Theor. Exp. Plant Physiol. (2021) 33:369–383
https://doi.org/10.1007/s40626-021-00219-9

Physiological and biochemical responses to water deficit and recovery of two olive cultivars (*Olea europaea* L., Arbequina and Empeltre cvs.) under Mediterranean conditions

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Received: 9 February 2021 / Accepted: 13 August 2021 / Published online: 30 August 2021

Abstract The main objective of the present study was to evaluate the responses to water deficit stress and recovery capacity of young potted trees from two olive cultivars, Empeltre cv. and the widely planted Arbequina cv. The experiment was carried out under semiarid environmental conditions at the experimental field of the University of Balearic Islands in Mallorca, Spain. Two-year-old plants in 22 L pots were exposed to three water availability regimes (full capacity [FC]; 50% FC; 30% FC). Growth, gas exchange, intrinsic water use efficiency, C^{13} discrimination and biochemical parameters (total soluble sugars, proline, starch, total soluble protein, pigments and phenolic fraction) were evaluated. Plants were rewetted and assessed again after the re-watering period. Drought stress reduced photosynthesis, mainly by regulating stomatal conductance (g_s). Arbequina cv. exhibited a more conservative water use strategy than Empeltre, with greater reductions in g_s, accompanied by significant reductions in vegetative growth. Non-significant differences in intrinsic water use efficiency were observed between cultivars and treatments. However, C^{13} discrimination analysis showed better water use efficiency in Empeltre than Arbequina in all treatments. Water deficit stress caused an increase of proline and total soluble solids and a reduction of starch, total soluble protein and chlorophylls concentrations of both cultivars. Empeltre showed a higher total phenol concentration than Arbequina during non-water deficit stress conditions. Cultivar-specific differences in the mechanisms to deal with drought were observed. Empeltre cv. exhibits a higher capacity to tolerate drought and it continues growing under water stress and recovery.

Keywords Drought · Water deficit stress · Water use efficiency · Recovery

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40626-021-00219-9.

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1 Introduction

Agriculture is strongly affected by climate change, which leads to low-resource environments. In Mediterranean regions, climate change is leading to a reduction in the annual number of precipitation days, as well as annual precipitation, thus implementation of water-saving strategies is essential (IPCC 2014).

The olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean basin. The good performance of olive trees grown under drought and high temperature conditions implies morpho-anatomical, physiological and biochemical characteristics and mechanisms that enable the plant to regulate water consumption and overcome water deficit stress. Knowledge of these adaptive processes and regulatory mechanisms is crucial to understand how these responses affect water use efficiency.

Stomatal regulation is one of the key plant responses to water deficit (Chaves et al. 2009). Tight stomatal control and progressive stomata closure have been described in olive trees in response to water deficit stress (Tognetti et al. 2009; Boughalleb and Hajlaoui 2011). Additionally, morphological changes (i.e., in leaf anatomy or area reduction) have also been identified in response to water deficit stress in olive trees (Bacelar et al. 2004; Sofo et al. 2007). At the metabolic level, osmotic adjustment is an important mechanism that enables plants to cope with drought (Chaves et al. 2003) and has been identified as a crucial process in olive trees during dry periods (Boussadia et al. 2013). Finally, olive trees also regulate their antioxidant system as a strategy to cope with the oxidative damage induced by drought (Sofo et al. 2005; Bacelar et al. 2007a). These responses of olive trees to the adverse conditions of climate change and related adaptive defence strategies have recently been reviewed by Brito et al. (2019).

The olive tree is an ancient crop with wide genetic variability, thus selecting cultivars with a better response to drought could be one strategy to mitigate the effects of climate change. Investigation of cultivar-dependent responses to drought has revealed genotypic variation in morphoanatomical characteristics, photosynthetic capacity and regulation, osmotic adjustment capacity, hydraulic properties, water use efficiency, phenolic composition and antioxidant activity among cultivars (Bacelar et al. 2004, 2007b, 2009; Ben Ahmed et al. 2009; Petridis et al. 2012; Trentacoste et al. 2018).

Moreover, under climate change scenarios, especially in Mediterranean areas, plants will be continuously exposed to drought and/or rewetting during their life cycle (Fischlin et al. 2007). Therefore, the ability to recover after a dry period will increasingly play a fundamental role in the growth and survival of plants. However, the combined responses of plants to drought and re-watering—and the mechanisms involved—are poorly studied. Photosynthesis recovery depends on the rate and degree of inhibition of photosynthesis during water shortage and also on the plant species (Flexas et al. 2009), and ranges from rapid and complete after moderate stress to depression or incomplete after severe stress (Chaves et al. 2009). Some reports have suggested olive plants recover good water status after re-watering, based on indicators of leaf water potential and stomatal conductance. However, the velocity and degree of recovery varied under different stress conditions (Torres-Ruiz et al. 2013, 2015; Trentacoste et al. 2018), suggesting recovery depends on plant genotype-specific characteristics, as well as the intensity and duration of stress. Thus, investigation of how olive cultivars cope with adverse climate change variables, such as alternating drought and rewetting events, is a crucial issue.

The aim of this work was to clarify the responses to water deficit stress and recovery capacity of young potted trees from two olive cultivars, the cultivar Empeltre (traditionally growing in Mallorca) and the widely planted cultivar Arbequina. We hypothesize that Empeltre has a better performance and greater stress tolerance than Arbequina under drought.

2 Material and methods

2.1 Plant material and site description

The experiment was carried out at the University of Balearic Islands (Palma, Spain) during the spring and summer of 2018. Two-year-old own-rooted olive trees (*Olea europaea* L.), of the 'Arbequina' and 'Empeltre' cultivars were transplanted into 22 L plastic pots filled with inert coco fibre and substrate (2/8) and grown outside (Table 1).

The two cultivars were submitted to three water regimes during the experiment. At the end of May (1st

Table 1 Monthly mean minimum (Tmin) and maximum (Tmax) temperatures, relative humidity (RH), photosynthetic solar radiation (PAR) and accumulated rainfall and evapotranspiration (ETo) throughout the experiment

Month	T_{min} (°C)	T_{max} (°C)	RH (%)	PAR (μmol m^{-2} s^{-1})	Rainfall (mm)	ETo (mm)
April	5.36	26.33	69.38	1551.13	62.3	91.22
May	7.23	27.95	74.45	1681.36	23.9	106.79
June	12.87	32.89	66.83	1626.53	12.5	137.92
July	18.30	34.69	60.10	1652.16	0.0	147.30

day of the experiment, see. Figure 1), the plants were separated into three different groups. Five plants of each cultivar used as controls were watered every day to maintain the soil water content close to field capacity (FC). Five plants of each variety were subjected to water withholding until 50% of field capacity (50% FC), considered moderate drought, and five other plants were maintained without irrigation until 30% of field capacity (30% FC), considered severe drought. After approximately two weeks, all plants reached the desired treatment conditions (50% FC and 30% FC). From this point onwards, the pots were weighed daily and the amount of water consumed was replenished to maintain the same level of drought during 7 d. The plants were then rewatered to FC to evaluate the recovery capacity of each cultivar. The new treatments after rewatering were named 100% FC, 50% FC rewatered (50R) and 30% FC rewatered (30R) (see Fig. 1 for more detail).

