for 3.5-21% and linolenic acid for <1%. The relative contents of oleic, linoleic and linolenic acids depend mainly on the cultivar but also on pedoclimatic and management conditions (Beltrán et al., 2004).

Fatty acid biosynthesis in higher plants starts in the plastid, yielding primarily palmitoyl-acyl carrier protein (ACP) and stearoyl-ACP by successive addition of two carbon atoms from acetyl-CoA (Harwood, 2005). Still in the plastid, the desaturation of stearoyl-ACP

is carried out by the soluble stearoyl-ACP desaturase (SAD) to produce oleoyl-ACP, which is the main product of the plastidial fatty acid biosynthesis. The oleoyl-ACPs are then cleaved by specific thioesterases to free fatty acids, which are then activated to acyl-CoAs and incorporated into the glycerolipid biosynthetic pathway, where can be further desaturate to linoleic and linolenic acids. Two sets of $\omega 6$ and $\omega 3$ membrane-bound fatty acid desaturases have been described, which differ in their cellular localization, lipid substrates, and electron donor system (Shanklin and Cahoon, 1998). The microsomal $\omega 6$ and $\omega 3$ desaturases (FAD2 and FAD3, respectively) are located in the endoplasmic reticulum (ER) and use phospholipids as acyl substrates, as well as NADH, NADH-cytochrome b5 reductase, and cytochrome b5 as electron donors. On the other hand, the plastidial $\omega 6$ and $\omega 3$ desaturases (FAD6 and FAD7/8, respectively) are located in the chloroplast and use primarily glycolipids as acyl carriers, as well as NAD(P)H, ferredoxin-NAD(P) reductase, and ferredoxin as electron donors.

In olive, three genes encoding the plastidial SAD have been isolated and characterised: *OeSAD1* (Haralampidis et al., 1998), *OeSAD2* and *OeSAD3* (Parvini et al., 2016). Among them, *OeSAD2* resulted to be the main gene contributing to the oleic acid content of the olive fruit and, therefore, of the virgin olive oil (Parvini et al., 2016). Regarding membrane bound desaturases, two genes encoding microsomal oleate desaturases (*OeFAD2-1* and *OeFAD2-2*) has been reported by Hernández et al. (2005), whereas only one *OeFAD6* gene has been identified so far (Banilas et al., 2005; Hernández et al., 2011). Hernández et al. (2009) suggested that *OeFAD2-2* is the main gene that determines the linoleic acid content in the olive mesocarp and, therefore, in the virgin olive oil. On the other hand, four members of the olive linoleate desaturase gene family have been isolated and characterised, 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). Gene expression analysis and lipid profiling during olive fruit development and ripening revealed that the *OeFAD7-1* and

OeFAD7-2 genes contribute mainly to the linolenic acid present in the olive oil (Hernández et al., 2016).

Concerning the effect of irrigation on the olive oil quality, different and even opposite behaviours have been reported (Dag et al., 2015; Issaoui et al., 2013; Stefanoudaki et al., 2009; Tovar et al., 2002), indicating that the meteorological conditions and the growing area could have a greater effect on the olive oil fatty acid profile than the irrigation treatment. Recently, Fernández et al. (2013) tested the impact of a fully irrigated (FI) and two RDI treatments with the same RDI strategy but different irrigation levels, replacing 60% (60RDI) and 30% (30RDI) of the water supplied in FI, on a SHD olive orchard cv. Arbequina. The 30RDI strategy was the closest to an optimum irrigation management for oil production, since it improved physical oil extraction and led to the virgin olive oil with higher content of phenolic compounds and greater oleic/linoleic ratio (Fernández et al. 2013, García et al. 2017). However, no information regarding fatty acid composition of total lipids and lipid classes of mesocarp tissue during olive fruit development and ripening has been reported so far. In addition, with the exception of a previous paper from our group on how two different water regimes (rain-fed and irrigated) affect transcript levels of olive *FAD2* and *FAD6* genes (Hernández et al. 2009), no data related to the effect of water deficit on the fatty acid desaturase gene expression levels in olive mesocarp tissue has been reported up to date.

In this sense, the aim of this work was to investigate how the three different irrigation treatments applied by Fernández et al. (2013) in the ‘Arbequina’ SHD orchard affect the oil content, fatty acid composition of total lipids and lipid classes, and fatty acid desaturase genes expression levels during olive fruit development and ripening in relation to olive oil quality.

2. Materials and Methods

2.1. Orchard Characteristics and Irrigation Treatments

The study was conducted in 2012 at the Sanabria orchard, a commercial SHD olive (*O. europaea* L., cv. Arbequina) orchard near Seville, south west Spain. Trees were planted in 2007, in rows north-northeast to south-southwest oriented, and at 4 m x 1.5 m (1667 tress ha⁻¹). Climate in the area is Mediterranean, with mild rainy winters and hot, dry summers. Most of the annual rainfall occurs between late September and May. For the studied year, values in the area of potential evapotranspiration (ET_c) and precipitation (*P*) were 1598.2 mm and 404.6 mm, respectively.

