



Article

Firmness and Cell Wall Changes during Maturation of ‘Arbequina’ Olive Fruit: The Impact of Irrigation

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Abstract: The olive tree (*Olea europaea* L.) has been cultivated around the Mediterranean basin since ancient times, ‘Arbequina’ being one of the most widely grown varieties. To improve the knowledge on ripening-related firmness changes in olive fruit, cell wall metabolism was studied in irrigated and rain-fed ‘Arbequina’ olives grown at ‘Les Garrigues’, a Protected Designation of Origin (PDO) in Catalonia (NE Spain) where harsh environmental conditions occur during fruit development. Fruit samples were picked periodically from September to January. Time-course dynamics of firmness loss during maturation were characterised by a first phase of rapid firmness loss followed by a second phase of moderate change. Compositional changes in cell walls and related enzyme activities were studied in fruit samples. Fruit firmness was significantly higher in rain-fed than in irrigated olives. Neutral sugar loss, an early event in ripening-related cell wall modifications, was lower in rain-fed samples, which, moreover, retained higher uronic acid amounts in the chelator-soluble fraction, thus resulting in attenuated firmness loss in these fruits.

Keywords: ‘Arbequina’; cell wall; firmness; fruit ripening; irrigation; olive



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“*Olea prima arborum omnium est*”.
Lucius Iunius Moderatus, a.k.a. *Columella*
De Arboribus

1. Introduction

The olive (*Olea europaea* L.) tree has been farmed at the Mediterranean and Asia Minor areas for thousands of years [1]. Nowadays, olive trees are cultivated in other areas of the world as well, such as southern Africa, Australia, California, Japan, China and Argentina, even though Mediterranean countries remain the strongest olive producers. The largest part of olive production (90% approximately) is intended for the oil industry and the rest is devoted to the manufacture of table olives. ‘Arbequina’ is one of the most important varieties in Spain, and its fruits are used for both purposes. The tree has low vigour, which makes it suitable for super-high-density orchards [2] and for the implementation of mechanical harvesting procedures, with the consequent reduction in production costs.

The mechanical properties of fruits have an impact on textural attributes, eating quality and consumers’ perceptions, as well as on the susceptibility to rots, infestations and bruises, as well as on the efficiency of oil extraction. Fruits typically undergo noticeable softening along the ripening process, leading to textural modifications. Fruit softening results largely from changes in cells walls and middle lamellae, driven by a plethora of pectolytic and non-pectolytic proteins [3] and resulting in the solubilisation of cell wall polysaccharides. In many fruit species, including olive, polysaccharide depolymerisation also occurs [4,5]. Non-enzymatic factors, such as ascorbic acid (AA) and its derivatives, may also contribute to the oxidative disassembly of cell wall polymers in some fruit species [6–9].

Cell wall composition changes during olive fruit ripening have received limited attention. Cell wall materials solubilised extensively along the ripening of ‘Koroneiki’ olives [10],

and ripening-related fruit softening was reportedly associated with noticeable arabinose losses in ‘Hojiblanca’ [11] and ‘Negrinha do Douro’ [12]. Cell-wall-related enzyme activities and cell wall gene expression levels increased progressively during maturation in ‘Hojiblanca’ [13] and ‘Picual’ [14] olives. In agreement with those previous reports, the removal of neutral sugars from pectins during the maturation of ‘Arbequina’ fruits was related to increased α -L-arabinofuranosidase (AFase) activity [15]. This observation was confirmed in a recent study [16] on ‘Arbequina’ and eight additional olive cultivars (‘Argudell’, ‘Empeltre’, ‘Farga’, ‘Manzanilla’, ‘Marfil’, ‘Morrut’, ‘Picual’ and ‘Sevillena’). Insoluble cell wall materials decreased during fruit ripening, and the concomitant decline in fruit firmness was associated to higher ascorbate content and AFase and β -galactosidase (β -Gal) activities, leading to important losses of neutral sugars.

Olive trees are often cultivated in harsh environmental conditions, such as low water availability in combination with high temperatures and UV irradiation. Irrigation helps alleviating these stress factors, but it may also impact fruit metabolism and quality attributes. The influence of water availability on olive crops has been studied in regard to oil yield and quality [17–19] and phenolic content [20,21], but we are not aware of any published study on the impact of irrigation on changes in cell wall metabolism along olive ripening, in spite of their relevance to the mechanical properties of fruit. In this work, therefore, cell wall modifications were assessed during the ripening of ‘Arbequina’ olives grown under irrigated and rain-fed conditions.

2. Materials and Methods

2.1. Plant Material and Assessment of Fruit Firmness

‘Arbequina’ olives were hand-collected at a commercial grove placed at El Soleràs (41°24’N; 0°40’E; altitude 450 m), located within the geographical area covered by the Protected Designation of Origin (PDO) ‘Les Garrigues’ (Catalonia, NE Spain) and submitted to the usual cultural procedures at that producing region. The geographical area is characterised by dry, continental Mediterranean climate. Total annual rainfall in 2017 was 318 mm, and took place mainly in March (78 mm), June (47 mm) and September (48 mm), with extremely dry July and August (Figure 1).

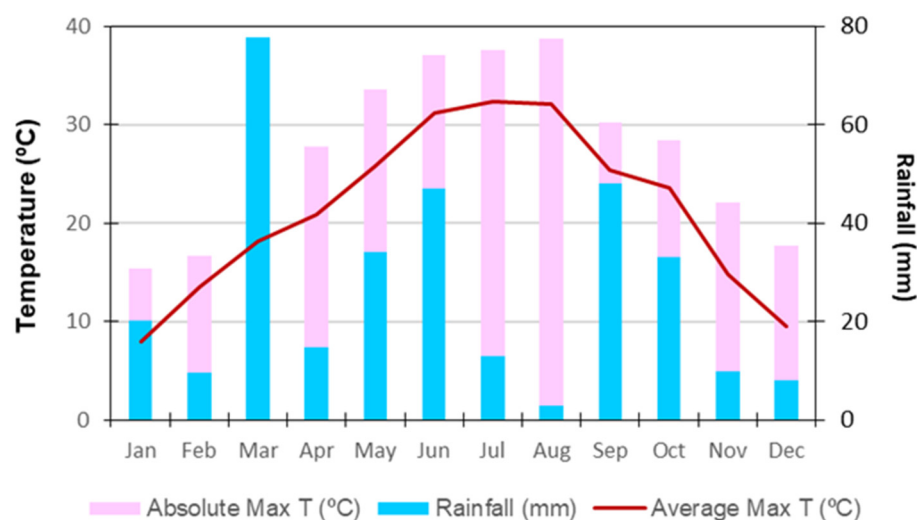


Figure 1. Rainfall and maximal (absolute and average) temperatures at the growing site (El Soleràs; 41°24’N; 0°40’E; altitude 450 m) in 2017.

