Ripening-related cell wall modifications in olive (*Olea europaea* L.) fruit: A survey of nine genotypes

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ABSTRACT

The production of olive (*Olea europaea* L.) is very important economically in many areas of the world, and particularly in countries around the Mediterranean basin. Ripening-associated modifications in cell wall composition and structure of fruits play an important role in attributes like firmness or susceptibility to infestations, rots and mechanical damage, but limited information on these aspects is currently available for olive. In this work, cell wall metabolism was studied in fruits from nine olive cultivars ('Arbequina', 'Argudell', 'Empeltre', 'Farga', 'Manzanilla', 'Marfil', 'Morrut', 'Picual' and 'Sevillenca') picked at three maturity stages (green, turning and ripe). Yields of alcohol-insoluble residue (AIR) recovered from fruits, as well as calcium content in fruit pericarp, decreased along ripening. Cultivar-specific diversity was observed in time-course change patterns of enzyme activity, particularly for those acting on arabinosyl- and galactosyl-rich pectin side chains. Even so, fruit firmness levels were associated to higher pectin methylesterase (PME) activity and calcium contents. In turn, fruit firmness correlated inversely with ascorbate content and with α-L-arabinofuranosidase (AFase) and β-galactosidase (β-Gal) activities, resulting in preferential loss of neutral sugars from cell wall polymers.

Keywords: cell wall; cultivars; enzymes; firmness loss; maturity stage; minerals; *Olea europaea* L.

O Love! What hours were thine and mine,
In lands of palm and southern pine,
In lands of palm, of orange-blossom,
Of olive, aloe, and maize and vine!

Alfred, Lord Tennyson (*The Daisy*)

1. Introduction

Olive (*Olea europaea* L.) tree was one of the first crops to be domesticated by humans (Besnard, Terral, & Cornille, 2018), and olive growing has outstanding economic relevance in countries around the Mediterranean basin. While the largest part of total production is devoted to oil extraction, a smaller amount thereof is intended for consumption as table olives, and hence fruit texture and mechanical properties are very relevant for the eating quality of the final product. Furthermore, high susceptibility of fruit to mechanical damage restricts the use of mechanical harvesting in table olive orchards, which hampers the reduction of production costs. In the case of cultivars used mainly for oil extraction, textural and mechanical factors may also influence extraction efficiency, while postharvest changes may have an impact on final oil quality (Vichi, Romero, Gallardo-Chacón, Tous, López-Tamames, & Buixaderas, 2009).

During the ripening process, textural changes occur which result from compositional and structural modifications in cell walls and middle lamellae. These changes arise largely from solubilisation and rearrangements of the constituent polysaccharides, carried out by pectolytic and non-pectolytic proteins (Goulao & Oliveira, 2008). Polysaccharide depolymerisation may also occur in some fruit species, including olive according to a few reports (Marsilio, Lanza, Campestre, & De Angelis, 2000; González-Cabrera, Domínguez-Vidal, & Ayora-Cañada, 2018). Non-enzymatic factors may also contribute to ripening-related cell wall alterations, and experimental evidence of a role for ascorbic acid (AA) and its derivatives in the oxidative disassembly of cell wall polysaccharides has been found for banana (*Musa* spp.) (Cheng et al., 2008), longan (*Dimocarpus longan* Lour) (Duan, Zhang, Zhang, Sheng, Lin, & Jiang, 2011) and sweet cherry (*Prunus avium* L.) (Belge, Comabella, Graell, & Lara, 2015).

In spite of the importance of ripening-associated modifications in cell wall composition and structure on key attributes such as firmness and susceptibility to mechanical damage, infestations or rots, very few published studies have addressed this topic during olive fruit maturation. Some information is however available for a few cultivars. For example, cell wall-related enzyme activities and cell wall gene expression levels have been reported to increase with maturity stage in 'Hojiblanca' (Fernández-Bolaños, Heredia, Vioque, Castellano, & Guillén, 1997) and 'Picual' (Parra, Paredes, Sánchez-Calle, & Gómez-Jiménez, 2013) fruit, respectively. Extensive solubilisation of cell wall materials occurred during ripening of 'Koroneiki' olives (Vierhuis, Schols, Beldman, & Voragen, 2000). Similarly, ripening 'Negrinha do Douro' fruit showed progressive cell-to-cell separation and strong losses of arabinosyl residues resulting in noticeable firmness diminution (Mafra et al., 2001). Accordingly, substantial loss of neutral sugars from pectins was observed for 'Arbequina' olives during fruit ripening, linked to the progressive increase in α-L-arabinofuranosidase (AFase) activity (Lara, Albrecht, Comabella, Riederer, & Graell, 2018). We were therefore interested in broadening these studies on a wider choice of cultivars, in order to improve current understanding of the biochemical mechanisms underlying texture changes in olive fruit during maturation.

2. Materials and methods

2.1. Plant material

Fruits of nine local Spanish olive cultivars ('Arbequina', 'Argudell', 'Empeltre', 'Farga', 'Manzanilla', 'Marfil', 'Morrut', 'Picual' and 'Sevillenca') were hand-collected in 2016 at three different maturity stages based on skin colour (green, turning and ripe) from trees supplied with support irrigation grown at an experimental orchard located at IRTA-Mas Bové (Constantí, Spain, 41° 09'N; 1° 12'E). Picking period was September to December. The main part of total annual rainfall in the producing area (500 mm in 2016) took place during spring (April-May). Cultural practices and fertilization were the standard ones used in commercial orchards around the sampling site. The selected cultivars included oil- and table-olive representatives of very early ('Empeltre', 'Manzanilla'), early ('Sevillenca'), medium ('Arbequina', 'Argudell', 'Farga', 'Picual') and late ('Marfil', 'Morrut') ripening patterns (Tous & Romero, 1993).