2.2 Substrate water content and plant water status

Substrate water content was measured volumetrically. Five substrate samples from each treatment and cultivar were collected using a plastic cylinder of known volume (v) and weighed (FW). The samples were then dried in an oven at 66 °C for 72 h and dry mass (DM) was recorded. The substrate water content was calculated as follows: SWC (% vol) = ((FM − DM)/v)·100.

Stem water potential ($_{stem}$) was measured at midday using a Scholander Pressure Chamber (pms-1000; Corvallis, OR, USA). Three expanded leaves were covered with opaque plastic envelopes at least 1 h before the measurement.

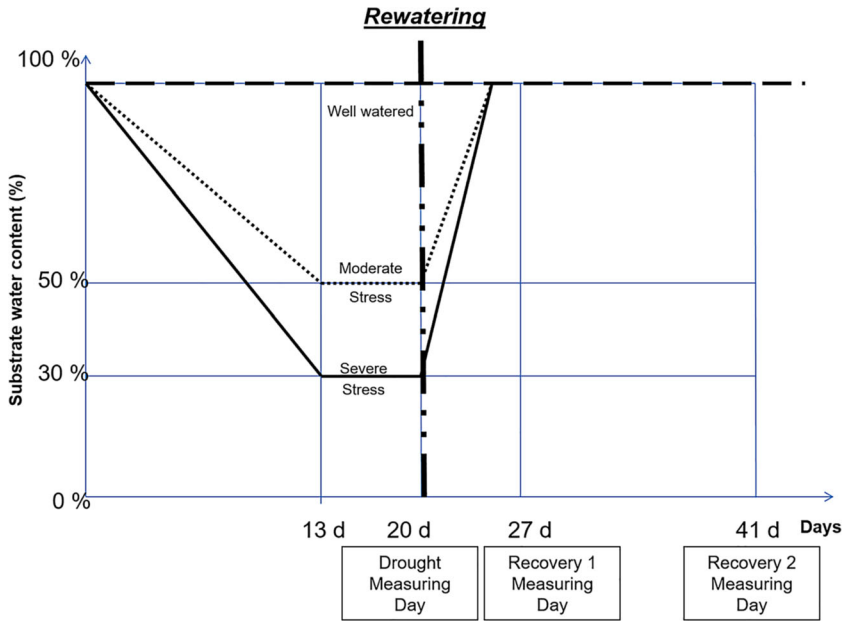

Fig. 1 Experiment diagram

Leaf relative water content (RWC) was calculated based on the fresh (FM), turgid (TM) and dry mass (DM) for each treatment and cultivar using the equation:

$$\mathrm{RWC}(\%) = (\mathrm{FM} - \mathrm{DM})/(\mathrm{TM} - \mathrm{DM}) \cdot 100$$

2.3 Growth parameters and leaf morphological traits

Leaf appearance rate (LAR) was calculated by tagging two branches on each tree and counting the number of new leaves that appeared during the drought period and after a further 7 and 21 d of rewatering.

The total number of leaves per plant was estimated by tagging two branches on each plant and counting the number of leaves and then multiplying the average of leaves number by the total number of branches per plant. Total leaf area per plant (LA; m^2 $plant^{-1}$)) was estimated by determining the average single leaf area, collected randomly and multiplying the average leaf area by the total number of leaves on each plant.

Leaf mass area (LMA) was calculated by dividing the dry mass of a leaf by its leaf area (g m^{-2}) according to Groom and Lamont (1999).

2.4 Gas exchange and water use efficiency

Net photosynthesis (A_N), stomatal conductance (g_s) and CO_2 concentration at the sub-stomatal cavity (C_i) were measured around midmorning (10–12:00 h) on a fully expanded leaf (one leaf of each plant, i.e., five leaves per treatment) using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA). All measurements were performed under saturated light (1200 µmol photon m^{-2} s^{-1}) and an air CO_2 concentration of 400 mol CO_2 mol^{-1}. Intrinsic water use efficiency (WUEi) was calculated as the ratio between A_N and g_s.

The carbon isotope composition of leaf dry matter was measured as an estimate of long-term water-use efficiency (WUE) in leaf. Five replicates were dried for 48 h in an oven at 60 °C, ground into a powder and the isotope ratios ($\delta^{13}C$) of 2 mg samples were determined. Samples were combusted in an elemental analyser (Carlo-Erba, Rodano, Italy) and the CO_2 was separated by chromatography and directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus, Bremen, Germany). Peach leaf standards were run every eight samples. $\delta^{13}C$ was calculated as (Farquhar and Richards 1984):

$$\delta^{13}\mathrm{C\ sample}(‰) = ((\mathrm{R\ sample}/\mathrm{R\ standard}) - 1) \cdot 1000.$$

2.5 Biochemical parameters

For the biochemical determinations, 20–40 leaves were sampled at mid-morning, immediately frozen in liquid nitrogen and stored at −80 °C until chemical analysis.

2.6 Total soluble sugars, proline and starch

To determine total soluble sugars (TSS), 0.2 g frozen leaf samples were mixed with 5.0 mL of 80% methanol in covered glass tubes and boiled in a water bath at 70 °C for 30 min. Sugar concentration was determined in phenol–sulphuric acid medium; 1 mL samples were extracted using a mix of 1 mL of 5% phenol and 5 mL sulphuric acid 98%, vortexed, cooled and the absorption values were measured at 640 nm using a spectrophotometer (DU730, Beckman Coulter, Inc.). Sugar concentrations were calculated using l-glucose as a standard.