Fruit samples were harvested from either rain-fed trees or from trees supplied with drip irrigation ($1.01 \text{ L m}^{-2} \text{ day}^{-1}$, corresponding to 100% of daily crop evapotranspiration estimated for the area during the irrigation period). Irrigation period was April to October, according to the usual practice at the PDO. Olives were picked periodically from

September 2017 to January 2018. Samples were coded P1-P8, corresponding to successive picking dates.

Fruit firmness was determined as the maximum strength (N) required to achieve surface breakage in a penetration test, using a 1 mm diameter cylindrical probe descending at 1 mm s^{-1} . Ten olives per sampling date and irrigation regime were assessed individually using a TA-TX2 texture analyser (Stable Micro Systems, Goldaming, U.K.).

2.2. Extraction, Fractionation and Analysis of Cell Wall Materials

After stone removal, cell wall materials were extracted as the alcohol-insoluble residue (AIR) ([22]) from 50 g fruit per sampling date and irrigation regime. Samples were blended in ethanol (80%, *v/v*) to obtain a 10% (*w/v*) suspension, heated (20 min at 80°C), cooled down to room temperature, and filtered through Miracloth® (Merck Life Science S.L.U., Madrid, Spain). The solid residue was re-extracted twice more in 80% (*v/v*) ethanol, once in 96% (*v/v*) ethanol and once in acetone, and the slurry filtered through Miracloth® each time. After drying at 50°C , the AIR was stored at -20°C until fractionation. AIR yields were expressed as $\text{g } 100 \text{ g}^{-1}$ fresh weight (FW).

For the fractionation of AIR samples (0.5 g), a modification of a previous procedure [23] was used as described in [16]. Samples were extracted sequentially in distilled water, 0.1% (*w/v*) sodium oxalate (pH 5.6), 0.05 mol L^{-1} sodium carbonate and 4 mol L^{-1} potassium hydroxide to recover the water-, sodium oxalate-, sodium carbonate- and potassium hydroxide-soluble fractions (W_{sf} , NaOx_{sf} , $\text{Na}_2\text{CO}_{3\text{sf}}$ and KOH_{sf} , respectively). The supernatants of each fractionation step were concentrated in a rotary evaporator and precipitated with ethanol (96%, *v/v*). The precipitates were washed three times in ethanol (96%, *v/v*), dried completely at 50°C , and weighed. All the extractions were performed in triplicate, and yields expressed as $\text{g } 100 \text{ g}^{-1}$ AIR.

Total sugar and uronic acid contents in each recovered fraction were determined by the phenol-sulfuric acid assay [24] and the m-hydroxyphenyl method [25], respectively. For the estimation of neutral sugar amounts, uronic acid content was subtracted from that of total sugars. Analyses were done in triplicate, and data were expressed as $\text{g } 100 \text{ g}^{-1}$ fraction.

The procedure for the analysis of the degree of methyl esterification (d.e.) of pectins was based on [26]. Briefly, AIR samples (15 mg) were shaken in 1 mol L^{-1} KOH (2 h at room temperature) to remove the methyl groups. Released methanol was then oxidised enzymatically in the presence of alcohol oxidase and, after incubating the samples with 0.02 mol L^{-1} pentane-2,4-dione (2 h at 60°C), the absorbance at 412 nm was read. Analyses were carried out in triplicate, and results given as the molar ratio (%) of methanol to uronic acid content.

2.3. Cell Wall-Related Enzyme Activities

The assays of cell wall-related enzyme activities were undertaken on crude extracts obtained from samples (100 mg) of acetone powder (AP) prepared from fruit pericarp as described in [13], with slight modifications. In short, destoned fresh olives (60–80 g) were homogenised in cold acetone (10% (*w/v*) suspension). After filtration, the solid was washed three more times in acetone, dried at room temperature and kept at -20°C until the activity assays. The extraction buffers and activity assays for pectin methylesterase (PME; EC 3.1.1.11), polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.1.2.15), pectate lyase (PL; EC 4.2.2.2), α -L-arabinofuranosidase (AFase; EC 3.2.1.55), β -galactosidase (β -Gal; EC 3.2.1.23), endo-1,4- β -D-glucanase (EGase; EC 3.2.1.4) and β -xylosidase (β -Xyl; EC 3.2.1.37) were as described elsewhere [27]. Total protein content in the extracts was estimated by the Bradford method [28], with bovine serum albumin (BSA) as the standard. Results were given as specific activity (U mg protein^{-1}).

2.4. Antioxidant Properties

Fifty olives per sampling date and irrigation regime were disinfected in 1% (*v/v*) Triton X-100, rinsed with deionised water and pitted. Samples were then lyophilised, milled and stored at -80°C until analysis. For the determination of radical scavenging activity (RSA), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used, which expresses the antioxidant capacity as the percentage of DPPH reduction in sample extracts in comparison with a control (DPPH without sample). Total phenolics were extracted in methanol solution (80%, *v/v*) and determined as mg gallic acid equivalents g^{-1} dry weight (DW) by the colorimetric Folin–Ciocalteu assay. Anthocyanins were extracted in methanol-HCl-water (50:1:49, *v/v/v*) and estimated spectrophotometrically as mg cyanidin equivalents g^{-1} DW. All procedures were carried out as in [29].

Total (TAA) and reduced (AA) ascorbic acid were estimated colorimetrically [30]. Dehydroascorbic acid (DHA) content was calculated as the difference between TAA and AA. Results were expressed as nmol g^{-1} DW.

2.5. Statistical Analysis

Multifactorial analysis of variance (ANOVA) and the LSD test ($p \leq 0.05$) were used to separate the means, with sampling date and irrigation regime as the factors, using the JMP[®] Pro 13 software. In order to aid in the interpretation of results, partial least square regression (PLSR) was used as a predictive method to relate fruit firmness (*Y*-variable) to a set of potentially explanatory variables (*X*). Data were weighed previously by the inverse of the standard deviation of each variable to prevent dependence on the measuring units, and full cross-validation was run as a validation procedure. PLSR models were developed with the Unscrambler software (version 9.1.2, CAMO ASA, Oslo, Norway).