The maturity index (0-7) was determined on 50 olives per cultivar and maturity stage based on the visual evaluation of fruit skin and flesh colour according to the usual practice by the olive industry, and values indicate the weighted average of the 50 fruits assessed. Oil content was determined jointly on 50 fruits per cultivar and maturity stage by nuclear magnetic resonance (NMR) spectroscopy after drying samples in the oven at 105 ºC till constant weight. For the evaluation of fruit firmness, a penetration test was run on 10 olives per cultivar and maturity stage with an INSTRON texture analyzer (Model 3344, Instron, Bucks, UK) equipped with a 1-mm diameter cylindrical probe descending at 1 mm s^{-1}. The maximum strength (N) and deformation (mm) to achieve surface breakage were recorded. Fruit skin colour was also assessed on 10 fruits with a desktop colorimeter (Chroma Meter CR-300, Minolta Corp., Osaka, Japan) using CIE illuminant D_{65} with 8-mm aperture diameter and 10º observation angle. Results were expressed as CIELAB colour space coordinates (L*, a*, b*). The incidence of some alterations (olive fly infestation, infection by *Camarosporium dalmaticum*, bruised and wrinkled fruits) was also assessed visually on 50 fruits per cultivar and maturity stage, and data shown as a percentage.

2.2. Determination of mineral content

Fifty olives per cultivar and maturity stage were washed in 1% (v/v) Triton X-100, rinsed in deionised water (Fernández-Hernández, Mateos, García-Mesa, Beltrán, & Fernández-Escobar, 2010) and pitted. Flesh samples were then vacuum-dried in a lyophilizer (Telstar® Cryodos, Azbil Group, Tokyo, Japan), milled and kept at -80 ºC until analysis.

A muffle furnace (Carbolite CWF 1100, Carbolite Gero Ltd., Hope, UK) was used to obtain the ashes from lyophilized samples: temperature was raised during 12 h to 550 ºC, kept at 550 °C for 12 h and then cooled down to room temperature. In order to hydrolyse pyrophosphates formed during incineration, samples were submitted to dry digestion in 6 mL of an aqueous HCl solution (1:1, v/v), and then kept in a sand bath at 70 °C until complete dryness. Finally, samples were resuspended in Milli-Q® water and filtered through Whatman® 40 ashless paper prior to injection into an inductively coupled plasma-mass spectrometry (ICP-MS) equipment (Agilent 7700X, Agilent Technologies Inc., Santa Clara, CA, USA) for quantification (mg kg^{-1} DW) of boron (B), magnesium (Mg), potassium (K), calcium (Ca), manganese (Mn) and iron (Fe) contents.

2.3. Extraction, fractionation and analysis of cell wall materials

Cell wall materials were extracted as the alcohol-insoluble residue (AIR) as described in Voragen, Timmers, Linssen, Schols, & Pilnik (1983). Destoned olive fruit samples (50 g) were homogenised in 80% (v/v) ethanol in a domestic blender to obtain a 10% (w/v) suspension, and then heated at 80 ºC for 20 minutes. After cooling down to room temperature, samples were filtered through Miracloth® (Merck Life Science S.L.U., Madrid, Spain). The solid residue was shaked three times in 80% ethanol for 30 minutes, then 5 minutes in 96% ethanol, and finally 5 minutes in acetone, and filtered through Miracloth® after each step. The final solid residue was dried at 50 ºC and stored at -20 ºC until fractionation and analysis. AIR yields were expressed as g 100 g^{-1} fresh weight (FW).

The methodology for AIR fractionation was modified from a previous work (Lefever, Vieuille, Delage, d'Harlingue, de Monteclerc, & Bompeix, 2004). AIR samples (0.5 g) 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 obtain the water-, sodium oxalate-, sodium carbonate- and potassium hydroxide-soluble fractions (W_{sf}, $NaOx_{sf}$, Na_2CO_{3sf} and KOH_{sf}, respectively). After each fractionation step, the supernatant was concentrated in a rotary evaporator and precipitated by adding 96% (w/v) ethanol. The sediment was then washed three times in water, and dried at 50 ºC to determine fraction yields. Each extraction was done in triplicate, and yields given as g 100 g^{-1} AIR.

Total sugar and uronic acid contents were analysed respectively by the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the *m*-hydroxyphenyl method (Blumenkratz & Asboe-Hansen, 1973). Neutral sugar amount was calculated by subtracting the content of uronic acids from that of total sugars. Results were given as g 100 g^{-1}.

The degree of methyl esterification of pectins was determined according to Klavons & Bennet (1986) with some modifications. Methyl groups were removed by adding 1 mL 1 M KOH and 5 mL Milli-Q® water to AIR samples (15 mg), which were then kept at room temperature for 2 h. After neutralising with 0.49 mol L^{-1} H_3PO_4, released methanol was oxidised enzymatically (1 U mL^{-1} alcohol oxidase) before adding 2 mL 0.02 mol L^{-1} pentane-2,4-dione and incubating at 60 ºC for 2 h. When mixture cooled down, the absorbance at 412 nm was read. The degree of methyl esterification was calculated as the molar ratio (%) of methanol to uronic acid content.

2.4. *Cell wall-related enzyme activities*

Enzyme activities were determined on acetone powder (AP) obtained from fruit pericarp samples as described by Fernández-Bolaños et al. (1997), with small modifications. Briefly, flesh tissue samples were homogenised in cold acetone (10% suspension, w/v) with a domestic blender and filtered. The solid residue was washed three times in acetone, filtered, allowed to dry at room temperature, and stored at -20 ºC. Enzyme assays were carried out in triplicate on AP samples (100 mg) mixed in 1 mL of the appropriate extraction buffer.

Extraction buffers and activity assays for α-L-arabinofuranosidase (AFase; EC 3.2.1.55), β-galactosidase (β-Gal; EC 3.2.1.23), 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), 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 in Ortiz, Graell, & Lara (2011), and references therein. Total protein content in the extracts was determined with the Bradford (1976) method, using BSA as a standard, and data expressed as specific activity (U mg protein^{-1}).