Proline concentration was determined following the method of Troll and Lindsley (1955) with some modifications. Briefly, 0.2 g frozen leaf powder samples were mixed with 4.0 mL of 40% methanol in covered glass tubes, boiled in a water bath at 100 °C for 30 min, 1 mL of each extract was mixed with 2 mL glacial acetic acid and 2 mL reagent mixture (120 mL distilled water, 300 mL glacial acetic acid, 80 mL orthophosphoric acid, 1 mL of 25 mg mL^{-1} ninhydrin solution), boiled at 100 °C for 1 h, cooled, 4 mL toluene was added, and the mixtures were vortexed for 15–20 s. The chromophore-containing toluene was separated, and the absorption values were read at 528 nm using toluene as a blank. Proline concentrations were calculated using l-proline as standard.

Starch concentration was determined by grinding 0.1 g frozen leaf samples into a fine powder, 500 µL distilled water was added, vortexed, boiled at 100 °C for 15 min, cooled, 300 µL of the supernatant was transferred to a microfuge tube, 900 µL absolute ethanol was added and mixed well, 1 mL of distilled

water and 50 µL of iodine solution was added and the absorption values were read at 595 nm.

2.7 Total soluble protein and photosynthetic pigments

To determine total soluble protein, 0.1 g frozen leaf samples were ground into powder in a mortar with liquid nitrogen. Extraction buffer (50 mM bicine, 20 mM $MgCl_2$, 1 mM EDTA pH 8.0, 50 mM β-mercaptoethanol, 20 mM HCO_3) was added and the mixture was ground until thawed. The extracts were centrifuged at 20,000 x g for 2 min at 4 °C. Leaf soluble protein concentration was determined spectrophotometrically according to Bradford (1976).

To determine pigments concentrations, 950 µL of pure ethanol was added to 50 µL aliquots of crude protein-prepared extracts, incubated for 10 min in the dark, and centrifuged at 12,000 rpm for 2 min. Chlorophylls *a* and *b,* and carotenoids were quantified spectrophotometrically using the equations described by Lichtenthaler and Wellburn (1983).

2.8 Total phenol concentration and flavonoids

The total phenol concentration was determined spectrophotometrically at 760 nm using Folin–Ciocalteu reagent (Škerget et al. 2005). Briefly, 0.25 g frozen powder samples were mixed with 10 mL of 80% methanol in covered glass tubes, boiled in a water bath at 100 °C for 30 min, 125 µL of the extracts were combined with 2.5 mL Folin–Ciocalteu reagent, 375 µL distilled water and 2 mL of 75 g L^{-1} sodium carbonate, incubated in a water bath at 50 °C for 5 min, cooled to room temperature, and optical density at 760 nm was measured with a spectrophotometer. Gallic acid was used to prepare a standard curve.

Flavonoids were determined using the colorimetric assay of Kim et al. (2003). Distilled water (4 mL) was added to 1 mL of olive leaf extract, 5% sodium nitrite solution (0.3 mL) was added, followed by 0.3 mL of 10% aluminium chloride solution, incubated at ambient temperature for 5 min, 2 mL of 1 M sodium hydroxide was added, the volume was adjusted to 10 mL with ddH_2O, the mixture was vortexed thoroughly and the absorbance value of the pink colour that developed was determined at 510 nm. The total flavonoid concentration was expressed as mg catechin equivalents per g dry matter.

2.9 Data analysis

Data were subjected to two-way ANOVA followed by the Tukey-HSD post-test to examine the effects of cultivar and water regime on the parameters. The data obtained at each time-point were analysed separately. When necessary, the variables were logarithmically transformed to adjust to the requirements of normality and homogeneity of variances. The analysis was performed using R 3.5.1 (R Core Team 2017).

3 Results

3.1 Substrate water content and water relations

Significant decreases in substrate water content (SWC) were observed in the drought treatments ($P < 0.0001$), but no differences were detected between cultivars or treatment by cultivar interaction (Table 2; Table S1). The changes in SWC during drought generated significant differences in water relations parameters (RWC and stem) between treatments ($P < 0.0001$). However, RWC between treatments were not significant for Arbequina cv. (Table 2). On the contrary, RWC reduced significantly from 77.44% to 51.90% in Empeltre during severe drought treatment (30% FC; Table 2). The stem data reflected the decreases in SWC in both cultivars in response to drought (Table 2). stem significantly reduced in Empeltre in both the 50% FC and 30% FC treatments, while stem only significantly reduced in Arbequina under severe drought (30% FC; Table 2). The measurements during the recovery period indicated SWC, RWC and stem totally recovered 7 days after rewatering (Table 2; Table S1[supplementary).

3.2 Vegetative growth and leaf morphological traits

Within each cultivar, the tree height and trunk diameter were not significantly different at the beginning of the drought treatments (spring 2018; data not shown). Hence, the drought treatments were initiated with a homogeneous group of trees from each cultivar. In the well-watered treatment, LAR was significantly

Table 2 Soil water content (SWC), relative water content (RWC) and stem leaf water potential (ψ_{stem}) for Arbequina and Empeltre plants under different water availability treatments (100% FC, 50% FC and 30% FC) and at 7 d after re-watering

Sampling	Treatment	Cultivar	SWC (%)	RWC (%)	ψ_{stem} (bars)
Drought	100% FC	Arbequina	37.96 ± 1.35a	70.81 ± 6.12ab	− 16.00 ± 1.58a
		Empeltre	41.13 ± 3.80a	77.44 ± 1.51a	− 15.60 ± 3.14a
	50% FC	Arbequina	24.88 ± 1.95b	78.13 ± 5.23a	− 22.10 ± 2.24ab
		Empeltre	21.88 ± 1.51b	77.48 ± 1.52a	− 26.80 ± 0.58b
	30% FC	Arbequina	4.19 ± 0.91c	56.29 ± 8.01ab	− 42.60 ± 1.74c
		Empeltre	8.50 ± 2.14c	51.90 ± 4.81b	− 42.20 ± 1.95c
Re-watering	100% FC	Arbequina	34.32 ± 0.92a	77.29 ± 3.34a	− 9.50 ± 1.43a
		Empeltre	33.28 ± 0.26a	71.90 ± 7.74a	− 11.00 ± 1.92a
	R50	Arbequina	34.42 ± 1.90a	82.98 ± 3.50a	− 8.50 ± 1.39a
		Empeltre	33.30 ± 0.22a	76.24 ± 2.11a	− 10.00 ± 1.87a
	R30	Arbequina	35.36 ± 1.18a	72.47 ± 3.04a	− 11.10 ± 1.34a
		Empeltre	33.03 ± 0.31a	78.99 ± 2.74a	− 8.40 ± 1.74a

Values represent the mean ± SE of five replicates per treatment. Different letters indicate significant differences ($P < 0.05$) between irrigation treatments and cultivars

higher in Arbequina than Empeltre (Fig. 2a). Water deficit stress significantly reduced LAR in Arbequina, with no differences between the 50% FC and 30% FC treatments (Fig. 2a). However, neither of the drought stress treatments significantly reduced the LAR in Empeltre (Fig. 2a).