3. Results

3.1. Physical and Chemical Characteristics

Some morphological and physical characteristics of olive fruits are shown. Fruit weight, size and flesh-to-stone (F:S) ratio were higher for irrigated than for rain-fed olives (Table 1). Substantial firmness loss was found during fruit maturation (Figure 2). Fruit firmness levels were significantly higher in rain-fed than in irrigated olives for most of the sampling period, picking dates spanning mid-to-late October being the only exception. For rain-fed samples, firmness decreased sharply over the first four sampling points and showed limited variation thereafter (November to January). The same trend was found for irrigated fruits, but the accentuated phase of firmness loss lasted longer, up to mid-November. Higher firmness in fruit from non-irrigated plants, particularly during the latter part of the experimental period, may be related partially to lower humidity in these fruits (Figure 3). Water content in rain-fed fruits decreased steadily after October, in parallel to weight loss (Table 1), but also in irrigated fruits after the end of the irrigation period (late October). Even so, it was significantly lower in fruits from rain-fed plants until January, when no significant differences were found. Overall firmness loss along the experimental period amounted, respectively, to 76.5 and 63.0% for irrigated and rain-fed fruits.

Table 1. Physical and chemical characteristics during fruit ripening of irrigated and non-irrigated ‘Arbequina’ olives.

Picking	Date	Irrigation Regime	Weight (g)		Length (mm)		Diameter (mm)		F:S Ratio *		Anthocyanin (mg g ⁻¹ DW)		Phenols (mg g ⁻¹ DW)		RSA * (%)		AA * (nmol g ⁻¹ DW)		DHA * (nmol g ⁻¹ DW)	
1	Sep 18	Irrigated	1.23	e A	14.18	d A	12.66	f A	3.80	c A	0.4	e A	19.8	a B	95.0	a A	0.10	bc B	0.15	a B
2	Oct 2		1.62	cd A	15.41	bc A	14.00	cde A	4.57	b A	0.4	de A	11.1	d B	83.7	b A	0.10	bc A	0.13	ab A
3	Oct 16		1.81	bc A	16.01	a A	14.77	a A	5.55	a A	0.3	e B	16.0	b B	95.5	a A	0.07	d B	0.06	d B
4	Oct 30		1.86	ab A	15.60	abc A	14.08	cd A	5.58	a A	0.6	cd B	13.0	c B	80.7	bc B	0.11	b A	0.08	c B
5	Nov 13		2.03	a A	15.90	ab A	14.52	ab A	4.69	b A	0.7	c B	11.3	d B	72.6	c B	0.11	b A	0.05	de B
6	Nov 28		1.84	ab A	15.48	bc A	14.21	bc A	4.46	b A	0.8	bc A	9.4	e B	38.0	e B	0.09	c A	0.04	e B
7	Dec 11		1.58	d A	15.20	c A	13.68	de A	4.55	b A	1.0	b A	14.4	bc B	53.7	d A	0.10	bc A	0.06	d B
8	Jan 15		1.59	d A	15.40	c A	13.61	e A	3.61	c A	3.1	a A	14.5	bc A	74.1	c A	0.16	a B	0.12	b A
1	Sep 18	Non-irrigated	1.08	d B	12.90	c B	11.63	e B	3.19	e B	0.2	f B	25.3	a A	97.0	a A	0.13	b A	0.21	a A
2	Oct 2		1.38	b A	13.54	b B	12.12	cd B	3.66	cd B	0.2	ef B	17.0	bc A	94.0	a A	0.07	de B	0.10	d A
3	Oct 16		1.23	c B	13.32	bc B	11.86	de B	3.63	cd B	0.4	de A	24.5	a A	88.9	ab A	0.08	d A	0.09	d A
4	Oct 30		1.42	b B	13.73	b B	12.53	bc B	4.48	a B	1.0	b A	24.2	a A	96.3	a A	0.13	b A	0.15	b A
5	Nov 13		1.52	a B	14.59	a B	12.96	ab B	3.86	bc A	1.0	b A	19.2	b A	82.1	bc A	0.11	c A	0.10	d A
6	Nov 28		1.28	c B	14.49	a B	12.69	ab B	3.41	de B	0.4	d B	16.4	bc A	58.7	e A	0.06	e B	0.07	e A
7	Dec 11		1.56	a A	14.40	a B	12.59	abc B	4.02	b B	0.8	c A	18.2	b A	66.4	de A	0.10	c A	0.10	d A
8	Jan 15		1.54	a A	14.30	a B	13.04	a A	3.46	de A	2.9	a A	14.1	c A	73.7	cd A	0.19	a A	0.12	c A

Length and diameter data represent means of 10 individual fruits. Weight and F:S ratio correspond to means of two 10-fruit replicates. For chemical properties, values represent means of three replicate analyses undertaken on lyophilised pericarp tissue. Different capital letters denote significant differences between irrigated and rain-fed samples for a given picking date, and different lower-case letters stand for significant differences among sampling dates for a given irrigation regime, at $p \leq 0.05$ (LSD test). * Abbreviations: F:S ratio, flesh-to-stone ratio; RSA, radical-scavenging capacity; AA, reduced acid ascorbic; DHA, dehydroascorbic acid.

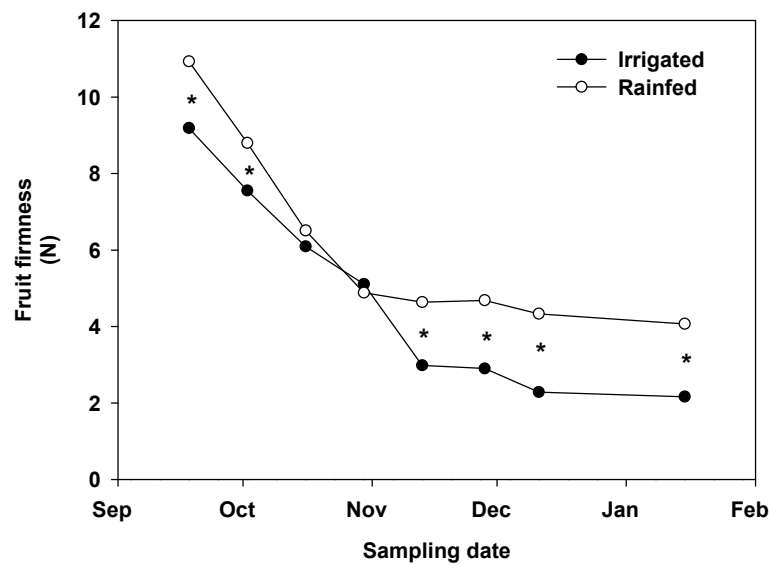


Figure 2. Fruit firmness of ‘Arbequina’ olives during on-tree ripening under irrigated and rain-fed conditions. Values represent means of ten individual fruits. Asterisks stand for significant differences between irrigated and non-irrigated trees at $p \leq 0.05$ (LSD test).