2.5. *Antioxidant properties*

All analyses were undertaken on lyophilised pericarp tissue. Radical scavenging activity (RSA) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, in which the antioxidant ability of sample extracts is expressed as the percentage of DPPH reduction in comparison with the control (DPPH without sample). Total phenolics were extracted in methanol, quantified colorimetrically, and results given as mg gallic acid equivalents g^{-1} DW. Anthocyanin content was estimated as cyanidin-3-rutinoside equivalents in extracts obtained from lyophilised tissue and expressed as mg cyanidin equivalents g^{-1} DW. All procedures were as described elsewhere (Lara, Camats, Comabella, & Ortiz, 2015).

The contents of total (TAA) and reduced (AA) ascorbic acid were measured with the colorimetric ascorbate assay (Gillespie & Ainsworth, 2007), and data given as nmol g^{-1} dry weight (DW). Dehydroascorbic acid (DHA) content was taken as the difference between those in TAA and AA.

2.6. *Statistical analysis*

Means were submitted to multifactorial analysis of variance (ANOVA), with cultivar and maturation stage as the factors, and separated by Student's *t* test ($p \leq 0.05$). JMP® Pro 13 and Sigma Plot 11.0 (Systat Software Inc.) software packages were used for statistical analyses. In order to relate dependent *Y*-variables to a set of potentially explanatory *X*-variables, partial least square regression (PLSR) was employed as a predictive method. The Unscrambler version 9.1.2 software (CAMO ASA, Oslo, Norway) was used for PLSR model development. Data were weighed by the inverse of the standard deviation of each variable and full cross-validation was run as a validation procedure**.**

3. Results and discussion

Physical characteristics of olive fruits used in this study are shown (**Table 1**). Additional phenotypical data including fruit size and water content are also included as supplementary material (**Supplementary Table 1**). Colour parameters showed increasing values of a* along maturation accompanied by concomitant decreases in those of b* and L*, which reflect the progressive shift in fruit surface colour from green to purple or black hues. Maturity indices (MI) ranged between 0.04 and 5.88, contingent upon cultivars and harvest date. In accordance with colour changes, total anthocyanins increased significantly along fruit maturation (**Supplementary Table 2**) with the exception of 'Marfil' samples, which turn white rather than black owing to blockage of anthocyanin synthesis. The highest levels were observed for ripe 'Manzanilla' fruits (8.0 mg g^{-1} DW), while they were unsurprisingly very low in ripe 'Marfil' samples (0.3 mg g^{-1} DW).

Fruits softened significantly along ripening as indicated both by a decrease in the maximum strength required to induce surface breakage (henceforth, "firmness") and by augmented deformation values indicative of increasing skin elasticity, excluding 'Morrut' samples for which no significant differences were observed in the latter indicator. 'Marfil' fruits displayed the largest differences in firmness levels between the green and the ripe stages (73.8%), while ripe 'Empeltre' olives lost only 36.9% firmness in relation with values at the green stage: these fruits showed the lowest firmness levels when sampled in September (**Table 1**) consistent with their very early ripening pattern (Tous & Romero, 1993). Both 'Empeltre' and 'Manzanilla' fruits suffered from the most severe incidence of *Bactrocera oleae* infestation (**Supplementary Table 3**).

Some chemical characteristics related to antioxidant properties were also assessed in fruit samples (**Supplementary Table 2**). The content of total phenols ranged from 12.3 mg g^{-1} DW (ripe 'Sevillenca' samples) to 49.9 mg g^{-1} DW (green 'Morrut' fruits). A previous study on cultivars 'Dhokar' and 'Chemlali' reported increased content of total phenolics along fruit ripening (Jemai, Bouaziz, & Sayadi, 2009). In this work, though, this increasing trend was observed for 'Farga' uniquely. In contrast, results indicate cultivar-related differences in the evolution of total phenols: while no significant changes were found for 'Manzanilla' and 'Marfil', contents decreased along fruit ripening in fruit samples from the rest of the cultivars assessed (**Supplementary Table 2**). Total amount of phenolics showed no apparent relationship with RSA in fruit samples. RSA levels were very high in all cases, ranging from 79.2% to as much as 98.9%. RSA was particularly high in 'Marfil', 'Manzanilla' and 'Morrut' fruits, with values above 90% regardless of maturity stage (**Supplementary Table 2)**. Limited ripening-related variation in RSA was found, with the exception of 'Farga' and 'Marfil' samples, for which significant increases were observed, in agreement with reports on 'Dhokar' and 'Chemlali' olives (Jemai et al., 2009). Ascorbic acid (AA) is a major antioxidant buffer in plant apoplasts (Pignocchi & Foyer, 2003). Because of increased permeability of cell membranes along fruit ripening, ascorbate is released into the apoplast (Fry, 1998), where it can be oxidised to dehydroascorbic acid (DHA). DHA has to be returned back to the cytosol for subsequent reduction. In five out of the nine cultivars considered in this study ('Empeltre', 'Farga', 'Manzanilla', 'Morrut' and 'Picual'), AA levels detected in fruit pericarp increased with maturity stage. In contrast, significant decreases were found along maturation for 'Marfil' and 'Sevillenca', while limited change was observed for 'Arbequina' and 'Argudell' (**Supplementary Table 2**). The observation of increased AA contents along maturation is interesting in the light of a previous work showing that D-galacturonic acid released as a consequence of cell wall solubilisation may be a major precursor for ascorbic acid biosynthesis in fruits (Agius, González-Lamothe, Caballero, Muñoz-Blanco, Botella, & Valpuesta, 2003). In this work, indeed, firmness loss along fruit maturation was paralleled by decreased yields of insoluble cell wall materials and by progressive solubilisation of cell wall constituents as shown by higher yields of the water-soluble fraction in more mature samples (**Table 2**). These ripening-related cell wall modifications were hence considered more in detail.