After rewatering, the 100% FC treatment in Arbequina still had a significantly higher LAR than the R50 and R30 treatments. The differences observed between cultivars in LAR were maintained in the recovery period (Figs. 2b and c).

No changes in total LA were observed in Empeltre plants during all the experiment. On the other hand, a slightly non-significant reductions were observed in Arbequina plants under water deficit stress. Even no changes were observed during the rewatering period within each cultivar, Empeltre plants had higher total LA than Arbequina ones in the R50 treatment (Fig. 2d–f). During the drought period Arbequina plants had higher number of leaves than Empeltre ones in all treatments. No changes were observed in Empeltre during the water stress imposition. However, a reduction in the number of leaves was observed in Arbequina being significant at 30% FC treatment (Fig. 2g). No re-growth was observed after 7 days of rewatering (Fig. 2h), moreover, Arbequiona plants from 50R and R30 treatment still presented less number of leaves than control treatment after 21 days of rewatering (Fig. 2i).

Drought did not affect the LMA, with no significant differences between cultivars and treatments (Supplementary Figure S1; Table S1). However, after rewatering, Empeltre exhibited a significantly higher LMA than Arbequina after 7 d and 21 d of rewatering (both $P = 0.03$; Table S1). Nevertheless, the differences between the cultivars were not significant within each treatment (Figure S1).

3.3 Gas exchange and water use efficiency

As expected, water deficit stress reduced net photosynthesis (A_N) and stomatal conductance (g_s) in both cultivars (Figs. 3a, c; Table S1). Moderate water deficit stress (50% FC) affected A_N similarly in both cultivars, with a 45.2% reduction in Arbequina and 47.7% reduction in Empeltre. However, severe water deficit stress (30% FC) seemed to affect Arbequina more severely than Empeltre. For Arbequina, the 30% FC treatment reduced A_N by 80.4% compared to control plants, with a significant difference between the 30% FC and 50% FC treatments (Fig. 3a). In comparison, 30% FC led to a 72.4% reduction in A_N in Empeltre compared to controls, with no significant differences between the 50% FC and 30% FC treatments. Similar trends were observed for g_s: both

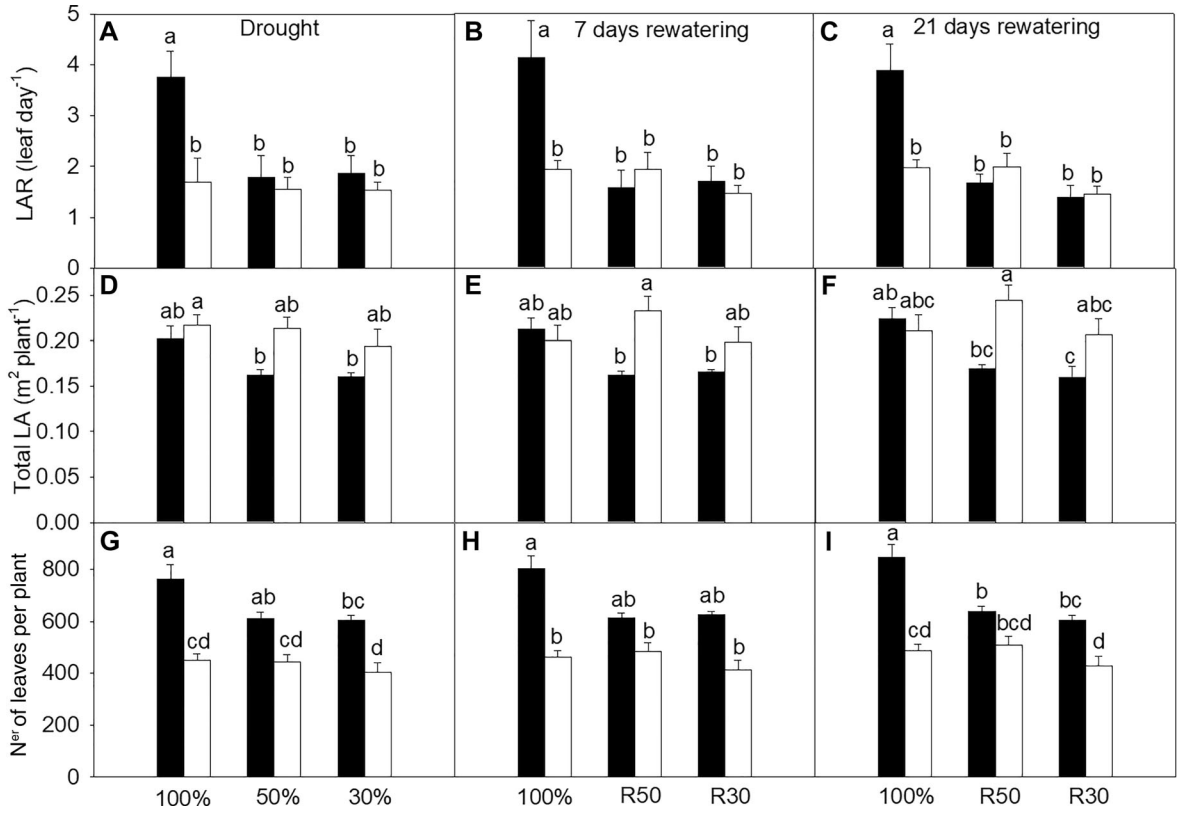

Fig. 2 Leaf appearance rate per day (**a–c**); Total leaf area per plant (d–**f**) and number of leaves per plant (**g–i**) in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC) and at 7 and 21 d after re-watering. Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

50% FC and 30% FC significantly reduced g_s in Arbequina, whereas only 30% FC significantly reduced g_s in Empeltre (Fig. 3c).

The substomatal CO_2 concentration of the leaves (Ci) did not vary significantly between cultivars and among treatments (Table S1; Fig. 3e).

Treatment, cultivar or their interaction had no significant effect on A_N and g_s and Ci after rewatering (Table S1). A_N and g_s totally recovered in the R50 and R30 treatments 7 days after rewetting (Figs. 3b, d, f).