The irrigation season lasted from June 5 to October 22. The harvesting was made on November 13, but irrigation was not needed from October 22 because rainfall was enough to replace ET_c. We had a FI treatment and two RDI treatments, as described by Fernández et al. (2013). Basically, the FI trees were irrigated daily all along the irrigation season, to replace the irrigation needs (IN), calculated as $IN = ET_c - P_e$, being ET_c the maximum potential crop evapotranspiration calculated with the crop coefficient approach and *P_e* the effective precipitation calculated as 75% of the precipitation recorded in the orchard (Orgaz and Fereres, 2001). See Fernández et al. (2013) for details on the crop coefficients. In the RDI treatments, daily irrigation was applied just at two periods of the irrigation season when the crop is highly sensitive to water stress (Fernández, 2014), which in 2012 were in June and from late August to mid-September, i.e. at periods 2 and 3 defined by Fernández et al. (2013). At period 1 (bloom) rainfall was enough and irrigation was not needed. The RDI treatments were scaled to a total irrigation amount of 60% (60RDI) and 30% (30RDI) of IN. For details on the supplied irrigation

amounts in each treatment, see Fernández et al. (2013). Basically, in all treatments water was supplied with a pipe per tree row located close to the trunks and with a 2 L h⁻¹ dripper every 0.5 m. All treatments were fertilized with the same amounts, by injection the calculated dose in the irrigation system. The amounts of fertilizer were adapted every month to match the crop needs (Troncoso et al., 2001). We used a randomized block design with four 12 m x 16 m plots per treatment. Each plot contained eight central trees surrounded by 24 border trees. For each biological sample, the olive mesocarp tissue was collected from 50 different olive fruits harvested from the eight central trees contained in each of the four plots per treatment, frozen in liquid nitrogen and stored at -80 °C. Olive mesocarp tissues were sampled at different times after blooming, corresponding to different developmental stages of the olive fruit. Specifically, we analysed olive fruit mesocarp immediately after the lignification of the stone at 13 weeks after flowering (WAF), when the olive fruit was completely green (16 WAF), when the olive fruit was yellowish (22 WAF), at the onset of ripening when the olive fruit was turning from green to purple (29 WAF) and finally when the olive fruit was completely purple and matured (33 WAF).

2.2. Total RNA Extraction and cDNA Synthesis

Total RNA isolation was performed as described by Hernández et al. (2005) using 1.5 g of frozen olive mesocarp tissue. RNA quality verification, removal of contaminating DNA and cDNA synthesis were carried out according to Hernández et al. (2009).

2.3. Quantitative Real-Time PCR (qRT-PCR)

Gene expression analysis was performed by qRT-PCR as previously described by Hernández et al. (2009). Primers for gene-specific amplification were previously designed

using the Primer3 program (<http://bioinfo.ut.ee/primer3/>) by Parvini et al. (2016) for *SAD* genes, Hernández et al. (2009) for ω 6 desaturase genes, and Hernández et al. (2016) for ω 3 desaturase genes. The housekeeping olive ubiquitin2 gene (*OeUBQ2*, AF429430) was used as an endogenous reference for normalization (Hernández et al. 2009). The qRT-PCR data were calibrated relative to the corresponding gene expression level in 13 WAF mesocarp tissue from FI treatment (100%). The relative expression level of each gene was calculated following the $2^{-\Delta\Delta C_t}$ method for relative quantification (Livak and Schmittgen 2001). The data are presented as means $\pm$ standard deviation (SD) of three reactions performed in different 96-well plates, each having two replicates in each plate.

2.4. Lipid Analysis

Olive fruit mesocarp tissue was heated at 70 °C for 30 min with isopropanol to inactivate endogenous lipase activity. Lipids were extracted as described by Hara and Radin (1978), and lipid separation was carried out by thin layer chromatography according to Hernández et al. (2008). Fatty acid methyl esters of the different lipid preparations were produced by acid-catalysed transmethylation (Garcés and Mancha, 1993), and analysed by gas chromatography (Román et al., 2012). Heptadecanoic acid was used as internal standard to calculate the lipid and fatty acid content in the samples, by comparing the peak area of a known amount of heptadecanoic acid with the peak area of the different fatty acids. Total lipid content was calculated as the sum of the different fatty acids. Results are expressed in mol percent of the different fatty acids, and are presented as means $\pm$ SD of three independent determinations.

3. Results

For the whole irrigation season, the irrigation amounts were 4787 m³ ha⁻¹ for FI, 2913 m³ ha⁻¹ for 60RDI and 1346 m³ ha⁻¹ for 30RDI. These amounts were, respectively, 102%, 63% and 29% of IN, which means that the management of irrigation was precise enough for the actual irrigation amounts to be very close of the aimed amounts. The fertilizers supplied to each treatment, for the whole fertirrigation period, amounted to 310 kg ha⁻¹ N and K, 117 kg ha⁻¹ of P, and 195 kg ha⁻¹ of B and Fe. These amounts were enough to avoid nutrient deficiencies, as confirmed from leaf analyses (Fernández et al., 2013). The severe water stress, recorded as stem water potential values at midday (MPa)², was only detected at 16 WAF in 60 RDI trees, and at 13 and 16 WAF in the case of 30 RDI trees (Fernández et al., 2013).