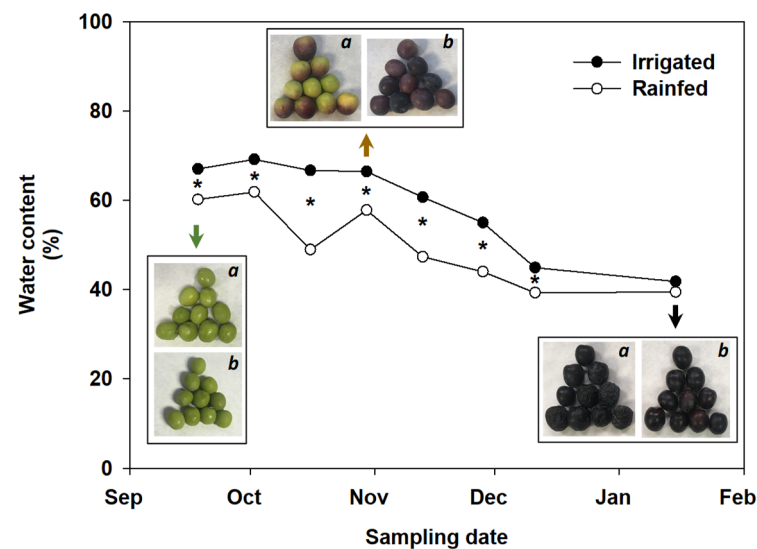


Figure 3. Water content (%) and appearance of ‘Arbequina’ olives during on-tree ripening under irrigated and rain-fed conditions. Letters (a) and (b) on the images identify fruits from irrigated and non-irrigated trees, respectively. Water content values represent the average of 3 replicates (10 fruits/replicate). Asterisks stand for significant differences between irrigated and non-irrigated trees at $p \leq 0.05$ (LSD test).

Some antioxidant properties were also analysed throughout fruit maturation (Table 1). Anthocyanin content increased, reflecting the ripening-related shift in skin colour. The amount of total phenolics was significantly higher in rain-fed than in irrigated olives. This observation might relate to environmental stress possibly imposed by lower water availability on rain-fed trees, as suggested in previous studies [20,21] on phenolic compounds in olive tree leaves under drought stress conditions. In agreement, higher anthocyanin levels were found in rain-fed than in irrigated fruits at P3–P5, concomitantly with decreased rainfall and the concurrence of high temperatures at the producing area (Figure 1), and colour change in rain-fed fruits took place earlier (Figure 3). Phenolic contents generally declined over maturation, as observed in a previous study [16], even though a later increase

was observed at P7 regardless of irrigation. Time-course changes in RSA paralleled those in the content of total phenolics and of ascorbic acid (AA), consistent with the antioxidant properties of these compounds. Significantly higher RSA values were found in rain-fed than in irrigated fruits at P4–P6 (November), coincident with a noticeable drop in rainfall in comparison with the preceding months (Figure 1). No clear differences in AA content were observed between irrigated and rain-fed samples, but significantly higher DHA levels were found in rain-fed than in irrigated fruits (Table 1). In contrast with the observations for ‘Arbequina’ described herein, both the content of phenolics and the antioxidant capacity increased along fruit ripening of ‘Dhokar’ and ‘Chemlali’ olives [31]. These discrepancies, however, agree with substantial cultivar-related differences in the evolution of total phenols, RSA and AA content, which were found in a previous study spanning nine olive genotypes [16].

3.2. Cell Wall Composition and Ripening-Related Changes

In order to investigate the mechanisms underlying differences in firmness levels and in firmness loss along fruit ripening between irrigated and rain-fed olives (Figure 2), ripening-related changes in cell walls were examined. It has been reported that firmness loss along fruit maturation of ‘Arbequina’ olives is accompanied by the progressive solubilisation of cell wall polysaccharides [16]. In that work, though, the sampling period was shorter, as the last picking of ‘Arbequina’ olives took place in late November. In contrast, a wider sampling period was considered in the present study, fruit samples being taken and analysed up to January (Table 1). Time-course changes in AIR amounts over the experimental time were similar for irrigated and rain-fed samples, even though yields were generally higher in the latter. AIR yields declined along maturation until late October (Table 2), in parallel with the phase of steady firmness loss observed during the first sampling points (Figure 2). Contrarily, AIR yields increased thereafter, due to a substantial decrease in water content (Figure 3) leading to higher AIR percentages over fresh weight.

Limited differences in AIR fraction yields were found between irrigated and rain-fed samples. Decreased AIR amounts over the first sampling dates (P1–P4) were accompanied by higher yields of the water-soluble fraction (W_{sf}) of cell wall materials and lower recoveries of the final insoluble residue (Table 2), indicative of gradual solubilisation of cell wall polysaccharides, which contributed to substantial loss of fruit firmness (Figure 2). For later sampling dates (P5 to P8), W_{sf} yields were even higher, particularly for irrigated fruit, which showed significantly higher values as compared to rain-fed olives. Contrarily, a decreasing trend was observed for the rest of AIR fractions isolated, particularly for the Na_2CO_3 - and the KOH-soluble fractions, consistent with the idea of sustained solubilisation of cell wall materials along fruit ripening.

The different cell wall fractions isolated were then analysed for the content of uronic acids and neutral sugars. A steady decline in neutral sugar contents in the alcohol-insoluble materials was observed during the ripening of rain-fed olives (Table 3), while in contrast limited changes were found for irrigated samples, with the exception of a transient peak at the P4 sampling (end of October). The results suggest that rapid fruit softening over the first sampling dates (P1 to P4) was linked to loss of neutral sugars (Table 3): increasing neutral sugar contents were observed in the W_{sf} in parallel to lowered levels in both the Na_2CO_3 - and the KOH-soluble fractions isolated from AIR. This correlation was particularly significant for irrigated samples, for which the initial phase of sharp firmness loss was more accentuated and lasted longer, up to the P5 stage (Figure 2): correlation coefficients between neutral sugar content in the W_{sf} and those in the Na_2CO_{sf} and the KOH_{sf} were -0.76 and -0.79 , respectively, during that period. Rain-fed samples, which incidentally showed higher firmness levels (Figure 2), also retained higher neutral sugar contents in AIR during the same period.

Table 2. Yield of alcohol-insoluble residue (AIR) and AIR fractions recovered during fruit ripening of irrigated and non-irrigated ‘Arbequina’ olives.