No changes in intrinsic water use efficiency WUE = AN/gs were observed during water deficit stress in either cultivar (Fig. 4a, Table S1). Water use efficiency estimated as ^{13}C did not vary significantly between treatments. However, two-way ANOVA revealed the cultivar had a significant effect (Fig. 4c; Table S1): Empeltre showed lower (higher discrimination against ^{13}C) values than Arbequina, and these differences were maintained after re-watering (Fig. 4c, d).

3.4 Total soluble sugars, proline and starch

The water regimes significantly affected TSS, proline and starch accumulation (all $P < 0.0001$; Table S1). TSS progressively accumulated in both cultivars under both water deficit stress treatments, but these changes were only significant in Arbequina under severe stress conditions (30% FC) compared to controls (13.47 and 9.43 μg g^{-1} DW respectively; Fig. 5a).

Proline accumulation was not significant under the moderate stress treatment (50% FC) in comparison to control plants in either cultivar. However, severe stress (30% FC) significantly increased proline accumulation in both cultivars (Fig. 5c). Water deficit stress reduced the starch concentration: 30% FC led to a significantly lower starch concentration in Empeltre

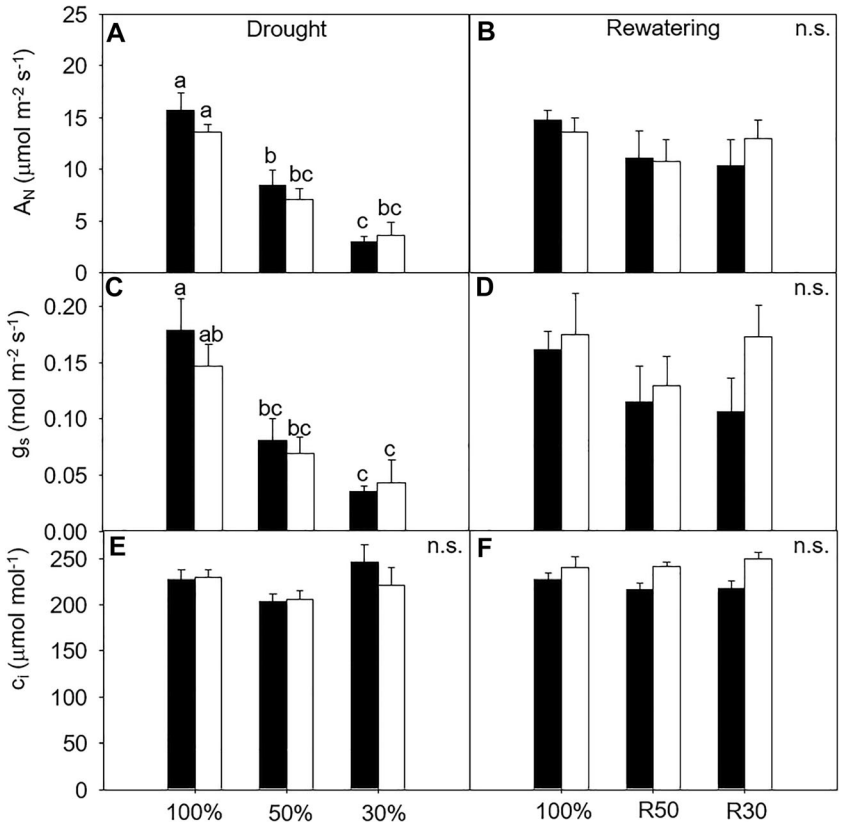

Fig. 3 Net photosynthesis rate (**a**, **b**), stomatal conductance (**c**, **d**) and internal CO_2 concentration (**e**, **f**) measured in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC—see material and methods) (**a**, **c**, **e**) and at 7 d after re-watering (**b**, **d**, **f**). Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

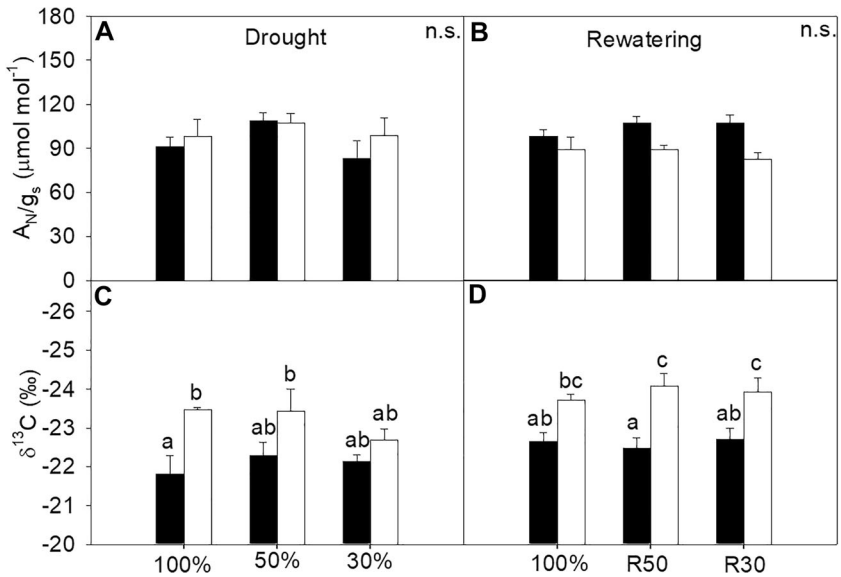

Fig. 4 Instantaneous water use efficiency (**a**, **b**) and C13 discrimination (**c**, **d**) measured in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC) and at 7 d after re-watering. Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

compared to controls (Fig. 5e), but not in Arbequina (Fig. 5e). At 7 days after re-watering, the levels of TSS, proline and starch were not significantly different between treatments or cultivars (Figs. 5b, d and f).

