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Water stress at the end of the pomegranate fruit ripening stage produces earlier harvest and improves fruit quality

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ABSTRACT

Pomegranate (*Punica granatum* L.) is a drought tolerant crop, which thrives in the face of scarce water resources, this fact underlines the importance of determining the optimum harvest time to improve the quality of pomegranate fruits. This research was focused on the crop responses to drought stress during the phenological period of fruit ripening. Special attention was paid to the effects on plant productivity of water restrictions during fruit ripening and whether such restrictions have secondary effects on fruit characteristics and composition. Control plants were irrigated above crop water requirements while deficit irrigation treatments were irrigated as control plants except for 6 days (fruit late ripening), 15 days (second half fruit ripening), 25 days (fruit ripening), and 36 days (end fruit growth and late ripening) before harvest, when irrigation was withheld. The results indicated that the water stress integral, calculated from leaf conductance, leaf water potential, stem water potential and fruit water potential data, differed in their assessment of the cumulative water deficit reached by the plants. Also, pomegranate fruit ripening was confirmed as a critical period because irrigation is clearly essential during most of this phenological period to achieve maximum yield. Moreover, a very short period of irrigation restriction (around 6 days) at the end of ripening period comes early harvest time, saves irrigation water, enhances the bioactive compounds content (anthocyanins, phenolic compounds, punicalagin and ellagic acid) and increases the price of the fruit without affecting marketable yield and fruit size. This suggests that the sensitivity to water stress during such a critical phenological period is not constant and/or that for productivity to be adversely affected it is necessary to exceed a threshold level of water stress.

1. Introduction

Pomegranate (*Punica granatum* L.) plants are equipped with xeromorphic characteristics such as a high leaf relative apoplastic water content and the ability to develop complementary stress avoidance and stress tolerance mechanisms to confront drought (Rodríguez et al., 2012). That means that it is able to thrive in arid and semi-arid areas, even under desert conditions (Aseri et al., 2008). Nevertheless, to reach optimal growth, yield and fruit quality for commercial production, the crop requires regular irrigation throughout the dry season (Prasad et al., 2003; Shaliendra and Narendra, 2005; Sulochanamma et al., 2005; Holland et al., 2009).

The commercial production of pomegranate in the Mediterranean Basin is characterized by high quality fruits (Stover and Mercure, 2007; Holland et al., 2009) with high bioactive compounds content (Gil et al., 2000; Poyrazoğlu et al., 2002; Mena et al., 2011) and a correspondingly

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high antioxidant capacity and beneficial health effects (Lansky and Newman, 2007).

All Mediterranean agrosystems must cope with water scarcity, and any policy involving greater use of the water (Pereira et al., 2002). In this sense, pomegranate farming must be directed towards the use of deficit irrigation strategies, allowing significant water savings, and the profitable production of high quality fruits. Sustained deficit irrigation (SDI) is an irrigation strategy in which the amount of water applied at any moment of the season is lower than that needed to satisfy the full crop water requirements (English and Raja, 1996). Regulated deficit irrigation (RDI) is another irrigation strategy designed to save water while having a minimum impact on yield and fruit quality (Goldhamer, 1989; Naor, 2006). This requires precise knowledge of the crop response to drought stress during different phenological periods when adverse effects on productivity are minimal (non-critical periods) or maximal (critical periods).

Reports on the effect of irrigation management on pomegranate fruit yield and quality are very scarce. The first results indicated that it is possible to control the desired ripening time in pomegranates by applying different irrigation regimes (Sonawane and Desai, 1989). Also, Prasad et al. (2003), Shaliendra and Narendra (2005), and Sulochanamma et al. (2005) showed that irrigation has a positive effect on pomegranate vegetative growth, yield, and fruit weight. Recently, Galindo et al. (2014a) indicated that SDI applied throughout the pomegranate season reduces total yield per tree, the number of fruits per tree and the size of the fruits; however, such a strategy can advance the availability of fruits from late flowerings, which despite their smaller size are of high interest for the pomegranate industry due to their very high content of bioactive compounds. In contrast, Mellisho et al. (2012) concluded that SDI, under moderate water stress, showed some changes in colour and chemical characteristics, which reflected earlier ripening. However, Mena et al. (2013) indicated that pomegranate juice from trees submitted to SDI that produces severe water stress levels was of lower quality and less healthy than that from fully irrigated trees. On the other hand, Peña-Estévez et al. (2015) concluded that pomegranates from SDI trees had good sensory qualities, a higher content of most bioactive compounds, and suffered less chilling injury during cold storage and shelf-life than fully irrigated fruits. Recently, Laribi et al. (2013) showed that pomegranates from SDI trees, submitted to mild water stress during flowering and fruit set and more severe water stress during the linear phase of fruit growth and ripening, had a redder peel and higher level of total soluble solids in the juice.