To investigate how the different irrigation levels affected olive lipid biosynthesis, we analyzed the lipid composition of olive fruit mesocarp at different stages of development and ripening in fruits from FI, 60RDI and 30RDI trees. We observed that the total lipids content increased during mesocarp development and ripening in the three treatments, with no significant differences between RDI and FI, apart from a slight reduction observed in both 60RDI and 30RDI compared to FI at the beginning of mesocarp development (Fig. 1). The fatty acid composition analysis revealed small changes in the total unsaturated fatty acids content during the mesocarp development and ripening in the three irrigation treatments (Fig. 2a). However, we observed that oleic acid, as well as linolenic acid, decreased during the mesocarp development and ripening, unlike linoleic acid content that showed an increase (Fig. 2b). At the beginning of the mesocarp development, when the olive fruit was still green (13 and 19

WAF), the oleic acid contents in the 60RDI and 30RDI treatments were lower than in FI treatment. However, when the olive fruit was yellowish before maturation (22 WAF), the oleic acid contents in 60RDI and 30RDI were higher than in FI. During olive fruit ripening (29 to 33 WAF), the oleic acid contents were similar in 60RDI and FI, and lower than in 30RDI. These results were in agreement with the linoleic and linolenic acid trends. The linoleic acid content was similar in the three irrigation treatments at the beginning of mesocarp development, and lower in 60RDI and 30RDI than in FI at 22 WAF. During olive mesocarp ripening (29 to 33 WAF) the linoleic acid content was lower in 30RDI than in 60RDI and FI, with no differences among these two treatments. On the other hand, the linolenic acid contents in 60RDI and 30RDI were higher than in FI at 13 and 19 WAF, while the olive mesocarp was green. Later on, no significant differences were detected between the three treatments.

To gain further insight into the effect of the different irrigation treatments on the olive mesocarp lipid biosynthesis, we analysed the amount and fatty acid composition of the different lipid classes during the mesocarp development and ripening of fruits from all treatments (Table S1). We did not detect any significant effect of the irrigation treatment on the amount of any lipid class. With respect to the fatty acid composition, however, we observed that the linoleic acid content was lower in TAG in 30RDI mesocarp compared to FI at 22, 29 and 33 WAF. The same effect was observed earlier in the same season for phosphatidylcholine (PC), phosphatidylethanolamine and diacylglycerol, actually, when the olive fruit was still completely green (19 WAF). On the other hand, the increase in the linolenic acid content in the 30RDI mesocarp was only detected in TAG, at 13 WAF.

Subsequently, we looked for possible correlations between the differences found in the fatty acid composition of the olive fruit subjected to different irrigation treatments and changes in the expression levels of the olive fatty acid desaturase genes, in order to identify the genes responsible for the observed alterations. To do that, we analyzed the expression levels of olive

desaturase genes in mesocarp tissue during the olive fruit development and ripening, in fruits of the three treatments. First, we analyzed the expression levels of the olive *SAD* genes, responsible for the desaturation of stearyl-ACP in the plastid to produce oleoyl-ACP. qRT-PCR expression analyses of *OeSAD1*, *OeSAD2* and *OeSAD3* genes in mesocarp tissue from the three irrigation treatments are shown in Fig. 3. No significant differences in *SAD* genes expression levels were found between RDI and FI fruits, with the exception of *SAD1* transcript levels in the 30RDI fruits at 29 WAF, which were lower than those in 60RDI and FI fruits. Subsequently, we analyzed the expression levels of olive oleate desaturase genes (Fig. 4), responsible for the linoleic acid synthesis. Although higher levels of *OeFAD2-1* were observed in 60RDI at the beginning of fruit development, the expression levels of this gene were similar in the three irrigations treatments from 22 WAF onwards. In contrast, transcript levels of the other microsomal oleate desaturase gene (*OeFAD2-2*) and the plastidial one (*OeFAD6*) were similar in the three irrigation treatments throughout fruit development and ripening. Finally, we studied the expression levels of linoleate desaturase genes (Fig. 5), involved in linoleic acid desaturation to produce linolenic acid. The expression levels of the two microsomal linoleate desaturase genes (*OeFAD3A* and *OeFAD3B*) were similar in the three irrigation treatments during olive fruit development and ripening, apart from a higher transcript level of both genes at 13 WAF only in the 60RDI treatment. Finally, the expression analysis of plastidial linoleate desaturases (*OeFAD7-1* and *OeFAD7-2*) revealed higher transcript levels of both genes at the beginning of fruit development in 30RDI as compared to FI. This effect was also observed in 60RDI, although to a lesser extent.