3.1. Cell wall modifications along fruit ripening

With the exception of 'Empeltre', AIR yields decreased significantly throughout fruit maturation (**Table 2**), in agreement with earlier reports on 'Hojiblanca' and 'Negrinha do Douro' olives (Jiménez, Rodríguez, Fernández-Caro, Guillén, Fernández-Bolaños, & Heredia, 2001; Mafra et al., 2001). When AIR were fractionated further, the percentage of water-soluble materials (W_{sf}) over total AIR was generally found to increase over ripening, reflecting progressive solubilisation of cell wall polymers. In contrast, no consistent trends in change dynamics over fruit ripening were observed across all nine cultivars considered regarding yields of the chelator-soluble ($NaOx_{sf}$), the sodium carbonate-soluble (Na_2CO_{3sf}) or the potassium-soluble (KOH_{sf}) fractions, enriched in non-covalently linked pectins, covalently-linked pectins and matrix glycans, respectively (**Table 2**).

When the content in neutral sugars was analysed in AIR, little variations were found over fruit ripening (**Table 3**), which suggest that sugars were reallocated among AIR fractions. Indeed, substantial loss of neutral sugars along fruit ripening was shown for the KOH-soluble fraction, sometimes paralleled by significant increases in neutral sugar content in the Na_2CO_3-soluble fraction. These rearrangements might account for the erratic trends in fraction yields observed during fruit ripening (**Table 2**).

The analysis of uronic acid percentage in the different AIR fractions isolated showed significant decreases in the water-soluble fraction during fruit ripening (**Table 4**). Together with the observation that total W_{sf} yields increased with maturity stage (**Table 2**), this finding clearly suggests that neutral sugars, rather than uronic acids, were solubilised preferentially from cell wall polymers. For some of the cultivars considered ('Arbequina', 'Argudell', 'Manzanilla', 'Morrut', 'Picual' and 'Sevillenca'), this is also supported by the observation of augmented proportions of uronic acids in the chelator-soluble fraction of more mature samples (**Table 4**). Substantial uronic acid losses from the Na_2CO_3-soluble fraction were found during 'Arbequina' fruit ripening, in accordance with a previous report (Lara et al., 2018). This trend was observed also for 'Morrut', 'Picual' and 'Sevillenca' fruits, suggesting a link to firmness loss.

3.2. Cell-wall modifying enzyme activities along fruit ripening

Changes in cell wall fraction yields and composition suggested important losses of neutral sugars from cell wall polymers along ripening, and thus pointed out to a relevant role for enzyme activities acting on pectin side-chains. Because arabinose stands out quantitatively in olive fruit pectins (Mafra et al., 2001), levels of AFase activity were considered. AFases remove arabinosyl residues from galacturonans, and so contribute to cell wall disassembly by promoting pectin solubilisation and by facilitating the access of other enzymes to their galacturonan backbone substrate. AFase activity levels in 'Arbequina' as well as in 'Empeltre', 'Farga', 'Manzanilla' and 'Sevillenca' fruits increased significantly with maturity stage (**Table 5**), as reported previously for 'Arbequina' (Lara et al., 2018). However, genotype-related differences existed as to time-course changes in AFase activity, since no variation or even decreased activity levels were observed for the rest of the cultivars considered. Even though galactose is less abundant in cell walls of olive fruit (Mafra et al., 2001), β-Gal-catalysed removal of galactosyl residues might also contribute to the reallocation of neutral sugars to the water-soluble fraction and to pectin rearrangements during ripening. The change patterns observed for this enzyme activity were also variable across the nine olive cultivars assessed, significant ripening-associated increases being found for 'Arbequina', 'Argudell', 'Manzanilla' and 'Marfil' uniquely (**Table 5**).

For eight out of the nine cultivars considered, the highest levels of PME activity corresponded to green fruits (**Table 5**), and accordingly the degree of methyl esterification of pectins was generally lower in these samples in comparison with ripe fruits (**Table 2**). The declining trend observed for PME activity during ripening suggests an early role in cell wall modifications leading to olive fruit softening, in agreement with previous works (Mafra et al., 2001; Lara et al., 2018). PME modulates cell wall structure in different ways. On a side, PME demethylating action may favour cell wall reinforcement through the establishment of calcium bridges between free carboxyl groups (Goulao & Oliveira, 2008). Yet PME action also leads to lowered pH in the apoplast, thus providing a regulatory mechanism for additional cell wall-related enzymes. Negatively charged polyuronides will also favour pectin hydration and may thus modify protein diffusion and activity (Grignon & Sentenac, 1991). Furthermore, PME-catalysed cleavage of methyl groups from α-D-GalUA-rich polymers is a requirement for subsequent action of other pectolytic enzymes such as PG and PL, which will remove demethylated residues uniquely, respectively through hydrolysis or β-elimination.

With the exception of 'Marfil', PG and PL activity assays showed reduced activity levels along olive ripening (**Table 5**). Even though the time-course trend was similar across cultivars, noticeable variation in specific activity levels were observed, ranges spanning from 16.1 (green 'Picual') to 0.2 (ripe 'Arbequina') unit mg^{-1} protein for PG, and from 5.8 (green 'Picual') to 0.4 (ripe 'Arbequina') unit mg^{-1} protein for PL (**Table 5**). It has been suggested (Jiménez et al., 2001) that degradation of cell wall polysaccharides during ripening of 'Hojiblanca' olives may be sequential, the metabolism of pectic polysaccharides being more active at the onset of the ripening process, while neutral polysaccharides would be metabolised more intensively at subsequent stages. This would be consistent with data herein showing opposite trends for enzyme activities acting on the pectin backbone (PG, PL, PME) and those acting on the neutral sugar-rich sidechains (AFase, β-Gal). This would also agree with the apparently preferential loss of neutral sugars along ripening (**Tables 2, 3**).