Picking	Date	Irrigation Regime	AIR (g 100 g ⁻¹ FW)	d.e. (%)	AIR Fractions (g 100 g ⁻¹ AIR)										
					W _{sf} *		NaOx _{sf} *		Na ₂ CO _{3sf} *		KOH _{sf} *		Insoluble Residue		
1	Sep 18	Irrigated	9.79	75.26	b A	1.52	ef A	7.89	c B	1.33	d B	1.53	bc B	87.7	a A
2	Oct 2		5.78	61.41	c A	0.86	f B	7.91	c A	3.52	b A	1.20	bc A	86.5	b A
3	Oct 16		6.05	56.09	cd A	5.31	ab A	8.76	a A	3.98	a A	3.58	a A	78.3	e B
4	Oct 30		4.85	50.57	d A	3.85	c A	8.64	ab A	2.59	c A	2.99	a A	82.1	d B
5	Nov 13		9.25	88.43	a A	2.06	de B	8.64	ab A	1.13	d B	1.66	b A	86.6	b A
6	Nov 28		13.19	84.73	ab A	2.46	d A	8.31	bc A	1.00	d B	0.93	c A	87.3	ab A
7	Dec 11		13.87	49.54	de A	5.45	a A	8.11	c A	1.26	d A	0.93	c A	84.2	c B
8	Jan 15		14.50	39.88	e A	4.59	bc A	8.11	c A	1.40	d A	1.40	bc A	84.5	c B
1	Sep 18	Non-irrigated	12.00	58.85	bc A	1.59	d A	8.37	ab A	1.99	c A	2.92	a A	85.2	b B
2	Oct 2		8.82	57.88	bc A	1.33	d A	7.85	bc A	3.32	a A	1.33	bc A	86.2	b A
3	Oct 16		8.21	69.49	b A	3.86	a A	8.18	abc A	2.79	b B	3.26	a A	81.9	c A
4	Oct 30		8.57	42.67	d A	2.79	b B	8.63	a A	1.79	c B	1.53	b B	85.3	b A
5	Nov 13		9.42	92.43	a A	3.79	a A	8.26	abc A	1.73	c A	0.93	cd B	85.4	b A
6	Nov 28		11.81	90.08	a A	3.13	b A	8.59	a A	2.00	c A	1.07	bcd A	85.3	b A
7	Dec 11		16.15	53.42	cd A	1.33	d B	8.42	ab A	0.93	d A	0.80	cd A	88.5	a A
8	Jan 15		16.06	21.12	e B	2.12	c B	7.70	c B	1.20	d A	0.66	d B	88.3	a A

Alcohol-insoluble residue (AIR) was extracted from approximately 50 g olive fruit pericarp. Fraction yield values represent means of three extraction replicates. Different capital letters denote significant differences between irrigated and rain-fed samples for a given picking date, and different lower-case letters stand for significant differences among sampling dates for a given irrigation regime, at $p \leq 0.05$ (LSD test). * Abbreviations: AIR, alcohol-insoluble residue; d.e., degree of methyl esterification of pectins; W_{sf}, water-soluble fraction; NaOx_{sf}, sodium oxalate-soluble fraction; Na₂CO_{3sf}, sodium carbonate-soluble fraction; KOH_{sf}, potassium hydroxide-soluble fraction.

Table 3. Uronic acid and neutral sugar contents in AIR and in AIR fractions recovered during fruit ripening of irrigated and non-irrigated ‘Arbequina’ olives.

Picking	Date	Irrigation Regime	Uronic Acids (g 100 ^{−1} g)										Neutral Sugars (g 100 ^{−1} g)							
			AIR		W _{sf}		NaOx _{sf}		Na ₂ CO _{3sf}		KOH _{sf}		AIR		W _{sf}		Na ₂ CO ₃		KOH _{sf}	
1	Sep 18	Irrigated	6.40	c A	11.45	b A	4.15	c A	20.77	c A	3.80	c A	3.53	b B	19.87	b A	3.82	de B	27.67	c B
2	Oct 2		11.16	b A	18.98	a A	4.20	c A	8.56	g B	5.62	a A	3.51	b B	14.24	d B	15.69	a A	35.62	a B
3	Oct 16		12.38	a A	8.61	c A	6.72	a A	11.88	f A	3.04	d A	3.32	b B	17.61	c A	9.52	b B	36.70	a A
4	Oct 30		10.61	b A	7.01	e B	6.22	b A	8.69	g B	4.06	c B	8.91	a A	18.62	bc B	15.20	a A	32.70	b B
5	Nov 13		4.60	d A	7.75	d A	3.44	d B	19.48	d A	4.93	b A	4.25	ab A	20.18	b A	4.58	d B	24.81	e B
6	Nov 28		4.61	d A	7.78	d A	2.52	e A	27.94	a A	4.00	c A	2.19	b A	13.65	d B	nd		20.26	f B
7	Dec 11		4.14	de A	11.45	b B	1.80	f B	21.76	b A	4.07	c B	2.91	b A	10.03	e A	1.93	e A	25.54	de A
8	Jan 15		3.24	e A	11.48	b A	2.01	f B	16.74	e A	4.70	b A	3.54	b A	54.12	a A	6.62	c B	26.87	cd A
1	Sep 18	Non-irrigated	6.73	b A	11.13	b A	2.91	c B	12.43	cd B	2.52	g B	6.50	b A	16.79	c B	12.78	ab A	35.14	b A
2	Oct 2		7.62	a B	8.04	c B	4.05	b A	10.72	e A	3.08	e B	10.76	a A	18.88	bc A	4.86	de B	40.64	a A
3	Oct 16		7.58	a B	7.98	c B	4.58	a B	11.67	de A	2.80	f B	10.63	a A	17.58	bc A	10.77	b A	29.88	c B
4	Oct 30		6.63	b B	7.50	cd A	4.20	b B	13.38	c A	6.21	b A	4.18	c A	20.97	a A	8.02	c B	34.65	b A
5	Nov 13		5.43	c A	7.58	cd A	4.06	b A	16.37	b B	5.29	c A	5.97	bc A	17.21	bc B	7.04	cd A	41.96	a A
6	Nov 28		5.01	c A	7.26	d A	2.34	d A	11.22	e B	3.31	d B	2.19	d A	18.10	bc A	14.16	a A	25.66	d A
7	Dec 11		3.47	d B	18.95	a A	2.87	c A	22.30	a A	8.65	a A	1.14	d B	11.70	e A	3.63	e A	25.25	d A
8	Jan 15		2.98	d A	11.46	b A	2.34	d A	16.97	b A	3.27	de B	1.05	d B	14.05	d B	11.58	ab A	19.82	e B

Values represent means of three replicates. Different capital letters denote significant differences between irrigated and rain-fed samples for a given picking date, and different lower-case letters stand for significant differences among sampling dates for a given irrigation regime, at $p \leq 0.05$ (LSD test). Abbreviations: AIR, alcohol-insoluble residue; W_{sf}, water-soluble fraction, NaOx_{sf}, oxalate-soluble fraction; Na₂CO_{3sf}, sodium carbonate-soluble fraction; KOH_{sf}, potassium hydroxide-soluble fraction; nd, non-detectable.