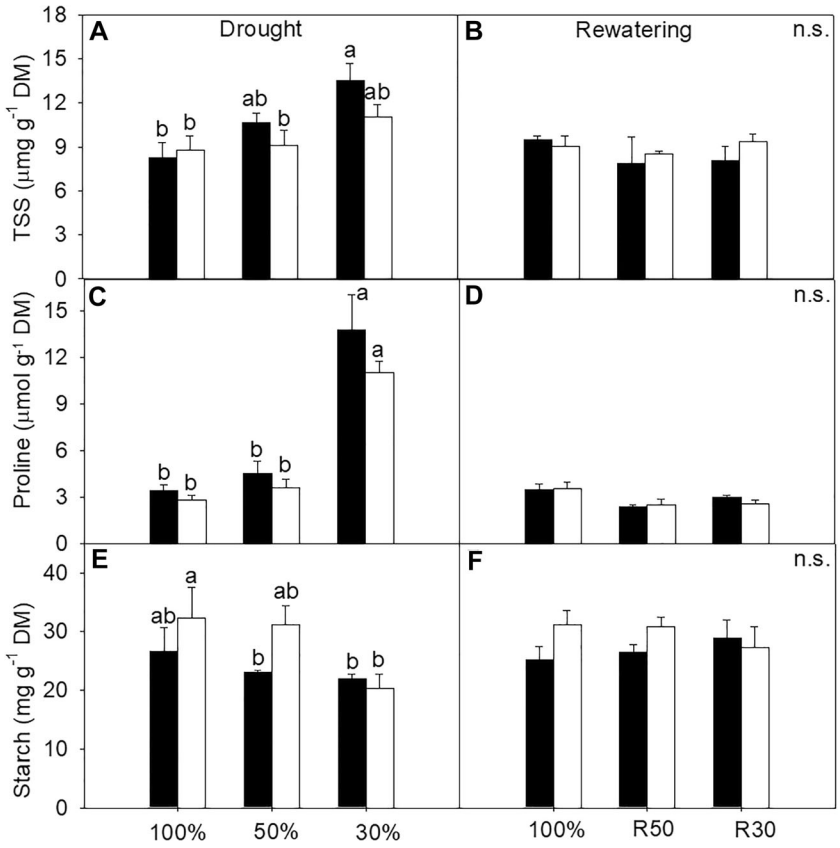

Fig. 5 Leaf total soluble sugar (**a**, **b**), proline (**c**, **d**) and starch (**e**, **f**) concentration in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC) and at 7 d after re-watering. Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

3.5 Total soluble protein and photosynthetic pigments

The drought treatments significantly reduced the total soluble protein (TSP) in both cultivars (Table S1); both moderate and severe drought significantly reduced TSP in Arbequina, while only severe stress treatment (30% FC) significantly reduced TSP in Empeltre (Fig. 6a).

Significant differences in the chlorophyll (Chl $a + b$) concentration were observed among cultivars ($P = 0.001$) and drought treatments ($P < 0.0001$; Table S1). Empeltre had a higher chlorophyll concentration than Arbequina under well-watered conditions (Table S1; $P = 0.001$), and 30% FC reduced the chlorophyll concentration of both cultivars to similar levels (Fig. 6c, Table S1: $P < 0.0001$).

The reduction in total Chl without changes in carotenoids indicates the C + cx/Chl $a + b$ ratio increased in plants under severe drought (30% FC) compared to the other treatments, especially for the Empeltre cultivar (Fig. 5e).

After re-watering, the TSP, Chl $a + b$ and C + cx/Chl $a + b$ values totally recovered in both cultivars (Fig. 6b, d, f; Table S1).

3.6 Total phenol concentration and flavonoids

Cultivar ($P < 0.0001$) and treatment ($P < 0.0001$) significantly affected the total phenol concentration (TPC) during water deficit stress. However, only treatment significantly affected the flavonoid concnetration (Table S1; $P < 0.0001$).

Empeltre had a higher TPC value than Arbequina under well-watered conditions and TPC increased in both cultivars under water deficit stress (Fig. 7a). Empeltre showed the highest TPC under severe drought treatment (30% FC). TPC significantly decreased after seven days of re-watering compared to the previous water deficit stress period for all treatments (Fig. 7b). However, 100% FC Empeltre plants maintained significantly higher levels of TPC than all other treatments during the rewatering phase, including all groups of Arbequina (Fig. 7b).

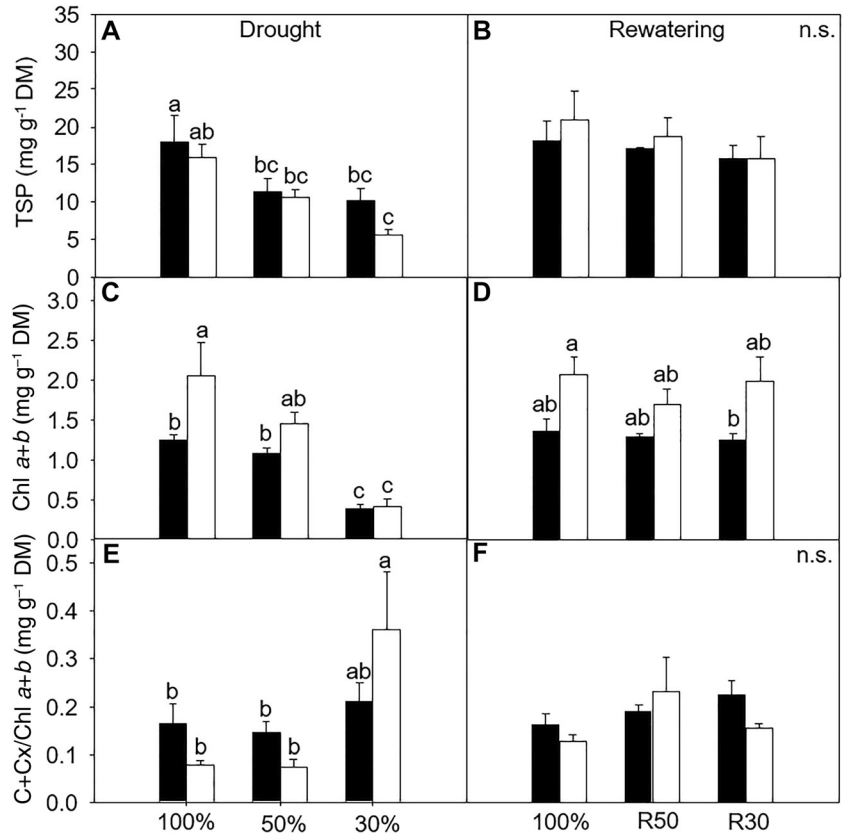

Fig. 6 Total soluble protein (**a**, **b**), chlorophyll $a + b$ (**c**, **d**) and carodenoid/chlorophyll ratio (**e**, **f**) in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC) and at 7 d after re-watering. Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