Two non-pectolytic enzyme activities, β-Xyl and EGase, were also analysed. Results revealed a general decreasing trend in activity values during fruit ripening. This observation may be reflecting an early role in cell wall modifications. For some of the cultivars assessed, lower β-Xyl activity levels were observed in samples displaying higher yields of KOH_{sf}, the cell wall fraction enriched in the matrix glycan substrates of those enzymes (**Table 2**). Even so, it should be pointed out that fraction yields were expressed as a percentage over AIR, and hence variations in other AIR fractions as well as in total AIR isolated will also affect the relative KOH_{sf} proportions observed. Additionally, certain amount of tightly-bound cell wall polymers may have not been extracted in KOH and have so remained in the final insoluble residue. Actually, yields of the insoluble residue remaining after sequential AIR extraction decreased over fruit ripening, expressed both as a percentage over AIR (g 100 g^{-1} AIR) and as a percentage over FW (g 100 g^{-1} FW) (**Table 2**). This would be in accordance with the observation of sharp decreases in the content of xylose, a quantitatively prominent sugar component of olive cell walls, in the final residue during ripening of 'Negrinha do Douro' olives (Mafra et al., 2001), as well as with increased W_{sf} yields over ripening found herein (**Table 2**).

3.3. Other potential factors: mineral content and antioxidant status

In addition to related enzyme activities, some studies have suggested a role for ascorbic acid in fruit ripening-associated cell wall disassembly and firmness loss (Cheng et al., 2008; Duan et al., 2011; Belge, Goulao, Comabella, Graell, & Lara, 2017). As a consequence of increasing cell membrane permeability upon fruit ripening, ascorbate is released into the apoplast, leading to the generation of hydroxyl (•OH) radicals (Fry, 1998). At physiological ranges, ascorbate can favour the oxidative scission of plant cell wall polysaccharides, and xyloglucans are reportedly more susceptible than pectins to ascorbate-induced scission (Fry, 1998), which might relate to the observed decline in yields of the insoluble residue during ripening (**Table 2**).

Evidence also exists that mineral deficiency may impact cell wall integrity through metabolic changes eventually affecting cell wall expansion, plant growth, crop yield and quality of the final product (Goulao, Fernandes, & Amâncio, 2017). Therefore, the content of some minerals in fruit samples was also studied. The highest contents observed corresponded to potassium (K), calcium (Ca) and magnesium (Mg) (**Supplementary Table 4**). In general, boron (B) concentrations observed were higher than those reported for other olive cultivars including 'Amfissis', 'Chondrolia Chalkidikis' and 'Picholine' (Chatzissavvidis, Therios, & Antonopoulou, 2004; Tekaya et al., 2014). Magnesium (Mg) and potassium (K) contents were also higher than those reported for other cultivars (Nergiz & Engez, 2000; Fernández-Poyatos, Ruiz-Medina, & Llorent-Martínez, 2019). In contrast, manganese (Mn) concentrations recorded were lower than those in other cultivars grown in Spain (Fernández-Hernández et al., 2010), while those of iron (Fe) were roughly as in earlier reports (Llorent-Martínez, Fernández-de Córdova, Ortega-Barrales, & Ruiz-Medina, 2014). The presence of Fe in green olives intended for manufacturing as table olives is considered undesirable, as this mineral sets up complexes with polyphenols naturally present in the fruit, which causes skin to blacken (Fernández-Poyatos et al., 2019), and from this point of view the low Fe levels in green 'Manzanilla' and 'Morrut' fruits would indicate that these cultivars are more suitable for this purpose than the rest of assessed cultivars.

A clear, general trend among cultivars was recognisable for Ca uniquely, which decreased with fruit ripening. Calcium content ranged widely from 306.0 to 1837.6 mg kg^{-1}DW (ripe 'Morrut' and green 'Marfil', respectively). The observation that it generally decreased along ripening is interesting in the light of its role in the preservation of fruit firmness and other quality-related aspects (reviewed in Lara, 2013). With the purpose of obtaining a global overview of the relationships among the many variables assessed, a partial least square regression (PLSR) model was developed, in which data on cell wall fractions, enzyme activities, ascorbate and mineral content were used as the set of *X*-variables potentially explaining firmness levels of fruit samples.

The corresponding correlation loadings plot (**Fig. 1**) shows that the two first principal components (PC) of the model accounted for up to 85% of total variability in fruit firmness. Firmness was associated to higher levels of PME activity and Ca concentrations, AIR yields, and content of neutral sugars in the KOH_{sf}. The association between PME activity and Ca levels to fruit firmness agrees with a reinforcing role for this enzyme activity at early stages of fruit ripening. Moreover, the mode of PME action on pectins, and hence the distribution of free carboxyl groups, is dependent on apoplastic pH (Denès, Baron, Renard, Péan, & Drilleau, 2000). In the apoplast of unripe fruits, with pH values close to neutrality, the enzyme acts through a single chain, multiple attack mechanism, leading to a blockwise distribution of de-esterified residues which confers pectins higher calcium affinity. In the presence of high calcium contents (**Supplementary Table 4**) this would result in cell wall stiftening and favour firmness retention as shown herein (**Fig. 1**).

Higher firmness levels were also associated to high levels of PG and PL activities (**Fig. 1**). However, it should be noted that high activities by themselves may be of little significance for actual cell wall disassembly. Factors such as cell wall porosity, apoplastic pH or cell wall hydration status may limit their activity or access to their pectin backbone substrate. Indeed, AFase and β-Gal activities were inversely correlated to fruit firmness, which suggest that the presence of highly branched sidechains in pectins of green fruits restricted actual PG and PL action in spite of high activity levels. Enzyme activity assays are usually performed in optimal conditions of pH, temperature and concentrations of substrates and cofactors, which often do not correspond with the real *in muro* conditions. Hence, *in vitro* activity may not match the actual *in planta* activity, and so some caution should be exerted when interpreting activity assay results. Furthermore, such data generally represent the joint activities of several isoforms, and change patterns in the activity of the ripening-specific isozyme(s) may be masked within total activity recorded.