4. Discussion

Recent evidence suggests that deficit irrigation could help to achieve sustainable balance between water saving, tree vigour and oil production in SHD olive orchards (Fernández, 2014). However, its effect on the olive oil technological, nutritional and organoleptic properties remains controversial. In this work, we have analysed the effect of 60RDI and 30RDI compared to FI on lipid content, fatty acid composition and desaturase genes expression, in order to determine how different irrigation treatments affected lipid biosynthesis during fruit development and ripening. In our study, the oil yield per milligram of mesocarp tissue in the RDI treatments was lower in comparison to FI during the olive fruit development, although no significant differences were detected in oil content between the different water treatments after fruit maturation. These results are in agreement with numerous studies, which reported that oil content on per fruit basis is not usually found to be affected by water application (d'Andria et al. 2004, Grattan et al. 2006, Lavee et al. 2007, Moriana et al. 2003). García et al. (2013) reported that olive trees with deficit irrigation treatments had lower fruit production than control trees. In this sense, Ben-Gal et al. (2001) reported that olive fruit yield increased with increasing irrigation. However, the major differences found in oil yield were according to the cultivar and the biennial bearing cycle. On the other hand, Rosecrance et al. (2015) suggested that moderate water stress significantly increased olive oil yield in a SHD 'Arbequina' olive orchard, in agreement with results obtained by García et al. (2013), Girona et al. (2002) and Iniesta et al. (2009). They found that the lower fruit production in the deficiently irrigated trees was compensated for by a more efficient physical extraction, because of the lower proportion of water in the fruit.

Interestingly, no significant change was detected in the content of any of the different lipid classes during the mesocarp development and ripening exposed to 60RDI, 30RDI and FI (Table S1). Distinct results were obtained when plant leaves were used (Liljenberg, 1992) including olive (Guerfel et al., 2008), where a decrease in galactolipids

(monogalactosyldiacylglycerol and digalactosyldiacylglycerol) and phospholipids (mainly PC) has been described in response to water deficit stress. The fact that in 60RDI and 30RDI there is not a complete water deficit could explain these contrasting results.

Regarding our results on the TAG fatty acid composition, no significant differences were detected in the total unsaturated fatty acid content between the RDI treatments and FI. This agrees with findings by Dabbou et al. (2015), who reported that the unsaturated/saturated ratio was not considerably affected by irrigation in cultivar Coratina. This result is also in accordance with the expression levels of olive *SAD* genes detected in this work, which did not show significant differences between RDI conditions and FI, with the exception of *SAD1* transcript levels in the 30RDI treatment at 29 WAF. However, *OeSAD2* and not *OeSAD1* was the gene known to be the main responsible for the content of oleic acid and its polyunsaturated derivatives in the olive mesocarp and, therefore, in the olive oil (Parvini et al., 2016). In addition, our results suggest that *OeSAD1* and *OeSAD3* genes are not related to olive tree water stress, since their expression levels and the unsaturated fatty acid content in lipid classes were similar under the different irrigation regimes.

Although the total unsaturated fatty acid content was not affected by water supply, we observed differences among treatments in the linoleic acid content. The 30RDI mesocarp showed a lower linoleic acid content than the 60RDI and FI treatments after fruit maturation, indicating that only the most severe water stress strategy had an effect on the linoleic acid content. This agrees with the already reported high oleic/linoleic ratio found in the oil obtained from the same 30RDI trees (García et al., 2017). Changes in linoleic acid content are usually the most consistently effect of irrigation on the fatty acid composition. Berenguer et al. (2006) reported a reduction in the linoleic acid content for severely stressed trees (15-25% water of control) in a SHD ‘Arbequina’ orchard. A similar behaviour was observed by Gómez-del-

314 Campo et al. (2013), who reported a reduction in the linoleic acid content when irrigation of
315 ‘Arbequina’ trees was 30% of control. Also, Caruso et al. (2014) detected lower levels of
316 linoleic acid in oils from deficit irrigated ‘Frantoio’ olive trees. However, in these last two cases
317 differences between water treatments resulted dependent on the climatic conditions. On the
318 other hand, a clear trend of reduction in oleic/linoleic ratio with increasing water stress was
319 observed in oils from ‘Koroneiki’ trees (Dag et al., 2015; Stefanoudaki et al., 2009). Therefore,
320 the effect of irrigation on fatty acid composition seems to depend on the cultivar and on
321 meteorological conditions. In this sense, Hernández et al. (2009) reported that water stress was
322 cultivar dependent, since irrigated olive trees of the cv. Arbequina showed a slight increase in
323 linoleic acid content in comparison to rain-fed trees. This effect was not observed in ‘Picual’
324 trees. Moreover, the increase in linoleic acid content observed in that study in cv. Arbequina
325 with irrigation correlated well with the higher expression level detected for *OeFAD2-2*, the
326 main gene responsible for the linoleic acid synthesis in the mesocarp (Hernández et al. 2009).
327 These data indicated that, in this cultivar, irrigation produced an increase in the *OeFAD2-2*
328 transcript level and, thus, in the linoleic acid content. However, in the present study we did not
329 find a correlation between the linoleic acid content in the different irrigation treatments and the
330 oleate desaturase genes expression levels, possibly because the water stress was not as severe
331 as that found under rain-fed conditions. This could explain that the expression levels of the
332 *OeFAD2-2* gene were not affected (Fig. 4). On the other hand, although *OeFAD2-1* and
333 *OeFAD6* genes have been shown to be transcriptionally regulated by different environmental
334 factors (Hernández et al., 2011), our results show that their expression levels do not appear to be
335 affected by differences on irrigation during fruit maturation. This is in agreement with results
336 previously reported by Hernández et al. (2009). Therefore, a possible explanation for the
337 decrease in linoleic acid content under RDI conditions could be the alteration of the transcript
338 levels of an un-identified oleate desaturase gene. Furthermore, the reduction in linoleic acid