To the best of our knowledge, there has been no scientific study evaluating the response of pomegranate to RDI, applying full irrigation in all the critical periods and deficit irrigation during the non-critical periods. However, Intrigliolo et al. (2013) and Laribi et al. (2013) studied pomegranate response to severe irrigation water restrictions applied during the phenological periods of (i) flowering, fruit set and early fruit growth, (ii) linear fruit growth, and (iii) the last part of fruit growth and ripening. These authors concluded that the phenological period comprising flowering and fruit set could be regarded as non-critical from the yield point of view. Moreover, Laribi et al. (2013) concluded that irrigation water restriction during pomegranate fruit growth and ripening enhances peel red colour intensity and total soluble solids in the juice, while irrigation water restriction during linear fruit growth period increased the concentration of many bioactive compounds in the juice, such as anthocyanins, that could be related to health and taste.

For this reason, the aim of this research was to (i) clarify whether the pomegranate fruit ripening phenological stage is a critical or non-critical period from the yield point of view, (ii) whether pomegranate yield response to water restriction during ripening depends on the exact point at which water stress takes place, and (iii) evaluate whether water restrictions during the ripening stage have secondary effects on fruit characteristics and composition.

2. Material and methods

2.1. Plant material, experimental conditions and treatments

The experiment was carried out in 2013 in a pomegranate (*Punica granatum* L.) orchard located near the city of Alhama de Murcia (Spain) (37°47′N, 1° 25′W). The trees were own-rooted 15 years old *Mollar de Elche* cultivar and the tree spacing was 3 m × 5 m. The soil of the orchard is a moderately saline silt loam (Hyposalic Calciorthid), with moderate lime content, very low organic matter content, low cationic exchange capacity, high available phosphorus levels and low available potassium. The irrigation water used had an electrical conductivity of between 0.9 and 1.3 dS m^{-1}. The chloride (Cl^-) concentration in the irrigation water ranged from 67 to 78 mg L^{-1} during the experimental period. Pest control and fertilization practices were those usually used by local growers, and no weeds were allowed to develop within the orchard.

Micrometeorological data, namely air relative humidity, air temperature, solar radiation, rainfall and wind speed 2 m above the soil surface, were collected by an automatic weather station located near the experimental site. Mean daily air vapour pressure deficit (VPD_m) and daily ETo, using the Penman–Monteith equation, were calculated as described by Allen et al. (1998).

During the growing season, control plants (T0) were irrigated above crop water requirements (123% ETc) in order to ensure non-limiting soil water conditions. Irrigation was performed daily during the night using a drip-irrigation system with a lateral pipe parallel to each tree row and 3 emitters per tree, each delivering 4 L h^{-1}. In-line water meters were used to measure the water supplied to each experimental unit. T1, T2, T3, and T4 treatments were irrigated as T0 except for 6 (DOY 277–283, fruit late ripening), 15 (DOY 268–283, second half fruit ripening), 25 (DOY 258–283, fruit ripening) and 36 (DOY 247–283, end fruit growth and late ripening) days before harvest (DOY 283), respectively, when irrigation was withheld. The total amount of water received by each treatment during the experimental period (DOY 247–283) was 128, 110, 86, 49 and 0 mm for T0, T1, T2, T3 and T4 treatments, respectively, without considering precipitation (basically the 84 mm that fell on DOY 271).

2.2. Plant water status

The water relations of the leaves and fruits were measured at midday (12 h solar time). Fruits and fully expanded leaves from the south-facing side and middle third of the tree of four trees per treatment were selected for measurements. Midday leaf conductance (g_{leaf}) was measured with a porometer (Delta T AP4, Delta-T Devices, Cambridge, UK) on the abaxial surface of two leaves per tree. Midday fruit water potential (Ψ_{fruit}), midday leaf water potential (Ψ_{leaf}), and midday stem water potential (Ψ_{stem}) were measured in two fruits or two leaves similar to those used for g_{leaf} using a pressure chamber (PMS 600-EXP, PMS Instruments Company, Albany, USA) (McFadyen et al., 1996; Galindo et al., 2014b). Leaves for Ψ_{stem} measurements were enclosed in a small black plastic bag covered with aluminium foil for at least 2 h before the measurements were made.