In contrast, data on uronic acid content suggest that uronic acid loss took place mainly at later maturity stages. Progressively reduced contents of uronic acids were found in AIR along fruit ripening (Table 3), in agreement with previous reports for ‘Arbequina’ as well as for other olive cultivars [10,15,16]. Whereas uronic acid loss was steady but moderate for rain-fed samples, a sharp decrease was observable for irrigated fruits from P5 sampling. At later picking dates (P5 to P8), rain-fed fruits retained significantly higher uronic acid levels in the NaOx-soluble fraction in comparison to those in irrigated samples. This fraction is enriched in pectins linked non-covalently to the cell walls, which might relate to the observation of higher firmness levels in comparison to irrigated olives (Figure 2).

3.3. Cell Wall-Modifying Enzyme Activities

Data gathered herein suggest that loss of neutral sugars be an early event during the ripening-related cell wall disassembly of ‘Arbequina’ olive fruits, which agrees with the observation that AFase activity was detectable at very early picking dates (Table 4). AFase cleaves arabinosyl residues from pectin side-chains, arabinose being the main neutral sugar component of olive fruit cell walls in quantitative terms [12]. AFase activity increased steadily along fruit maturation, and was generally higher in rain-fed than in irrigated samples, with the exception of the P6 sampling when no significant differences between both irrigation regimes were detected. Even though galactose is reportedly less abundant than arabinose in cell walls of olive fruit, β -Gal-catalysed cleavage of galactosyl residues may also contribute to the ripening-related loss of neutral sugars and their mobilization to the water-soluble fraction. β -Gal activity levels around 0.120 U mg^{-1} protein were detected already at P1 fruits irrespective of irrigation regime, which then declined to rise again from mid-October (Table 4). Interestingly, β -Gal activity levels were significantly higher in irrigated than in rain-fed fruits at more advanced maturity stages, which coincided with higher sugar loss as shown by superior yields of the water-soluble fraction (Table 2) and by higher neutral sugar content therein (Table 3). Accordingly, neutral sugar content in the NaCO_{sf} and the KOH_{sf} fractions were also lower in irrigated samples.

Enzyme activities acting on pectin side-chains are considered to favour the action of pectin backbone-acting enzymes by increasing cell wall porosity and hence facilitating access to their substrates. Yet, for PG- and PL-catalysed cleavage of galacturonic acid residues from cell wall pectins, previous demethylation of these residues is required, which is catalysed by pectin methyl esterases. The high PME activity levels (over 1000 U mg^{-1} protein) at the initial (P1-P2) picking dates (Table 4) agree with the idea of an early role in cell wall modifications leading to ripening-related firmness changes. This caused a decline in the degree of methyl esterification (d.e.) of pectins (Table 2), which may underlie the largely unchanged NaOx_{sf} yields (Table 2) and uronic acid contents in the NaOx_{sf} (Table 3) over the first sampling dates (P1 to P4): in the presence of calcium, PME action aids the establishment of inter- and intra-molecular calcium bridges between demethylated, negatively charged galacturonic acid residues in pectic polymers. This helps their retention in the cell wall [3], in contrast to the fate of neutral sugars. Even though no calcium content analyses were undertaken in this work, a recent study [16] reported around 1200 mg kg^{-1} DW in the pericarp of green ‘Arbequina’ fruit, which declined substantially at later maturity stages.

PME activity decreased noticeably at picking dates later than P2, with the exception of a transient increase during early December (P6-P7), which was preceded by a substantial upsurge in the degree of pectin esterification at P5-P6 (Table 2). Pectins are secreted into the cell wall in highly methyl-esterified forms [32] and are subsequently de-esterified. Therefore, the d.e. peak observed at P5-P6 samples suggests that new cell wall materials were being deposited at this stage, which agrees with data on fruit weight and size showing that fruits were still growing (Table 1). PME activity during the P6-P7 upsurge was significantly higher for rain-fed olives, while during the rest of the experimental period it was generally the opposite.

Table 4. Changes in cell wall-related enzyme activities (U mg^{−1} protein) during fruit ripening of irrigated and non-irrigated ‘Arbequina’ olives.

Non-Pectolytic																Pectolytic							
Picking	Date	Irrigation Regime	β-Xyl		EGase		Backbone-Acting						Side Chain-Acting										
							PME		PG		PL		AFase		β-Gal								
1	Sep 18	Irrigated	0.041	a A	0.787	a A	1246.23	a A	5.802	a A	3.949	b A	0.013	e B	0.129	c A							
2	Oct 2		0.015	d A	0.276	d A	1100.28	b A	0.544	d B	2.329	d A	0.023	d B	0.051	d A							
3	Oct 16		0.016	d B	0.328	c A	327.54	c A	0.892	c A	2.139	d A	0.020	d B	0.005	e B							
4	Oct 30		0.011	e B	0.432	b A	299.33	c A	1.922	b A	1.022	e B	0.033	c B	0.164	bc A							
5	Nov 13		0.030	b B	0.401	b A	151.31	d A	0.779	c A	4.661	a A	0.057	b B	0.235	b A							
6	Nov 28		0.017	d B	0.422	b B	310.32	c B	0.842	c B	3.081	c B	0.071	a A	0.331	a A							
7	Dec 11		0.014	de B	0.192	e B	186.01	d B	0.303	e B	4.132	b A	0.073	a B	0.105	cd B							
8	Jan 15		0.026	c B	0.394	b A	205.76	d A	0.770	c B	2.223	d A	0.077	a B	0.091	cd A							
1	Sep 18	Non-irrigated	0.031	c B	0.448	bc B	249.57	d B	2.265	ab B	2.909	c B	0.032	d A	0.121	c A							
2	Oct 2		0.012	e B	0.447	c A	1093.25	a A	1.106	d A	2.476	d A	0.035	d A	0.027	e A							
3	Oct 16		0.033	bc A	0.407	cd A	259.03	d B	0.716	d B	0.930	e B	0.061	c A	0.043	e A							
4	Oct 30		0.020	d A	0.255	e B	130.16	e B	0.764	d B	2.528	d A	0.057	c A	0.114	c A							
5	Nov 13		0.038	a A	0.341	de B	122.92	e B	0.804	d A	3.933	b A	0.065	c A	0.159	a B							
6	Nov 28		0.029	c A	0.545	ab A	485.76	b A	1.382	cd A	4.998	a A	0.074	b A	0.132	bc B							
7	Dec 11		0.038	a A	0.624	a A	344.62	c A	2.010	bc A	3.652	b B	0.096	a A	0.151	ab A							
8	Jan 15		0.036	ab A	0.352	cde B	99.48	e B	3.028	a A	2.249	d A	0.095	a A	0.084	d A							

Values represent means of three replicates. Different capital letters denote significant differences between irrigated and rain-fed samples for a given picking date, and different lower-case letters stand for significant differences among sampling dates for a given irrigation regime, at $p \leq 0.05$ (LSD test). Abbreviations: β-Xyl, β-xylosidase (EC 3.2.1.37); EGase, endo-1,4-β-D-glucanase (EC 3.2.1.4); PME, pectin methylesterase (EC 3.1.1.11); PG, polygalacturonase (EC 3.2.1.67 -exo- and EC 3.1.2.15 -endo-), PL, pectate lyase (EC 4.2.2.2); AFase, α-L-arabinofuranosidase (EC 3.2.1.55); β-Gal, β-galactosidase (EC 3.2.1.23).