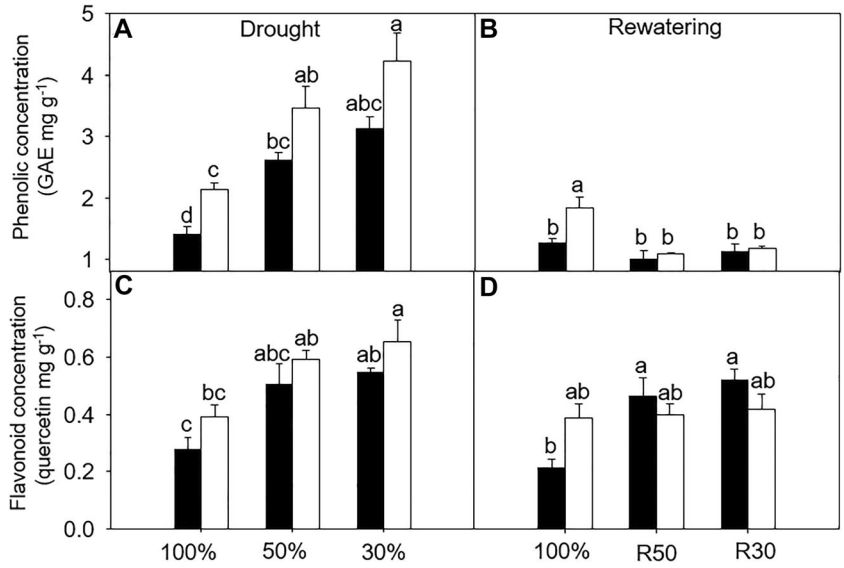

Fig. 7 Leaf phenolic concentration (**a**, **b**) and flavonoids concentration (**c**, **d**) measured in Arbequina (black bars) and Empeltre (white bars) olive plants under different water availability treatments (100% FC, 50% FC, 30% FC) and at 7 d after re-watering. Columns are mean VALUES (n = 5 plants) and BARS are standard errors. Different letters indicate significant differences ($P < 0.05$) between treatments and cultivars at the same evaluation date

Similar trends were observed for flavonoids, with a significant increase in flavonoids in the 30% FC treatment compared to the 100% FC treatment. The cultivar differences in flavonoids exhibited the same trends as TPC, but were not statistically significant (Fig. 7c). The flavonoids concentration was not affected by the re-watering in Arbequina plants, with very similar values in all treatments compared to the

drought period. However, a clear recovery to control values was observed in Empeltre R50 and R30 treatments after seven d of re-watering (Fig. 7d).

4 Discussion

Understanding the main effects and response mechanisms adopted by different olive tree cultivars to cope with water deficit is crucial to achieving a more sustainable and productive crop (Brito et al. 2019). The natural variability existing in cultivars that are locally better adapted to the Mediterranean region constitutes formidable genetic material to study contrasting behaviours or responses to climate change, particularly to water deficit stress and recovery capacity. In the present study, growth, morphological and physiological traits have been characterized in a traditional and a widespread cultivar. The indices of plant water status showed that both cultivars reduced Ψ_{stem} in response to drought stress due to depletion of SWC. However, the reduction in Ψ_{stem} in Arbequina was only significant under severe drought (30% FC), indicating lower water loss in this cultivar than Empeltre, probably due to the stronger reduction in g_s. Some genotype-specific differences in stomatal behaviour were previously reported in olive trees (Bacelar et al. 2007a, 2009; Brito et al. 2019). The present study also revealed genotype-specific differences in the stomatal response, as Arbequina exhibited a more conservative strategy with a greater reduction in g_s at moderate water deficit. The strong reduction in g_s in Arbequina under drought strongly impaired growth, as reflected by the reduction in the LAR and total LA in this cultivar. In addition, Arbequina possesses smaller leaves than Empeltre (i.e. higher number of leaves in similar LA), which improves heat dissipation and enables less water loss through transpiration cooling, as previously described (Jarvis and McNaughton 1986). The reduction in total leaf area can be considered a dehydration-avoidance mechanism, to minimize water loss by transpiration, and may enable the plants to resist long periods of water deficit while keeping the leaves photosynthetically active (Tardieu 2003). Based on these results, we conclude that Arbequina possesses a more conservative strategy of stomatal closure—as previously reported by Bacelar et al. (2009) for this cultivar—and is thus able to adopt an avoidance mechanism (Gulías et al. 2002). Nevertheless, our results showed that the reduction in g_s for Arbequina could not prevent the reduction in Ψ_{stem} observed at 30% FC, which probably resulted in growth inhibition. The growth impairment at 30% FC in Arbequina—reflected in low leaf appearance rate, total LA reduction and reduced number of leaves—was maintained, even at 7 and 21 days after rewatering. In contrast, Empeltre maintained LAR with no changes in the number of leaves under drought, even though, both parameters were lower in Empeltre than in Arbequina in all treatments. Leaves usually exhibit higher LMA under water deficit stress than under irrigation, but we did not observe any differences in LMA or leaf density (data not shown) between treatments. Probably because very low numbers of leaves developed during the drought period in both cultivars, and growth almost completely stopped in Arbequina. Nevertheless, differences between cultivars in other morph-anatomical traits as stomatal density or leaf pubescence cannot be discarded as genetic variability in these traits have been described before (Bacelar et al. 2004).

Our results suggest that the reduction in photosynthesis in olive plants under drought is mainly due to diffusional limitations, as a strong correlation (not shown) was maintained between A_N and g_s and no changes in Ci were recorded, indicating concomitant reductions in mesophyll conductance (Brodribb 1996; Flexas and Medrano 2002; Bota et al. 2004; Perez-Martin et al. 2009, 2014) but no metabolic impairment, since the predominance of non-stomatal limitation usually is reflected by increases of Ci (Flexas and Medrano 2002). Nevertheless, the chlorophyll and protein concentrations decreased significantly in leaves as the water deficit increased, indicating that some metabolic changes occurred as a consequence of or response to water deficit stress. In agreement with other studies, water deficit stress reduced leaf total protein concentration, Vaz et al. 2016). The decrease in the protein concentration and simultaneous accumulation of proline under drought conditions, observed in the present study, could be explained by enhanced proteolysis and decreased protein synthesis (Thakur and Thakur 1987). The reduction in Chl and absence of changes in carotenoids led to an increase in the C + cx/Chl ratio under severe drought in both cultivars. Xanthophyll and carotenes play essential roles in photoprotection (Takahashi and Badge 2011). Abdallah et al. (2018) also reported increases in the

Carotenes/Chl ratio under drought and salt stress in olive trees. Our results confirm that, especially in Empeltre, a high C + cx/Chl ratio under severe drought may act as a mechanism to protect, at least partly, the photosynthetic apparatus against photooxidation.