In order to assess the cumulative effect of the water deficit, the water stress integral (SI) was calculated from the g_{leaf}, Ψ_{leaf}, Ψ_{stem} and Ψ_{fruit} data according to the expression proposed by Myers (1988).

$$SI_A = \left|\sum (\overline{A}-H)n\right|$$

where A can be g_{leaf}, Ψ_{leaf}, Ψ_{stem} or Ψ_{fruit} and $\overline{A}$ is the average g_{leaf}, Ψ_{leaf}, Ψ_{stem} or Ψ_{fruit} value for any interval, H is the maximum value measured during each interval and n is the number of days in the interval.

2.3. Fruit physico-chemical analysis

In order to study any changes in pomegranate fruit due to the irrigation treatments, the samples from each treatment were picked on 16 October (DOY 283). Forty-eight fruits per treatments were harvested (four trees for treatments and twelve fruits per tree). All the fruits were transported to the laboratory and analyses were performed immediately. For each fruit, the following parameters were measured: maximum width or equatorial diameter, ED (mm), and fruit length from calyx to base, FL (mm), using a digital calliper/calliper (model CD-15 DC; Mitutoyo (UK) Ltd, Telford, UK) with 0.01 mm accuracy; fruit weight, FW (g) using a precision weighing balance (Mettler AJ50, Goettingen, Germany) with an accuracy of 0.0001 g. Then, the fruits were peeled by hand and the arils were weighted, homogenised, and half of the arils was squeezed, between two layers of muslin cloth, to extract the complete juice. The juice was centrifuged (1200*g*) at 4 °C and stored at −70 °C until the chemical analyses were conducted (total soluble solids (TSS), titratable acidity (TA) and pH), and the other half of the arils were immediately frozen in liquid nitrogen and later freeze-dried in an Alpha 2–4 freeze drier (Alpha 2–4; Christ, Osterode am Harz, Germany) for 24 h at a pressure reduction of 0.220 mbar. The temperature in the drying chamber was −25 °C, while the heating plate reached 15 °C. At the end of freeze-drying, the samples were powdered and packed under vacuum. Antioxidant activity (AA), total polyphenol content (TPC), total anthocyanin content (TAC), α-punicalagin, β-punicalagin and ellagic acid were analysed.

2.3.1. Total soluble solids, pH and total titratable acidity

TSS were measured with a digital Atago refractometer (model N-20; Atago, Bellevue, WA) at 20 °C with values being expressed as °Brix. The TA and pH were determined by acid–base potentiometer (877 Titrino plus; Metrohm ion analyses CH9101, Herisau, Switzerland), using 0.1 N NaOH up to pH 8.1; values were expressed as g citric acid L^{-1}. Finally, the maturity index (MI), which is a ratio of TSS to TA, was also calculated for each sample.

2.3.2. Colour determination

Pomegranate juice colour was determined and measured in glass cells of 2 mm path length (CT-A22). A Minolta CR 2000 colorimeter (Osaka, Japan) was used and results were expressed in the CIE L^*, a^*, b^* system, and the mean values of lightness (CIE L^*), red/greenness (CIE a^*) and blue/yellowness (CIE b^*) coordinates for each juice were calculated. The objective colour was calculated as chromaticity or chroma [$C^* = (a^{*2} + b^{*2})^{½}$] and hue angle [$H^\circ = \tan^{-1}(b^*/a^*)$].

2.3.3. Total polyphenols content, total anthocyanin content and antioxidant activity

Total phenolic compounds (TPC) were determined using Folin–Ciocalteu reagent. Briefly, an aliquot of filtered juice was diluted with 0.4 mL of phosphate buffer (50 mmol L^{-1}, pH = 7.8). Folin–Ciocalteu reagent (2.5 mL) was added and the content of the flask was mixed thoroughly. After 8 min, an Na_2CO_3 solution (10 mL, 10%, w/v) was added and the samples were incubated in a water bath at 50 °C for 5 min. The resulting blue colour was measured spectrophotometrically at 760 nm. The concentration of the total polyphenol compounds in juice was determined by comparison with the absorbance of gallic acid at different concentrations. Results were expressed as mg of gallic acid (GAE) L^{-1} of juice.

The total anthocyanin content (TAC) was determined by a pH differential method with two buffer systems, sodium acetate buffer, pH 4.5 (0.4 mol L^{-1}) and potassium chloride buffer, pH 1.0 (0.025 mol L^{-1}) (Giusti et al., 1999). Pomegranate juice (0.4 mL) was mixed with 3.6 mL of the corresponding buffers and read against water as blank at 510 nm and 700 nm. The absorbance (A) was calculated as $A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$. The total anthocyanin content was calculated by following the equation $TAC = (A \times MW \times DF \times 100/MA)$, where A is the absorbance, MW is the molecular weight (449.2), DF is the dilution factor (10), and MA is the molar absorptivity of cyaniding-3-glucoside (26.900). The result was expressed as mg cyaniding-3-glucoside (C3G) L^{-1} of pomegranate juice.