content was observed first in PC (19 WAF), where linoleic acid is synthesized in the ER, and phosphatidic acid and diacylglycerol, the precursors of TAG in the Kennedy pathway, and later on in TAG (22 WAF) (Table S1). These data support the hypothesis that an un-identified *FAD2* gene could be repressed by water stress, decreasing in PC the content of linoleic acid, which is then transferred to TAG. Nevertheless, the existence of post-transcriptional regulatory mechanisms of known *FAD2* genes cannot be discounted.

Differences in water conditions seem to have little effect on the linolenic acid content. Most authors reported either no significant differences in linolenic acid levels detected using different irrigation treatments (Caruso et al., 2014; Dabbou et al., 2011; Servili et al., 2007; Tognetti et al., 2007), or slight, inconsistent changes. While Berenguer et al. (2006) and Toplu et al. (2009) described an increase in the linolenic acid content in oils made from trees receiving increased amount of irrigation, the opposite effect was described by Dabbou et al. (2010) and Stefanoudaki et al. (2009). Our data reveal a gradual increase in linolenic acid content with decreasing irrigation, only at early stages of fruit development. After fruit ripening, when olive fruits are harvested to produce olive oil, we did not observe any difference in linolenic acid content between the different irrigation treatments. These results correlate well with the increased expression levels detected for *OeFAD7-1* and *OeFAD7-2* genes in 60RDI and 30RDI mesocarps, at the beginning of fruit development. Hernández et al. (2016) suggested that *FAD7-1* and *FAD7-2* are mainly responsible for the synthesis of the linolenic acid present in the virgin olive oil, since both plastidial $\omega 3$ desaturase isoforms catalyse the desaturation of linoleoyl-galactolipids to produce linolenoyl-galactolipids and then, linolenic acid is exported to the ER to be incorporated into TAG. Therefore, the higher transcript levels of *OeFAD7-1* and *OeFAD7-2* genes observed in 60RDI and 30RDI conditions caused an increase in linolenic acid in galactolipids, and this linolenic acid could be then transferred to TAG. However, this effect was observed at early stages of fruit development only. Since olive fruits are harvested

after fruit ripening, about 29 WAF, when *OeFAD7-1* and *OeFAD7-2* genes were not affected by irrigation, no significant differences were observed in the linolenic acid in oils obtained from different irrigation treatments, as previously reported (García et al., 2017).

5. Conclusions

Our results suggest that a deficit irrigation of 30% of the IN induced an increase in the oleic/linoleic ratio in olive mesocarp after fruit maturation and, therefore, in the olive oil. From this point of view, the 30RDI strategy resulted suitable for sustainable water saving and oil quality improvement. Although one year may be a limited period of time for drawing definitive management conclusions, it is noteworthy that this is the first detailed study that analyses responses of fatty acid composition to water stress at the gene level from field samples. The lack of correlation with the expression of known oleate desaturase genes (*FAD2-1*, *FAD2-2* and *FAD6*) and the decrease in linoleic acid in PC, where linoleic acid is synthesised in the ER, points out to an un-identified *OeFAD2* gene that could be repressed by water stress, although the existence of post-transcriptional regulatory mechanisms of known *FAD2* genes cannot be discarded. The increase in the linolenic acid content observed at the beginning of fruit development under 30RDI conditions could be explained by the observed induction of the *OeFAD7-1* and *OeFAD7-2* genes, which are the main responsible for the linolenic acid content in the olive oil. However, this effect was not noticeable in the olive oil since it was not observed during fruit ripening, when the olive fruits are harvested to produce the olive oil. This study constitutes a significant step towards the understanding of the molecular mechanisms involved in the response to water stress and related to fatty acid desaturation in oil fruits. For most farmers, however, the production target is not to obtain the olive oil of greatest quality, but the

most productive balance, in terms of net income, between olive yield and olive quality. Consequently, to decide on the irrigation management of a particular olive orchard will imply to combine our findings with those on the effect of the irrigation strategy on oil yield.

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Supplementary material

Table S1. Effect of RDI treatments on the content and fatty acid composition of lipid classes in mesocarp tissue from cultivar Arbequina during olive fruit development and ripening.