The differences observed in stomatal behaviour were not reflected in WUEi. Usually, drought can substantially increase WUEi in olive trees (Bacelar et al. 2007a; Abdallah et al. 2018; Trabelsi et al. 2019), but genotype variability in this trait has been much less explored. In the present study, no differences in WUEi among treatments or between cultivars were observed. Nevertheless, WUEi does not always reflect total plant water use efficiency (Bacelar et al. 2013; Brito et al. 2019). In fact, the absence of a significant association between WUEi and plant WUE was reported in olive trees by Bacelar et al. (2007a). On the other hand, carbon stable isotopes, usually reported as ($\delta^{13}C$), have been successfully used to estimate the growing season mean water-use efficiency (WUE) of C3 plants (Farquhar et al. 1989; Cabrera-Bosquet et al. 2009; Di Matteo et al. 2010). Empeltre exhibited a higher $\delta^{13}C$ than Arbequina under irrigation and during the rewatering phase. This characteristic could be a point to consider in crop selection and irrigation strategies.

Another player in stomatal regulation as a response to water availability is the osmotic adjustment. The osmotic adjustment was previously reported in olive trees and is considered to be dependent on the severity of stress (Sofo et al. 2004; Bacelar et al. 2006; El Yamani et al. 2019, 2020). In this sense, some slight differences in osmotic adjustment were observed between cultivars. The increase in TSS during water deficit stress was accompanied by a reduction in starch concentration under severe drought in both cultivars. Similar results were reported by Ben Ahmed et al. (2009), and the decrease in the starch concentration could be due to increased amylase activity under drought (Todaka et al. 2000). Moreover, water deficit stress can also alter carbon assimilate partitioning between sucrose and starch and translocation of carbon out of the leaves (Lemoine et al. 2013). The concomitant reduction in the starch concentration of the leaves and low assimilation rates suggest that—in these experiments—translocation within the leaves was not affected in any of the cultivars. Among the various osmoprotectants, proline has been widely studied and plays crucial roles in plant defence during abiotic stress (Kaur and Asthir 2015; Zulfiqar et al. 2020). Our results confirm that significant proline accumulation occurred during severe water deficit stress similarly in both cultivars. Proline exerts a protective action that prevents membrane damage and protein denaturation during severe drought stress (Ain-Lhout et al. 2001). This evidence may explain the non-significant reductions in TSP between the 50% FC and 30% FC treatments. Ben Ahmed et al. (2009) observed a general relationship between the A_N and proline concentration that varied among cultivars. Our data revealed a similar relationship between these parameters (A_N vs. proline) in both cultivars (Fig. 8). However, Empeltre accumulated proline at higher A_N values and achieved higher values of proline than Arbequina, which reinforces the suggestion of greater stress tolerance in the Empeltre cultivar.

Phenolics act as powerful antioxidants, and genotype-specific variation in TPC production was previously described (Boughalleb and Mhamdi 2011; Petridis et al. 2012; Ahmadipour et al. 2018). Empeltre exhibited a higher TPC than Arbequina, which can confer more antioxidant capacity and this difference between cultivars was more evident under non-water deficit stress conditions.

During the rewatering phase, we found that the plants recovered their water status with similar RWC and ψ_{stem} values in all treatments, in agreement with previous studies that reported good recovery capacity from water deficit in olive trees (Torres-Ruiz et al. 2013, 2015). Increases in A_N and g_s were observed in

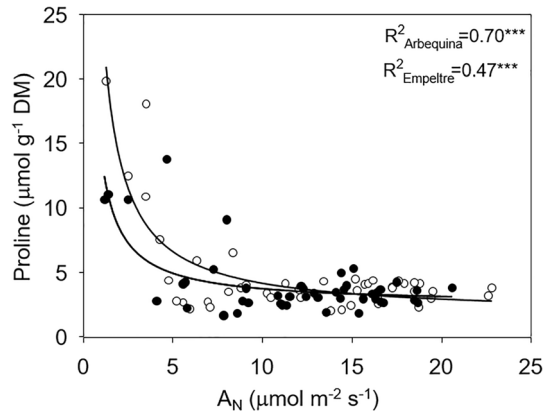

Fig. 8 Relationship between net photosynthetic rate (A_N) and leaf proline concentration in Arbequina (black symbols) and Empeltre (white symbols) olive leaves based on the entire experiment

the first seven days of rewatering, confirming predominant stomatal limitation occurs under moderately stressful conditions (Guerfel et al. 2009; Fernández 2014). As observed in the present study, Torres-Ruiz et al. (2015) also reported a slow recovery of g_s over a period of 18 h, with total recovery after six days of irrigation. In agreement with these results, the solutes related to osmotic adjustment during drought, such as TSS and proline, reached similar values to control plants in the rewatering phase, indicating that the repair mechanisms reduced to non-stress levels. Additionally, no differences in TSP, Chl, carotenoids or phenolic compounds were observed between the recovery treatments (R50, R30) and control plants, even though some genotypic-specific responses in the synthesis of phenolic compounds, including flavonoids, were observed. Empeltre had a higher concentration of phenolic compounds and exhibited a more dynamic response during recovery, suggesting the protective effects of phenolic compounds are more important in this cultivar.

The general recovery observed during the rewatering phase in this study suggests the photosynthetic apparatus was only slightly damaged and the plants were able to quickly recover their normal physiological and biochemical state after short-term rewatering (Torres-Ruiz et al. 2013; Torres-Ruiz et al. 2015; Trabelsi et al. 2019). However, Arbequina did not restore the vegetative growth rate during the rewatering phase, even after up to 21 days' irrigation.

The findings showed that there are cultivar-specific differences in terms of drought tolerance. Arbequina exhibited a more conservative water use strategy, which was associated with a strong reduction in vegetative growth. Empeltre exhibits more efficient water use ($\delta^{13}C$) than Arbequina under well-watered conditions and has a higher phenolic concentration, which may confer a higher capacity to tolerate drought. Rewatering led to a total recovery of most parameters and alleviated the damage caused by drought. However, the vegetative growth rate of Arbequina did not recover after 21 days of rewatering. Nevertheless, further long term studies under field conditions are needed to confirm these findings on olive trees growing in the field and to confirm the behavior of each genotype in different locations.

Author contributions Concept and design of the experiment: JB, JME, AB. Performed the experiments: AM, IM, HEA, JB. Analyzed the data: AM, JB, EB. Contributed reagents/materials/analysis tools: JB, JME. Wrote the paper: AM and JB.

Funding Ms Amira Melaouhi and Imen Mahjoub have been supported by an ERASMUS + Associated countries KA107 programme, call 2015–2017 co-funded by UIB and Erasmus + programme of the European Union. This work has been supported by the project PROCOE/1/2017 funded by the Conselleria Innovació, Recerca i Turisme and the European Regional Development Fund (FEDER).

Data availability The authors confirm that the data supporting the findings of this study are available in its supplementary materials.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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