Firmer fruits also showed higher percentage of uronic acids in the water-soluble fraction, which reflects the decrease in the uronic acids:neutral sugar ratio in this fraction as sugars become progressively solubilized as ripening proceeds. This is in agreement with the observation that the content of neutral sugars in the KOH_{sf} was also associated to higher fruit firmness (**Fig. 1**). The KOH_{sf} is enriched in polysaccharides collectively termed hemicelluloses, which among others include xyloglucans, xylans, glucomannans and arabinoxylans. The xyloglucan backbone is constituted of β-1,4-linked glucose residues, displays xylose- and galactose-rich sidechains, and forms cell wall-strengthening cross-links with cellulose. Decreasing trends for β-Xyl and EGase activities (**Table 5**), which act on these non-pectic polymers, may indicate an early role in the onset of ripening-related cell wall changes.

Ascorbic acid content was inversely correlated to fruit firmness and to PME (**Fig. 1**). This is also interesting on the basis of previous studies reporting that (a) de-esterified pectin is more susceptible than methyl-esterified pectin to ascorbate-induced scission (Dumville & Fry, 2003), and that (b) galacturonic acid released from cell walls is an important precursor for L-ascorbic acid biosynthesis in fruits (Agius et al., 2003). Higher susceptibility of de-esterified pectins to ascorbate would hint at an additional mechanism by which PME could impact on ripening-related firmness loss.

4. Conclusions

The comparative study reported herein pointed out a relevant role for some cell wall-related enzyme activities in the process of ripening-associated softening of olive fruit. Even though cultivar-specific diversity was observed in time-course trends of activity changes, some common patterns in ripening-related cell wall modifications were found. Progressive solubilisation of cell wall polysaccharides was reflected in increased yields of the water-soluble fraction. Fruit firmness in green fruits was associated to higher levels of PME activity and calcium levels, suggesting that the formation of egg-box structures between pectic polysaccharides led to cell wall reinforcement. Data also suggest that neutral sugars rather than uronic acids were lost from cell wall polymers, in agreement with the observation that AFase and β-Gal activities were correlated inversely with fruit firmness. Ascorbate levels might also play an aiding role in cell wall disassembly. A better comprehension of these ripening-associated modifications may allow improving orchard management and produce handling for the enhancement of fruit quality.

Author contributions

CD, JG, AR, AN and IL collected the samples. CD and AI carried out the biochemical analyses. AR and AN were responsible of the experimental orchards and the physicochemical characterization of fruit samples. TC was in charge of mineral composition analyses. FG contributed to sample processing. CD and IL conceptualized and wrote the manuscript. All the Authors revised and approved the manuscript.

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Conflict of interest

The authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1. Correlation loadings plot of PC1 *vs*. PC2 corresponding to a PLSR model for fruit firmness (*Y* variable) *vs*. cell wall composition, enzyme activities, ascorbate and mineral contents (*X* variables) of olive fruits.

Abbreviations: AIR, alcohol-insoluble residue; d.e., degree of methyl esterification of pectins; W_{sf}, $NaOx_{sf}$, $NaCO_{sf}$ and KOH_{sf}, yields of water-, sodium oxalate-, sodium carbonate- and potassium hydroxide-soluble fractions, respectively; UA(W), UA(Ox), UA (NaCO) and UA (KOH), uronic acid contents in W_{sf}, $NaOx_{sf}$, $NaCO_{sf}$ and KOH_{sf}, respectively; NS (NaCO) and NS (KOH), neutral sugar contents in $NaCO_{sf}$ and KOH_{sf}, respectively; d.e., degree of methyl esterification of pectins; β-Xyl, β-xylosidase; EGase, endo-1,4-β-D-glucanase; PG, polygalacturonase; PL, pectate lyase; PME, pectin methylesterase; AFase, α-L-arabinofuranosidase; β-Gal, β-galactosidase; Ca, Fe, Mg, content of calcium, iron and magnesium; AA, reduced ascorbic acid; DHA, dehydroascorbic acid; RSA, radical scavenging activity.