For the total antioxidant activity (TAA), a methanol extract was prepared with each sample to be analysed. Freeze-dried fruits (0.5 g) were mixed with 10 mL of MeOH/water (80:20, v/v) + 1% HCl, sonicated at 20 °C for 15 min and left for 24 h at 4 °C. Then, the extract was again sonicated for 15 min, and centrifuged at 15,000 rpm for 10 min. The radical scavenging activity was evaluated using the DPPH radical (2,2-diphenyl-1-picrylhydrazyl). Briefly, 10 mL of the supernatant were mixed with 40 mL of MeOH and added to 950 mL of DPPH solution. The mixture was shaken vigorously and placed in a dark room for 10 min. The decrease in absorbance was measured at 515 nm using a UV–vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). Calibration curves, in the range 0.01–5.00 mmol Trolox L^{-1}, were used for the quantification of antioxidant activity, showing good linearity ($r^2 \geq 0.998$). Results are expressed as mM Trolox.

2.3.4. Punicalagin isomers and ellagic acid

The punicalagin (isomers α and β) and ellagic acid contents were determined in freeze-dried fruits (0.3 g) diluted with 7 mL of MeOH/water (80:20, v/v) and 1% acetic acid and then centrifuged at 15,000 rpm for 20 min. Supernatants were filtered through a 0.45 μm Millipore filter and then injected into a Hewlett-Packard series 1200 HPLC equipped with a diode-array detector. Each sample (20 mL) was analysed on a LiChroCART 100 RP-18 reversed-phased column (250 mm x 4 mm, particle size, 5 μm; Merck, Darmstadt, Germany) equipped with a C18 pre-column (LiChrospher 100 RP-18, 5 mm; Merck, Darmstadt, Germany) using a mobile phase of 1% acetic acid in ultra-high purity deionised water (solvent A) and 1% acetic acid in MeOH (solvent B). Elution was performed at a flow rate of 1 mL min^{-1} using a gradient starting with 1% B for 5 min, and increasing to 60% B for 40 min. Punicalagin (α and β) and ellagic acid were detected at 360 nm. To confirm their identification, absorption spectra and retention times were compared with those obtained from chemical standards. Standard curves for pure punicalagins (Chengdu Biopurify Phytochemicals Ltd., Sichuan, China), with a concentration range of 0.05–0.80 g L^{-1}, as well as for ellagic acid (Tocris Bioscience, Ellisville, MO), with a concentration range of 0.0025–0.0200 g L^{-1}, were used for quantification. The results for individual isomer punicalagin (α and β) and ellagic acid are expressed as mg L^{-1}.

2.4. Statistical design and analysis

The design of the experiments was completely randomized with four replications, each replication consisting of three adjacent rows, each with thirteen trees. Measurements were taken on the inner-most trees of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross sectional area, height, ground shaded area, etc.), while the other trees served as border trees. Data were analysed using Statgraphics 5.1 for Windows (Statpoint Technologies, Warrenton, VA, USA). A basic descriptive statistical analysis was followed by an analysis of variance (ANOVA) test for means comparisons. Fisher's Least Significant Difference (LSD) procedure at a 95.0% confidence level was used to discriminate among the means (Multiple Range Test). Values for each replicate were averaged before the mean and the standard error of each treatment were calculated.

3. Results

3.1. Climate and plant water status

The experimental conditions were semi-arid, characterized by a

Table 1
Effect of irrigation treatments on leaf conductance (SI_{gleaf}, mmol m^{-2} s^{-1} x day), stem ($SI_{\Psi stem}$, MPa x day), leaf ($SI_{\Psi leaf}$, MPa x day) and fruit ($SI_{\Psi fruit}$, MPa x day) water stress integral.

Treatment	SI_{gleaf}	$SI_{\Psi stem}$	$SI_{\Psi leaf}$	$SI_{\Psi fruit}$
T0	5434.5c	10.5b	27.6c	8.5c
T1	5911.5c	15.0b	29.1c	15.1bc
T2	6414.0c	21.5b	31.1bc	22.7b
T3	9784.0b	49.5a	49.2ab	43.7a
T4	15215.3a	62.8a	53.5a	52.9a

Means within a column that do not have a common letter are significantly different by $LSD_{0.05}$ test.