High PG and PL activity levels were observed at early (P1) picking dates, which declined thereafter to rise again over the last stages of fruit maturation (Table 4). No clear irrigation-related differences were observed for PL activity, while PG activity in rain-fed olives augmented markedly after mid-November (P5), leading to significantly higher activity as compared with irrigated fruit over later picking points. High activity levels at P1 were possibly of little significance for the onset of ripening-related cell wall modifications, as the high degree of pectin esterification at that sampling point (Table 2) would prevent PG- and PL-catalysed hydrolysis and β -elimination, respectively, of galacturonic acid residues. In contrast, increased PG and PL activity levels at later picking points were associated to the onset of uronic acid loss (Table 3), which hints an actual role on subsequent cell wall disassembly events.

The activities of β -Xyl and EGase, as representatives of non-pectolytic enzymes, were also assessed (Table 4). These enzymes act on hemicelluloses, which comprise a variety of polysaccharides, such as xyloglucans, xylans, arabinoxylans and glucomannans, and are recovered mainly in the KOH-soluble fraction. As for other enzyme activities considered herein, high activity levels were found at early picking dates (P1), which declined subsequently and rose again at later stages of maturity. The observed trends were generally in accordance with recent reports [15,16]. However, a longer timespan was considered herein, and thus a more comprehensive dataset was obtained. The results, moreover, show irrigation-related differences in β -Xyl activity during the experimental time: activity levels were significantly higher in rain-fed than in irrigated samples from P3 sampling onwards. As to EGase, differences in activity levels between irrigated and rain-fed fruit appeared to arise from asynchronous time-course of activity changes rather than to actual significant differences in activity levels. Indeed, irrigated olives showed some delay in ripening, as indicated for example by later skin colour change (Figure 3). A recent study [16] on nine olive genotypes suggested an early role for these enzymes in the onset of ripening-related cell wall disassembly, based on the finding that activity levels were lower at the black than at the green stage. The longer experimental period considered herein, however, allowed for the observation of increased activity at very late sampling dates, which may relate to declining KOH_{sf} yields (Table 2).

3.4. Regression Model for Fruit Firmness

On account of the high dimensionality of the dataset, multivariate analysis procedures were used to help extract useful information. A PLSR model was developed for fruit firmness during on-tree maturation (P1 to P8), using cell wall fraction yields and composition and cell-wall-modifying enzyme activities as the set of potentially explanatory variables. Irrigated and rain-fed samples collected at the different picking dates (P1 to P8) were included in the model, the two first principal components (PC1 and PC2) of which explained together up to 94% of total variability among samples. The scores plot corresponding to this model (Figure 4A) showed that samples distributed along PC1 according mainly to picking date, while irrigation regimes separated along PC2, particularly for more mature samples (P5 to P8). The correlation loadings plot (Figure 4B) confirmed some relationships among variables. Fruit firmness was associated to yield and neutral sugar content of the sodium carbonate-soluble fraction, supporting the view that loss of neutral sugars from pectic polysaccharides was a relevant factor for ripening-related firmness loss. To a lesser extent, firmness was also related to KOH_{sf} yields and neutral sugar content. High levels of PME activity were also correlated with higher fruit firmness, which suggests that demethylation of galacturonic acid residues in pectic polymers helped reinforce egg-box structures, hence limiting cell wall disassembly. This agrees with data showing higher firmness and PME activity levels at early maturity stages (Figure 1, Table 4). Sufficient calcium levels would be required for reinforcing egg-box structures in more immature (firmer) fruit, in agreement with a recent report on ‘Arbequina’ olives [16].