REFERENCES

- Banilas, G., Moressis, A., Nikoloudakis, N., Hatzopoulos, P., 2005. Spatial and temporal expressions of two distinct oleate desaturases from olive (*Olea europaea* L.). *Plant Sci.* 168, 547-555.
- Banilas, G., Nikiforiadis, A., Makariti, I., Moressis, A., Hatzopoulos, P., 2007. Discrete roles of a microsomal linoleate desaturase gene in olive identified by spatiotemporal transcriptional analysis. *Tree Physiol.* 27, 481-490.
- Beede, R.H., Goldhamer, D.A., 1994. Olive irrigation management, in: Ferguson, L., Sibbett, G.S., Martin, G.C. (Eds.), *Olive Production Manual*. University of California, Division of Agriculture and natural Resources, Oakland, CA, Publication 3353, pp. 61-68.
- Beltrán, G., del Río, C., Sánchez, S., Martínez, L., 2004. Influence of harvest date and crop yield on the fatty acid composition of virgin olive oils from cv. Picual. *J. Agric. Food Chem.* 52, 3434-3440.
- Ben-Gal, A., Yermiyahu, U., Zipori, I., Presnow, E., Hanoch, E., Dag, A., 2001. The influence of bearing cycles on olive oil production response to irrigation. *Irrig. Sci.* 29, 253-263.
- Berenguer, M.J., Vossen, P.M., Grattan, S.R., Connell, J.H., Polito, V.S., 2006. Tree irrigation levels for optimum chemical and sensory properties of olive oil. *Hort. Sci.* 41, 427-432.

436 Caruso, G., Gucci, R., Urbani, S., Esposto, S., Taticchi, A., Di-Maio, I., Selvaggini, R., Servili,
 437 M., 2014. Effect of different irrigation volumes during fruit development on quality of
 438 virgin olive oil of cv. Frantoio. *Agric. Water Manage.* 134, 94-103.

439 Chalmers, D.J., Mitchel, P.D., Van-Heek, L., 1981. Control of peach tree growth and
 440 productivity by regulated water supply, tree density and summer pruning. *J. Am. Soc.*
 441 *Hortic. Sci.* 106, 307-312.

442 Connor, D.J., 2006. Towards optimal designs for hedgerow olive orchards. *Aust. J. Agric. Res.*
 443 57, 1067-1072.

444 Dabbou, S., Chehab, H., Faten, B., Dabbou, S., Esposto, S., Selvaggini, R., Taticchi, A., Servili,
 445 M., Montedoro, G.F., Hammami, M., 2010. Effect of three irrigation regimes on
 446 Arbequina olive oil produced under Tunisian growing conditions. *Agric. Water*
 447 *Manage.* 97, 763-768.

448 Dabbou, S., Dabbou, S., Chehab, H., Brahmi, F., Taticchi, A., Servili, M., Hammami, M., 2011.
 449 Chemical composition of virgin olive oils from Koroneiki cultivar grown in Tunisia
 450 with regard to fruit ripening and irrigation regimes. *Int. J. Food Sci. Technol.* 46, 577-
 451 585.

452 Dabbou, S., Dabbou, S., Taticchi, A., Servili, M., Hammami, M., 2015. Content of fatty acids
 453 and phenolics in Coratina olive oil from Tunisia influence of irrigation and ripening.
 454 *Chem. Biodiversity* 12, 397-406.

455 Dag, A., Naor, A., Ben-Gal, A., Harlev, G., Zipori, I., Schneider, D., Birger, R., Peres, M., Gal,
 456 Y., Kerem, Z., 2015. The effect of water stress on super-high-density Koroneiki olive
 457 oil quality. *J. Sci. Food Agric.* 95, 2016-2020.

458 d'Andria, R., Lavini, A., Morelli, G., Patumi, M., Terenziani, S., Calandrelli, D., Fragnito, F.,
 459 2004. Effects of water regimes on five pickling and double aptitude olive cultivars (*Olea*
 460 *europaea* L.). *J. Hortic. Sci. Biotechnol.* 79, 18-25.

461 Fernández, J.E., 2014. Understanding olive adaptation to abiotic stresses as a tool to increase
 462 crop performance. *Env. Exp. Bot.* 103, 158-179.

463 Fernández, J.E., Pérez-Martín, A., Torres-Ruiz, J.M., Cuevas, M.V., Rodríguez-Domínguez,
 464 C.M., Elsayed-Farag, S., Morales-Sillero, A., García, J.M., Hernández-Santana, V.,
 465 Diaz-Espejo, A., 2013. A regulated deficit irrigation strategy for hedgerow olive
 466 orchards with high plant density. *Plant Soil.* 372, 279-295.

467 Garcés, R., Mancha, M., 1993. One-step lipid extraction and fatty acid methyl esters preparation
 468 from fresh plant tissues. *Anal. Biochem.* 211, 139-143.

469 García, J.M., Cuevas, M.V., Fernández, J.E., 2013. Production and oil quality in Arbequina
 470 olive (*Olea europaea*, L.) trees under two deficit irrigation strategies. *Irrig. Sci.* 31, 359-
 471 370.

472 García, J.M., Morales-Sillero, A., Pérez-Rubio, A.G., Diaz-Espejo, A., Montero, A., Fernández,
 473 J.E., 2017. Virgin olive oil quality of hedgerow Arbequina olive trees under deficit
 474 irrigation. *J. Sci. Food Agric.* 97, 1018-1026.

475 Girona, J., Luna, M., Arbonés, A., Mata, M., Rufat, J., Marsal, J., 2002. Young olive trees
 476 responses (*Olea europaea* cv. Arbequina) to different water supplies. Water function
 477 determination. *Acta Hort.* 586, 277-280.