VPD_m ranging from 0.33 to 1.87 kPa, average daily maximum and minimum air temperatures of 28.0 and 14.8 °C, respectively, and accumulated ETo of 162 mm. Total rainfall was 88.4 mm: DOY 270 (3.5 mm), DOY 271 (84 mm) and DOY 272 (0.9 mm)

Table 1 describes the cumulative effect of the irrigation treatments on the pomegranate trees using SI_{gleaf}, $SI_{\Psi stem}$, $SI_{\Psi leaf}$ and $SI_{\Psi fruit}$ values, whose values in the different irrigation treatments tended to increase with the number of days irrigation was withheld. However, SI_{gleaf}, $SI_{\Psi stem}$ and $SI_{\Psi leaf}$ values in T0, T1 and T2 were statistically equivalent, whereas $SI_{\Psi fruit}$ values in these treatments showed differences, the $SI_{\Psi fruit}$ values in T2 being significantly higher than in T0 but similar to that in T1, which was also similar to that in T0 (Table 1). Moreover, $SI_{\Psi stem}$, $SI_{\Psi leaf}$ and $SI_{\Psi fruit}$ values in T3 and T4 plants presented similar values, whereas SI_{gleaf} values in T4 plants were significantly higher than those in T3 plants (Table 1).

3.2. Yield and fruit physical characteristics

The marketable yield of pomegranates was significantly reduced by the withholding of water: the longer the period without water, the lower the marketable yield (Table 2). In this sense, control (T0) plants and those from which water was withheld during late ripening (T1) showed similar yields (56.8 and 55.5 kg $tree^{-1}$, respectively) but higher than those of T2, T3 and T4 plants (35.2, 28.9 and 17.8 kg $tree^{-1}$, respectively), which were similar among themselves. Harvested pomegranate fruits affected by peel cracking and/or peel splitting (data not shown) were significantly higher in T3 and T4 (30.5 and 31.9 kg $tree^{-1}$, respectively), which had longer water withholding periods and lower in T0 plants (7.6 kg $tree^{-1}$) whereas T2 plants showed intermediate values (19.8 kg $tree^{-1}$) and similar to T1 plants (14.7 kg $tree^{-1}$), which at the same time were statistically similar to T0 plants. The effect of withholding water during the different phases of fruit ripening on average fruit weight was similar to the effect on fruit yield, with the characteristic that T1 fruit weight (258 g) was statistically equivalent to that measured in T0, T2, T3 and T4 (293, 252, 249 and 253 g, respectively) (Table 2). Fruits from T0 plants had the highest equatorial diameter (86.9 mm), whereas no differences in this value were observed in the other water withheld treatments (from 78.1 up to 81.6) (Table 2). Additionally, fruits from T1, T3 and T4 plants showed significant lower fruit length (69.7, 69.8 and 67.2 mm, respectively) than fruits from T0 plants (75.0 mm); whereas fruits from T2 plants showed an intermediate response with a fruit length similar to that observed in the other four treatments (71.4 mm) (Table 2).

Table 2
Effect of irrigation treatments on marketable pomegranate fruit yield (MY, kg $tree^{-1}$), average fruit weight (FW, g), fruit equatorial diameter (ED, mm), and fruit length (FL, mm).

Treatment	MY	FW	ED	FL
T0	56.8a	293a	86.9a	75.0a
T1	55.5a	258ab	80.4b	69.7b
T2	35.2b	252b	81.4b	71.4ab
T3	28.9b	249b	81.6b	69.8b
T4	17.8b	253b	78.1b	67.2b

Means within a column that do not have a common letter are significantly different by $LSD_{0.05}$ test.

Table 3
Effect of irrigation treatments on pomegranate peel and juice lightness (CIE L^*), red/greenness (CIE a^*), blue/yellowness (CIE b^*), chroma (C^*) and hue angle (H°) values.

	Treatment	L^*	a^*	b^*	C^*	H°
Peel	T0	64.2a	26.5b	31.2a	41.6b	50.1a
	T1	60.8ab	30.7a	30.5a	43.9a	45.3ab
	T2	60.1b	32.0a	30.1ab	44.5a	43.7abc
	T3	57.2bc	33.5a	27.6bc	43.9a	40.1bc
	T4	55.1c	34.6a	26.8c	44.3a	38.2c
Juice	T0	32.4b	8.3b	2.3b	8.6b	14.8b
	T1	33.1ab	10.5ab	3.1ab	10.9ab	16.5ab
	T2	33.1ab	9.6ab	2.7ab	9.9ab	15.3ab
	T3	33.9a	11.7a	3.6a	12.3a	17.2ab
	T4	33.7a	11.8a	3.9a	12.5a	18.2a

Means within a column for each fruit part that do not have a common letter are significantly different by LSD0.05 test.