Table 1. Maturity indicators and physical characteristics of olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	Sampling date	Maturity index	Oil content (g 100g^{-1}DW)	L*	a*	b*	Maximum strength (N)	Deformation (mm)
'Arbequina'	Green	Sept 29	0.26	39.3	38.11 a BC	-9.23 c A	23.16 a CD	6.39 a CD	0.94 b A
	Turning	Sept 29	2.14	43.3	28.34 b BC	4.12 b B	10.17 b B	4.01 b A	1.03 b CD
	Ripe	Nov 27	3.40	52.2	21.88 c B	11.01 a A	2.62 c B	2.99 c A	1.38 a B
'Argudell'	Green	Sept 29	0.26	39.8	29.84 a F	-9.97 b AB	16.19 b E	6.60 a BC	0.58 b D
	Turning	Nov 27	0.96	48.0	32.69 a B	-7.98 b D	19.23 a A	3.72 b A	1.13 a BC
	Ripe	Nov 27	2.36	50.1	16.76 b C	2.32 a B	-1.04 c C	2.55 c BC	1.03 a CD
'Empeltre'	Green	Sept 29	0.48	45.9	38.24 a B	-9.76 c AB	25.49 a B	4.04 a F	0.87 c AB
	Turning	Sept 29	3.58	45.5	18.45 b D	3.37 a B	-0.13 b C	2.57 b C	1.20 b B
	Ripe	Nov 27	5.00	56.1	17.88 b C	-0.02 b BC	-1.83 c C	2.55 b BC	1.68 a A
'Farga'	Green	Sept 29	0.36	36.4	35.56 a CD	-10.53 b ABC	21.76 a CD	6.29 a CD	0.64 b
	Turning	Sept 29	2.04	40.9	26.27 b C	-0.08 a C	8.82 b B	4.02 b A	0.80 ab E
	Ripe	Nov 27	4.40	51.2	16.35 c C	-0.20 a C	-1.85 c C	2.14 c DE	0.87 a D
'Manzanilla'	Green	Sept 29	0.12	45.0	34.12 a DE	-10.94 b BC	21.33 a D	5.96 a D	0.87 b AB
	Ripe	Nov 27	5.88	50.6	17.64 b C	1.71 a BC	-1.52 b C	2.37 b CD	1.25 a B
'Marfil'	Green	Sept 29	0.04	46.1	50.26 b A	-13.91 b D	30.97 a A	7.57 a A	0.69 b C
	Ripe	Dec 12	0.96	34.2	66.39 a A	-3.27 a D	20.79 b A	1.98 b E	1.03 a D
'Morrut'	Green	Sept 29	0.16	27.0	35.00 b D	-10.48 c ABC	21.18 a D	7.15 a AB	0.83 a B
	Turning	Nov 27	1.04	37.2	38.73 a A	-5.12 b D	21.36 a A	3.21 b B	0.96 a D
	Ripe	Jan 16	3.40	45.0	22.12 c B	10.28 a A	1.72 b B	2.70 c B	0.95 a D
'Picual'	Green	Sept 29	0.30	35.6	32.01 a EF	-10.83 c BC	21.17 a D	6.92 a BC	0.86 c AB
	Turning	Nov 27	2.84	48.6	15.59 b D	7.71 a A	0.64 b C	2.97 b BC	1.49 b A
	Ripe	Nov 27	3.88	55.4	17.32 b C	1.14 b BC	-1.74 c C	2.59 c BC	1.69 a A
'Sevillenca'	Green	Sept 29	0.32	43.8	35.73 a BCD	-11.67 b C	23.91 b BC	5.26 a E	0.68 b CD
	Ripe	Nov 27	3.16	57.0	17.71 b C	0.14 a BC	-1.85 a C	2.26 b D	1.24 a BC

Maturity indices represent the weighted average of 50 olives. Oil content was determined jointly for 50 fruits, and values reported represent the average of the 50 olives assessed. For CIELAB colour parameters, maximum strength and deformation, values represent means of 10 olives assessed individually. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's *t* test).

L*, a*, b*: coordinates of CIELAB colour space.

Table 2. Yield of alcohol-insoluble residue (AIR) (g 100 g^{-1} FW), degree of methyl esterification of pectins (%, molar ratio), yields of AIR fractions (g 100 g^{-1} AIR) and of the final insoluble residue (g 100 g^{-1} FW) isolated from olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	AIR	d.e.	AIR fractions				Final insoluble residue
				W_{sf}	$NaOx_{sf}$	Na_2CO_{3sf}	KOH_{sf}	
‘Arbequina’	Green	7.8	56.9 b DEF	0.7 b D	2.6 b C	0.9 c D	1.8 b EF	7.31 a D
	Turning	7.6	49.8 b D	2.5 a A	5.0 ab D	1.7 b C	2.4 a CD	6.72 b D
	Ripe	3.6	64.8 a B	3.5 a CD	7.8 a B	2.3 a BCD	0.9 c E	3.08 c G
‘Argudell’	Green	12.3	77.9 b B	1.1 b D	7.6 b AB	0.9 a D	2.0 b DEF	10.90 a B
	Turning	11.4	93.1 a A	1.5 a A	9.2 a A	2.0 a C	2.7 ab BC	9.62 b C
	Ripe	8.4	78.4 b A	1.3 ab G	8.5 b B	2.2 a BCD	3.4 a BC	7.12 c B
‘Empeltre’	Green	5.1	88.5 a A	4.1 ab A	3.7 b C	3.3 a AB	4.5 b A	4.26 b F
	Turning	3.8	48.9 b D	1.9 b A	5.8 b CD	3.8 a A	4.7 ab A	3.21 c F
	Ripe	6.4	31.9 c D	6.7 a A	8.7 a AB	3.4 a ABC	5.5 a A	4.82 a E
‘Farga’	Green	8.6	52.1 b EF	2.9 b B	6.3 a B	2.8 a BC	4.1 a AB	7.20 a D
	Turning	5.0	58.2 b CD	2.5 b A	6.7 a BC	2.7 a BC	3.5 a B	4.26 b E
	Ripe	4.0	71.2 a AB	5.2 a B	6.5 a B	1.5 b CD	3.3 a BC	3.36 c F
‘Manzanilla’	Green	4.0	62.4 a CDE	2.0 b C	8.8 a A	4.6 a A	3.3 a BC	3.25 a G
	Ripe	3.0	67.5 a AB	4.0 a C	11.9 a A	4.9 a A	2.3 a D	2.30 b I
‘Marfil’	Green	7.93	49.8 a F	1.9 a C	6.6 b B	3.6 a AB	4.1 b AB	6.64 a E
	Ripe	3.5	50.9 a C	2.4 a EF	9.7 a AB	3.7 a AB	5.3 a A	2.78 b H
‘Morrut’	Green	15.2	70.0 a BC	1.1 b D	9.0 b A	1.3 a CD	1.1 b F	13.29 a A
	Turning	12.7	76.6 a B	2.0 a A	10.2 a A	1.9 a C	1.7 b D	10.69 b A
	Ripe	6.2	74.3 a AB	2.0 a F	8.5 b B	1.7 a BCD	2.7 a CD	5.31 c D
‘Picual’	Green	8.4	58.3 b DEF	1.1 b D	8.7 a A	3.1 a ABC	2.9 a CD	7.07 b D
	Turning	11.6	68.1 ab BC	2.3 a A	7.5 b B	3.4 a AB	1.6 b D	9.90 a B
	Ripe	7.3	76.5 a AB	2.5 a EF	7.8 ab B	3.1 a ABCD	1.4 b E	6.24 c C
‘Sevillenca’	Green	10.1	62.7 a CD	1.1 b D	7.6 a AB	2.1 a BCD	2.3 b DE	8.75 a C
	Ripe	9.7	67.5 a AB	2.9 a DE	8.0 a B	1.1 a D	3.7 a B	8.17 b A

Alcohol-insoluble residue (AIR) was recovered jointly from approximately 50 g fruit pericarp, obtained from 15 to 50 olives contingent upon fruit size. Degree of esterification values and fraction yields represent means of three replicate determinations. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student’s *t* test).