478 Gómez-del-Campo, M., García, J.M., 2013. Summer deficit-irrigation strategies in a hedgerow
 479 olive cv. Arbequina orchard: effect on oil quality. *J. Agric. Food Chem.* 61, 8899-8905.

480 Grattan, S.R., Berenguer, M.J., Connell, J.H., Polito, V.S., Vossen, P.M., 2006. Olive oil
 481 production as influenced by different quantities of applied water. *Agric. Water Manag.*
 482 85, 133-140.

483 Guerfel, M., Baccouri, O., Boujnah, D., Zarrouk, M., 2008. Changes in lipid composition, water
 484 relations and gas exchange in leaves of two young 'Chamlali' and 'Chetoui' olive trees
 485 in response to water stress. *Plant Soil.* 311, 121-129.

486 Hara, A., Radin, N.S., 1978. Lipid extraction of tissues with a low-toxicity-solvent. Anal.
 487 Biochem. 90, 420-426.

488 Haralampidis, K., Milioni, D., Sanchez, J., Baltrusch, M., Heinz, E., Hatzopoulos, P., 1998.
 489 Temporal and transient expression of stearyl-ACP carrier protein desaturase gene
 490 during olive fruit development. J. Exp. Bot. 49, 1661-1669.

491 Harwood, J.L., 2005. Fatty acid biosynthesis, in: Murphy D.J. (Eds.), Plant Lipids. Blackwell
 492 Publishing, Oxford, UK, pp. 27-101.

493 Hernández, M.L., Guschina, I.A., Martínez-Rivas, J.M., Mancha, M., Harwood, J.L., 2008. The
 494 utilization and desaturation of oleate and linoleate during glycerolipid biosynthesis in
 495 olive (*Olea europaea* L.) callus cultures. J. Exp. Bot. 59, 2425-2435.

496 Hernández, M.L., Mancha, M., Martínez-Rivas, J.M., 2005. Molecular cloning and
 497 characterization of genes encoding two microsomal oleate desaturases (FAD2) from
 498 olive. Phytochemistry. 66, 1417-1426.

499 Hernández, M.L., Padilla, M.N., Mancha, M., Martínez-Rivas, J.M., 2009. Expression analysis
 500 identifies FAD2-2 as the olive oleate desaturase gene mainly responsible for the
 501 linoleic acid content in virgin olive oil. J. Agr. Food Chem. 57, 6199-6206.

502 Hernández, M.L., Padilla, M.N., Sicardo, M.D., Mancha, M., Martínez-Rivas, J.M., 2011.
 503 Effect of different environmental stresses on the expression of oleate desaturase genes
 504 and fatty acid composition in olive fruit. Phytochemistry. 72, 178-187.

505 Hernández, M.L., Sicardo, M.D., Martínez-Rivas, J.M., 2016. Differential contribution of
 506 endoplasmic reticulum and chloroplast ω -3 fatty acid desaturase genes to the linolenic
 507 acid content of olive (*Olea europaea*) fruit. Plant. Cell Physiol. 57, 138-151.

508 Iniesta, F., Testi, L., Orgaz, F., Villalobos, F.J., 2009. The effects of regulated and continuous
 509 deficit irrigation on the water use, growth and yield of olive trees. Eur. J. Agron. 30,
 510 258-265.

511 Issaoui, M., Flamini, G., Chehab, H., Cioni, P.L., Braham, M., Hammami, M., 2013. Irrigation
 512 regimes and growing area effects on Chétoui olive oil quality. *J. Food Biochem.* 37,
 513 185-1992.

514 Lavee, S., Hanoch, E., Wodner, M., Abramowitch, H., 2007. The effect of predetermined water
 515 deficit on the performance of cv. Muhasan olive (*Olea europaea* L.) in the eastern coastal
 516 plain of Israel. *Scientia Hort.* 112, 156-163.

517 Liljenberg, C.S., 1992. The effect of water deficit stress on plant membrane lipids. *Prog. Lipid*
 518 *Res.* 31, 335-343.

519 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
 520 quantitative PCR and the 2- $\Delta\Delta C_t$ method. *Methods.* 25, 402-408.

521 Moriana, A., Orgaz, F., Pastor, M., Fereres, E., 2003. Yield responses of a mature olive orchard
 522 to water deficit. *J. Am. Soc. Hortic. Sci.* 128, 425-431.

523 Orgaz, F. and Fereres, E., 2001. Riego, in: Barranco, D., Fernández-Escobar, R., Rallo, L.,
 524 (Eds), *El Cultivo del Olivo*, 4th ed. Coedition Mundi-Prensa and Junta de Andalucía,
 525 Madrid, 2001, pp. 285-306.

526 Parvini, F., Sicardo, M.D., Hosseini-Mazinani, H., Martínez-Rivas, J.M., Hernández, M.L.,
 527 2016. Transcriptional analysis of stearoyl-acyl carrier protein desaturase genes from
 528 olive (*Olea europaea*) in relation to the oleic acid content of the virgin olive oil. *J. Agric.*
 529 *Food Chem.* 64, 7770-7781.