A significant effect of water stress on pomegranate peel and juice colour was observed (Table 3). Thus, L^*, b^* and H° values of the peel tended to decrease with accumulated water stress effect (from 64.2 to 55.1, from 31.2 to 26.8 and from 50.1 to 38.2, respectively), while a^* and C^* values increased from 26.5 to 34.6 and from 41.6 to 44.3, respectively, leading to a fruit peel with higher redness and darkness values (Table 3). Coordinates L^*, a^*, b^*, C^*, and H° values of the pomegranate juice showed a tendency to increase as the days of water reduction increased, but no significant differences in L^*, a^*, b^* and C^* values were found between T0, T1 and T2 and in H° values between T0, T1, T2 and T3 (Table 3).

3.3. Fruit chemical characteristics

TSS values in T1, T2, and T3 fruits were similar and lower (~17°Brix) than in T0 fruits (17.8°Brix), while the TSS value in T4 fruits showed an intermediate value (17.5°Brix) similar to that measured in T0 and T1 and T3 fruits (Table 4). TA values in T0, T1, T2, and T4 fruits were similar (ranging between 2.11 and 2.17 g citric acid L-1); wile the TSS in T3 (2.23 g citric acid L-1) fruits was higher than in T1 and similar to that observed in T0, T2 and T4 fruits. Moreover, non-significant differences among treatments were observed in pH values, with MI values showing a similar trend to that mentioned for TA values, with non-significant differences among T0, T1, T2 and T4, but with a T3 value lower than in T0, and similar to those of the other treatments (Table 4).

TPC progressively decreased as the number of water withholding days increased (Table 5), reaching minimum values in T2, T3, and T4 fruits (1945, 1534, and 1589 mg GAE L-1, respectively, with no significant differences among these three treatments). In contrast, withholding water did not affect the TAA values, while its effects on TAC, α-

Table 4
Effect of irrigation treatments on pomegranate juice total soluble solids (TSS, °Brix), titrable acidity (TA, g citric acid L^{-1}), pH and maturity index (MI, TSS/TA).

Treatment	TSS	TA	pH	MI
T0	17.8a	2.17ab	4.9a	82.2a
T1	17.1bc	2.11b	4.8a	81.3ab
T2	17.1c	2.15ab	4.9a	79.6ab
T3	17.2bc	2.23a	4.7a	77.1b
T4	17.5ab	2.16ab	4.7a	81.2ab

Means within a column that do not have a common letter are significantly different by LSD0.05 test.

Table 5
Effect of irrigation treatments on pomegranate juice total polyphenols content (TPC, mg GAE L^{-1}), total anthocyanin content (TAC, mg L^{-1}), total antioxidant activity (TAA, mM Trolox), α- punicalagin, β-punicalagin, and ellagic acid (mg L^{-1}).

Treatment	TPC	TAC	TAA	α-punicalagin	β-punicalagin	Ellagic acid
T0	3133a	69.5b	12.1a	168.7ab	164.5b	19.0b
T1	2681b	123.1a	13.3a	184.2a	174.2a	19.6a
T2	1945c	76.1b	13.3a	169.5ab	172.0a	19.5a
T3	1534c	75.1b	11.9a	162.2b	170.2ab	19.5a
T4	1589c	75.1b	12.0a	157.7b	168.7ab	19.3ab

Means within a column that do not have a common letter are significantly different by LSD0.05 test.

punicalagin β-punicalagin and ellagic acid were not very pronounced, inducing (i) a significant TAC increase only in T1 fruits (123.1 mM Trolox), (ii) a slight but significant ellagic acid increase in fruits under water stress, although no significant differences among T1, T2, T3, and T4 (19.6, 19.5, 19.5 and 19.3 mg L^{-1}) were observed, and the value in T4 fruits (19.3 mg L^{-1}) was also similar to that in T0 fruits (19.0 mg L^{-1}), (iii) no significant differences in α-punicalagin values among treatments, except in T1 fruits (184.2 mg L^{-1}), which showed higher values than T3 and T4 (162.2 and 157.7 mg L^{-1}), but similar values to those in T0 and T2 (168.7 and 169.5 mg L^{-1}), and (iv) a significant increase in β-punicalagin in T1 and T2 fruits, which reached 172.0 and 174.2 mg L^{-1}, respectively (Table 5).