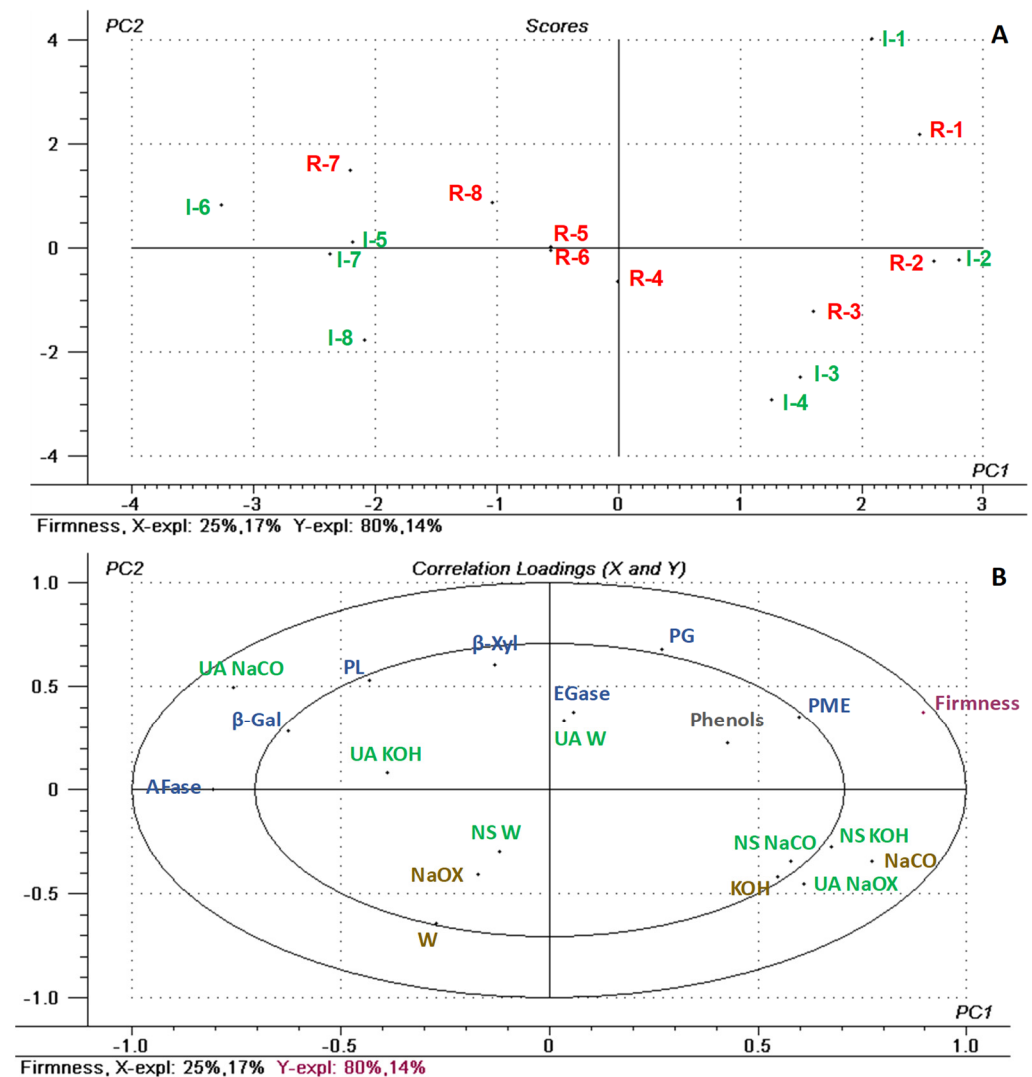


Figure 4. (A) Scores plot of PC1 vs. PC2 corresponding to a Partial Least Squares Regression (PLSR) model for 'Arbequina' fruit firmness (Y variable) vs. a set of potentially explanatory X variables. Codes 1 to 8 denote successive picking dates throughout on-tree fruit maturation under irrigated (I) and rainfed (R) conditions. (B) Correlation loadings plot of PC1 vs. PC2 for the same model (Abbreviations: W, water-soluble fraction; NaOX, sodium oxalate-soluble fraction; NaCO, sodium carbonate-soluble fraction; KOH, potassium hydroxide-soluble fraction; UA, uronic acid content; NS, neutral sugar content; AFase, β-Gal, β-Xyl, EGase, PG, PL, PME, α-L-arabinofuranosidase, β-galactosidase, β-xylosidase, endo-1,4-β-D-glucanase, polygalacturonase, pectate lyase and pectin methylesterase activities, respectively).

AFase and, to a lesser extent, β-Gal activity levels correlated inversely with $\text{Na}_2\text{CO}_{3\text{sf}}$ yields, which highlights the relevance of these enzyme activities for neutral sugar loss and firmness changes along fruit maturation. Similarly, PG and β-Xyl activity correlated inversely with NaOX_{sf} and KOH_{sf} yields, respectively, which hints an actual role in ripening-related modifications in polyuronides and hemicelluloses.

In this full-data model, though, the main factor for sample differentiation was maturity stage, while irrigation regimes separated along PC2, which accounted for only 14% of total variability. However, we were also interested in explaining fruit firmness differences between irrigated and rain-fed fruit, which were particularly conspicuous at later picking dates (Figure 2). For this reason, an additional PLSR model was developed for fruit firmness in which P5 to P8 stages uniquely were included, using cell wall fraction yields and composition as the set of potentially explanatory variables. When more immature

(firmer) samples were removed from the model, differences between irrigated and rain-fed fruit became apparent, and samples separated clearly along PC1 according to irrigation regime (Figure 5).

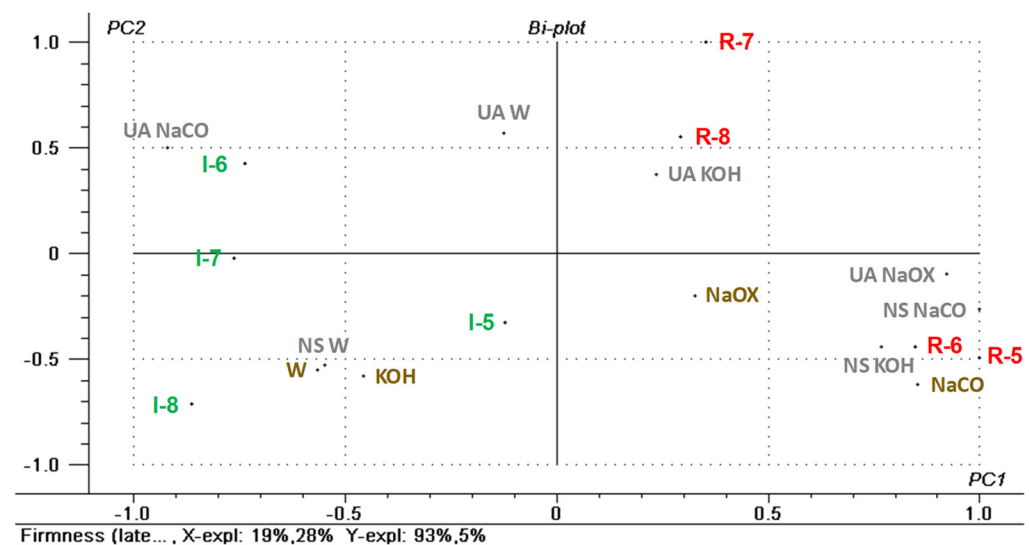


Figure 5. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a Partial Least Squares Regression (PLSR) model for ‘Arbequina’ fruit firmness (Y variable) vs. yield and composition of different cell wall fractions (X variables). Codes 5 to 8 denote successive picking dates throughout on-tree fruit maturation under irrigated (I) and rainfed (R) conditions (Abbreviations: W, water-soluble fraction; NaOX, sodium oxalate-soluble fraction; NaCO, sodium carbonate-soluble fraction; KOH, potassium hydroxide-soluble fraction; UA, uronic acid content; NS, neutral sugar content).

The results confirm the relevance of neutral sugar loss for firmness decline, since rain-fed samples, which were firmer (Figure 2), were characterised by higher yields of the sodium carbonate-soluble fraction, as well as by higher contents of neutral sugars in both the $\text{Na}_2\text{CO}_{3\text{sf}}$ and the KOH_{sf} . Accordingly, irrigated fruit displayed more intense solubilisation of cell wall polymers, as indicated by higher yields of the water-soluble fraction and neutral sugar contents therein. Interestingly, rain-fed samples also retained higher amounts of uronic acids in the chelator-soluble fraction, which agrees with the observation of higher PME activity levels in firmer fruit (Figure 4B) and supports the idea that, provided enough calcium is available, this enzyme activity may contribute to reinforce egg-box structures and hence to attenuate firmness loss.

Some caution has to be exerted when interpreting enzyme activity data: *in vitro* activity assays are usually performed in optimal conditions, which in most cases will not correspond with those met *in muro* (pH, substrate availability and accessibility, cell wall porosity and charge, additional enzyme activities, etc.). Yet, the information reported herein provides a good foundation for future research. Further studies should entail, among others, chromatographic analyses of individual sugars in cell wall fractions, activity of additional cell wall-related proteins, and their isolation and characterisation. Similarly, season-to-season variability and different irrigation strategies should also be addressed. These studies may help in improving the management of olive groves, particularly at sites characterised by harsh climatic conditions, such as those encountered at the Mediterranean basin and middle east areas, the main producers of olives and olive oil.

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