530 Poghosyan, Z.P., Haralampidis, K., Martsinkowsakaya, A.I., Murphy, D.J., Hatzopoulos, P.,
 531 1999. Developmental regulation and spatial expression of a plastidial fatty acid
 532 desaturase from *Olea europaea*. *Plant Physiol. Biochem.* 37, 109-119.

533 Román, A., Andreu, V., Hernández, M.L., Lagunas, B., Picorel, R., Martínez-Rivas, J.M.,
 534 Alfonso, M., 2012. Contribution of the different omega-3 fatty acid desaturase genes to
 535 the cold response in soybean. *J. Exp. Bot.* 63, 4973-4982.

536 Rosecrance, R.C., Krueger, W.H., Milliron, L., Bloese, J., Garcia, C., Mori, B., 2015. Moderate
 537 regulated deficit irrigation can increase olive oil yields and decrease tree growth in super
 538 high density ‘Arbequina’ olive orchards. *Sci. Hortic.* 190, 75-82.
 539 Sánchez, J., 1994. Lipid photosynthesis in olive fruit. *Prog. Lipid Res.* 33, 97-104.
 540 Servili, M., Esposto, S., Lodonili, E., Selvaggini, R., Taticchi, A., Urbani, S., Montedoro, G.,
 541 Serravalle, M., Gucci, R., 2007. Irrigation effects on quality, phenolic composition, and
 542 selected volatiles of virgin olive oils cv. Leccino. *J. Agric. Food Chem.* 55, 6609-6618.
 543 Shanklin, J., Cahoon, E.B., 1998. Desaturation and related modifications of fatty acids. *Annu.*
 544 *Rev. Plant Physiol.* 49, 611-641.
 545 Stefanoudaki, E., Williams, M., Chartzoulakis, K., Harwood, J.L., 2009. Effect of irrigation on
 546 quality attributes of olive oil. *J. Agric. Food Chem.* 57, 7048-7055.
 547 Tognetti, R., d’Andria, R., Sacchi, R., Lavini, A., Morelli, G., Alvino, A., 2007. Deficit
 548 irrigation affects seasonal changes in leaf physiology and oil quality of *Olea europaea*
 549 (cultivars Frantoio and Leccino). *Ann. Appl. Biol.* 150, 169-186.
 550 Toplu, C., Önder, D., Önder, S., Yildiz, E., 2009. Determination of fruit and oil characteristics
 551 of olive (*Olea europaea* L. cv. Gemlik) in different irrigation and fertilization regimes.
 552 *Afr. J. Agric. Res.* 4, 649-658.
 553 Tovar, M.J., Romero, M.P., Alegre, S., Girona, J., Motilva, M.J., 2002. Composition and
 554 organoleptic characteristics of oils from Arbequina olive (*Olea europaea* L.) trees under
 555 deficit irrigation. *J. Sci. Food Agric.* 82, 1755-1763.
 556 Troncoso A., Cantos M., Liñán J., Fernández J.E., 2001. Fertirrigación, in: Barranco, D.,
 557 Fernández-Escobar, R., Rallo, L. (Eds), *El Cultivo del Olivo*, 4th ed. Coedition Mundi-
 558 Prensa and Junta de Andalucía, Madrid, pp. 307–332.
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 560

FIGURE CAPTIONS

Figure 1. Effect of RDI treatments on the total lipid content in mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. At the indicated times, total lipid content was analysed as described in Materials and Methods. Data are mean $\pm$ SD from three independent experimental determinations.

Figure 2. Effect of RDI treatments on the unsaturated fatty acids percentage a), and the oleic, linoleic and linolenic acids percentage b), in the mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. At the indicated times, fatty acids were analyzed by gas chromatography as described in Materials and Methods. Data are mean $\pm$ SD from three independent experimental determinations. * indicates that 60RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test. ** indicates that 30RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test.

Figure 3. Effect of RDI treatments on the relative expression levels of olive *SAD* genes (*OeSAD1*, *OeSAD2* and *OeSAD3*) in the mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. At the indicated times, relative expression levels were determined by qRT-PCR as described in Materials and Methods. Data are mean $\pm$ SD from three independent experimental determinations. * indicates that 60RDI is significant different ($p \leq$

0.05) to FI according to two-way ANOVA with a Bonferroni post test. ** indicates that 30RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test.

Figure 4. Effect of RDI treatments on the relative expression levels of olive ω -6 desaturase genes (*OeFAD2-1*, *OeFAD2-2* and *OeFAD6*) in the mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. At the indicated times, relative expression levels were determined by qRT-PCR as described in Materials and Methods. Data are mean $\pm$ SD from three independent experimental determinations. * indicates that 60RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test. ** indicates that 30RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test.

Figure 5. Effect of RDI treatments on the relative expression levels of olive ω -3 desaturase genes (*OeFAD3A*, *OeFAD3B*, *OeFAD7-1* and *OeFAD7-2*) in the mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. At the indicated times, relative expression levels were determined by qRT-PCR as described in Materials and Methods. Data are mean $\pm$ SD from three independent experimental determinations. * indicates that 60RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test. ** indicates that 30RDI is significant different ($p \leq 0.05$) to FI according to two-way ANOVA with a Bonferroni post test.