4. Discussion

A detailed analysis of the effects of the irrigation water withholding treatments on plant and fruit water status were described in a previous manuscript from our research network (Galindo et al., 2014b). Bearing in mind the results from that article and those shown in Table 1, it is clear that, in spite of the rainfall events (occurring on DOY 271), the cumulative water stress tended to increase with the number of days irrigation was withheld, the treatments in which irrigation was withheld during late fruit ripening (T1) and during the second half of fruit ripening (T2) producing a similar and moderate water stress level and a more pronounced water stress level being observed in the treatments in which irrigation was withheld during ripening (T3) and at the end of fruit growth and ripening (T4). In addition, it is important to highlight the fact that SI_{gleaf}, $SI_{\Psi stem}$, $SI_{\Psi leaf}$ and $SI_{\Psi fruit}$ values showed some differences in describing the cumulative water deficit reached by the plants. $SI_{\Psi fruit}$ was the most reliable indicator to detect differences between the treatments at moderate water stress (T0 and T2), while SI_{gleaf} was the only indicator able to detect differences between the treatments at more pronounced water stress levels (T3 and T4).

The decrease in fruit yield in water stressed plants during the second half of fruit ripening (T2) and during fruit ripening (T3) (Table 2) confirmed the hypothesis that fruit ripening is a critical period from the yield point of view (Intrigliolo et al., 2013; Laribi et al., 2013). However, the fact that plants that were water stressed only at fruit late ripening stage (T1) showed similar marketable yield and fruit size to fully irrigated plants (T0) mean to clarify some aspects of the concept of phenological critical period (Goldhamer, 1989; Naor, 2006). In this sense, it is probable that sensitivity to water stress during a given critical phenological period is not constant and/or it is necessary to exceed a certain level of water stress to achieve adverse effects on productivity during a critical period. Whatever the case, although pomegranate trees are able to withstand severe drought conditions (Rodríguez et al., 2012; Galindo et al., 2014b), irrigation was essential during most of the ripening stage to achieve optimum yield. According to Galindo et al. (2014b), the decrease in the marketable yield in T2, T3 and T4 plants was due mainly to the incidence of the fruit cracking and/or fruit splitting disorders and to the decrease in fruit size, which can be attributed to a loss of fruit turgor, because a direct relation between turgor and growth has been found in many studies (Serpe and Matthews, 2000; Matthews and Shackel, 2005).

In agreement with the results reported by Laribi et al. (2013) in pomegranate and Collado-González et al. (2014) in jujube fruits, withholding irrigation water during the ripening phase increases redness and darkness of the fruit peel (Table 3). In this sense, the absence of data for peel pigments prevents any conclusion concerning whether the changes in peel colour were due to anthocyanin accumulation. Nevertheless, a negative correlation between lightness and pigment content is known, because as pigment levels increase, more light is absorbed, and lower values of luminosity are recorded. Moreover, considering that fruit peel from T1 plants, in which irrigation was withheld during late ripening for only 6 days, was also redder and darker than in fruits from T0 (Table 3), it is possible to rule out higher fruit exposure to sun-light as the only cause of colour changes (Gelly et al., 2004) because a significant reduction in the canopy characteristics is not very likely in only 6 days.

It is important to take remember that the first pomegranate fruits reaching the market fetch higher prices and, in this sense, 'Mollar de Elche' cultivar is often harvested when the peel has a sufficient red colouratiing (Manera et al., 2013). The significant increase in juice colour from T3 and T4 fruits (Table 3) is also very interesting for producers because pomegranate fruit attractiveness is primarily related to colour and taste parameters of the arils and their juice (Borochov-Neori et al., 2009). However, despite the fact that pomegranate colouration in pomegranates is predominantly due to anthocyanins (Shulman et al., 1984), TAC levels in T3 and T4 fruits were similar to that observed in T0 fruits (Table 5). Laribi et al. (2013) showed also similar behaviour in juice from trees submitted to severe water restrictions during the last part of fruit growth and ripening period.

The fact that (i) TAC juice levels increased only in T1 fruits, (ii) TAA levels were similar in juices from the different irrigation treatments, and (iii) redness significantly increased only in T3 and T4 fruit juices (Tables 3 and 5), confirmed the view that juice antioxidant capacity is not linearly correlated with the red colour intensity, meaning that the anthocyanins are not major contributors to the antioxidant capacity exhibited by the pomegranates and their juice (Borochov-Neori et al., 2009). Moreover, the fact that withholding water irrigation decreased TPC levels and did not affect TAA levels (Table 5) does not agree with the linear relationship between soluble phenolic levels and antioxidant capacity indicated by Borochov-Neori et al. (2009), who supported the idea that phenolic compounds are the main contributors to the antioxidant activity in pomegranate juice. In this sense, further analysis of fatty acids (Alcaraz-Mármol et al., 2015) and organic acids (Calín-Sánchez et al., 2013) must be conducted to fully understand the antioxidant capacity and bioactivity of pomegranate fruits subjected to deficit irrigation strategies.

The behaviour observed in TSS, TA, pH and MI juice values (Table 4) in response to irrigation withholding was not very clear and showed certain differences with respect to the results indicated by other authors in similar experiments. To be precise, Mellisho et al. (2012) indicated that arils from fruits exposed to water deficit during the second half of the linear fruit growth phase showed very similar overall chemical characteristics to arils from fully irrigated fruits, and Labiri et al. (2013) found a significant increase in TSS and TA levels in pomegranate juice from plants exposed to water deficit during the final

phase of fruit growth and ripening. Additionally, when the effect of SDI applied throughout the season on pomegranate fruit quality is considered, the results at first sight are ambiguous. Mena et al. (2013) indicated that an SDI strategy that induced severe water stress led to pomegranate juice of lower visual attractiveness and less healthy (more yellowish, lower antioxidant activity and lower total phenolic compound, punicalagin and total anthocyanin contents) than the juice from fully irrigated trees. In contrast, Galindo et al. (2014a) concluded that SDI inducing severe water stress led to fruits with similar bioactive quality but a darker and more intense garnet colour than fully irrigated fruits, bringing the optimal harvest time by about 7–8 days. Also, Mellisho et al. (2012) showed that SDI inducing moderate water stress throughout the season led to changes in colour and chemical characteristics, which reflected earlier ripening.

In this sense, it is well known that water stress influences the content of secondary metabolites in plant tissues, having also contradictory results in other crops. For example, Chaves et al. (2007) reported the substantial accumulation of anthocyanins in grape berries under water stress. In contrast, Kennedy et al. (2000, 2002) showed that osmotic stress had little or no effect on anthocyanin accumulation in grape berries. This, at first sight, confusing relation between water stress and the production of bioactive compounds could be attributed to the fact that most manuscripts are not meticulous when it comes to recording aspects of plant water stress (precise phenological period at which it takes place, water stress rate of development, duration of maximum water stress, incidence of partial recoveries and other aspects) although such information is essential for the characterisation of experimental water stress conditions. In addition, it is essential to underline that is not possible to establish a linear correlation between water stress and secondary metabolite contents (Mattsson and Haack, 1987; Gobbo-Neto and Lopes, 2007). For this reason, Horner (1990) proposed a quadratic model to predict the concentration of phenolic compounds as a function of plant water status. So, under a mild water stress, CO_2 assimilation could be maintained and carbon-based secondary metabolites will probably increase when carbohydrates exceed the amount required for growth. Thus, mild osmotic stress may lead to a reduction in plant growth, accompanied by an increasing concentration of non-nitrogenous secondary metabolites. When water stress increases, stomatal regulation takes place and CO_2 assimilation is reduced. In this situation, carbon will be preferentially allocated to the synthesis of primary metabolites to the detriment of the synthesis of secondary metabolites (Mellisho et al., 2012).

5. Conclusion

The present results indicated that the SI calculated from g_{leaf}, Ψ_{leaf}, Ψ_{stem} and Ψ_{fruit} data vary as regards their ability to describe the cumulative water deficit reached by plants. $SI_{\Psi fruit}$ was the most feasible indicator for detecting differences between the treatments at moderate water stress levels while SI_{gleaf} was the only indicator able to detect differences between the treatments at higher water stress levels. Moreover, pomegranate fruit ripening is a critical period from the yield point of view because irrigation is essential during most of this phenological period if maximum yields are to be achieved. Nevertheless, the fact that a very short irrigation restriction period (around 6 days) at the end of ripening bring the harvest time forward and so increase pomegranate fruit price, saves irrigation water and enhances the bioactive compound content (anthocyanin, phenolic compounds, punicalagin and ellagic acid) without affecting marketable yield and fruit size suggests that the sensitivity to water stress during a given critical phenological period is not constant and/or it is necessary to exceed a certain level of water stress to achieve adverse effects on productivity during a critical period. Moreover, the increase in fruits colouration as a result of water stress during fruit ripening may be considered as an interesting aspect because the appeal of pomegranate fruit is directly associated with colour. In spite of this, it is important to note that a very short irrigation restriction (around 6 days) at the end of the ripening period advances the harvest time, increases pomegranate fruit price, saves irrigation water and enhances the bioactive compound contents (anthocyanin, phenolic compounds, punicalagin and ellagic acid). Finally, the results confirmed the hypothesis that there is no a linear correlation between pomegranate water stress and secondary metabolite contents, because mild water stress may lead to a reduction in plant growth and a higher concentration of secondary carbon metabolites, whereas under a more pronounced water stress carbon are preferentially allocated to the synthesis of primary metabolites to the detriment of secondary metabolites.

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