Abbreviations: AIR, Alcohol-insoluble residue; d.e., degree of methyl esterification of pectins; Wsf, water-soluble fraction; NaOxsf, sodium oxalate-soluble fraction; Na2CO3sf, sodium carbonate-soluble fraction; KOHsf, potassium hydroxide-soluble fraction.

Table 3. Neutral sugar content (g 100^{-1} g) in the alcohol-insoluble residue (AIR), and in AIR fractions isolated from olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	AIR_{sf}	AIR fractions	
			Na_2CO_{3sf}	KOH_{sf}
'Arbequina'	Green	12.62 a A	nd	28.11 a D
	Turning	7.38 b DE	2.80 b C	20.68 b E
	Ripe	13.33 a AB	10.05 a DE	11.70 c D
'Argudell'	Green	12.82 a A	3.62 a D	29.32 b D
	Turning	13.72 a AB	8.56 a B	35.40 a C
	Ripe	14.42 a A	7.70 a E	29.01 b A
'Empeltre'	Green	13.68 a A	16.33 a C	39.33 a C
	Turning	11.61 a BC	14.67 a A	42.14 a B
	Ripe	13.47 a AB	5.68 b E	28.42 b A
'Farga'	Green	12.24 a A	4.98 b D	20.86 c E
	Turning	10.41 a CD	18.01 a A	48.02 a A
	Ripe	10.84 a BC	5.53 b E	29.17 b A
'Manzanilla'	Green	14.40 a A	14.92 a C	39.54 a C
	Ripe	14.38 a A	13.07 a CD	24.51 b B
'Marfil'	Green	2.06 b C	19.92 b C	58.44 a A
	Ripe	9.92 a C	29.06 a A	19.36 b C
'Morrut'	Green	14.71 a A	8.73 b D	43.40 a B
	Turning	14.96 a A	16.44 a A	36.20 b C
	Ripe	13.11 a ABC	15.68 a C	21.97 c BC
'Picual'	Green	5.84 a BC	26.51 a B	39.72 a C
	Turning	6.77 a E	16.30 b A	28.42 b D
	Ripe	6.50 a D	16.24 b C	23.91 b B
'Sevillenca'	Green	8.23 b B	36.29 a A	19.19 a E
	Ripe	11.04 a BC	21.36 b B	13.22 b D

Values represent means of three replicates (nd, non-detectable). Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's t test).

Abbreviations: AIR, alcohol-insoluble residue; Na_2CO_{3sf}, sodium carbonate-soluble fraction; KOH_{sf}, potassium hydroxide-soluble fraction.

Table 4. Uronic acid content (g 100^{-1} g) in the alcohol-insoluble residue (AIR) and in AIR fractions isolated from olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	AIR	AIR fractions			
			W_{sf}	$NaOx_{sf}$	Na_2CO_{3sf}	KOH_{sf}
'Arbequina'	Green	8.25 a CD	6.88 a D	2.96 b B	34.93 a A	1.95 b DE
	Turning	6.46 b BC	1.71 b C	3.20 a AB	17.46 b A	1.94 b A
	Ripe	8.67 a B	1.93 b F	4.36 a A	11.01 c BC	3.03 a C
'Argudell'	Green	9.43 b BC	20.55 a A	0.73 b DE	8.90 a CD	2.64 a B
	Turning	10.98 a A	11.80 b A	1.13 a C	8.75 a C	1.98 b A
	Ripe	6.86 c CD	12.93 b A	1.83 a EF	8.81 a D	2.40 ab D
'Empeltre'	Green	6.16 ab E	1.66 b E	3.78 a A	10.38 a BC	2.68 a B
	Turning	6.91 a B	2.66 a C	3.54 ab A	11.80 a B	2.16 ab A
	Ripe	4.38 b G	0.30 c G	2.45 b CD	12.25 a B	2.00 b E
'Farga'	Green	6.90 a DE	2.14 a E	4.04 a A	11.44 b B	1.66 b EF
	Turning	5.56 b C	0.47 b D	4.07 a A	11.03 b B	2.45 a A
	Ripe	6.20 ab DE	1.49 a FG	2.75 b C	15.80 a A	2.44 a D
'Manzanilla'	Green	14.27 a A	8.39 a D	0.77 b D	6.88 a D	2.49 b BC
	Ripe	12.79 a A	2.33 b EF	3.54 a B	5.49 a EF	4.54 a A
'Marfil'	Green	14.00 a A	9.41 a CD	2.84 a B	8.64 b CD	2.41 a BC
	Ripe	7.25 b C	5.16 b C	1.94 b DEF	10.53 a C	2.58 a D
'Morrut'	Green	5.55 a E	13.31 a BC	0.50 b E	8.10 a D	3.25 a A
	Turning	3.95 b D	10.69 a A	1.33 a C	5.08 b D	2.43 b A
	Ripe	5.71 a EF	6.84 b B	1.54 a FG	4.85 b F	2.38 b D
'Picual'	Green	10.37 a B	17.27 a AB	1.30 c C	8.20 a D	1.51 c F
	Turning	6.69 b B	6.86 b B	2.01 b BC	5.04 b D	2.53 b A
	Ripe	6.13 b DE	3.52 c DE	2.21 a CDE	5.02 b F	4.04 a B
'Sevillenca'	Green	8.34 a CD	15.96 a B	0.98 b D	11.22 a B	2.11 a CD
	Ripe	4.97 b FG	3.62 b D	1.14 a G	6.61 b E	1.28 b F

Values represent means of three replicates. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's *t* test).

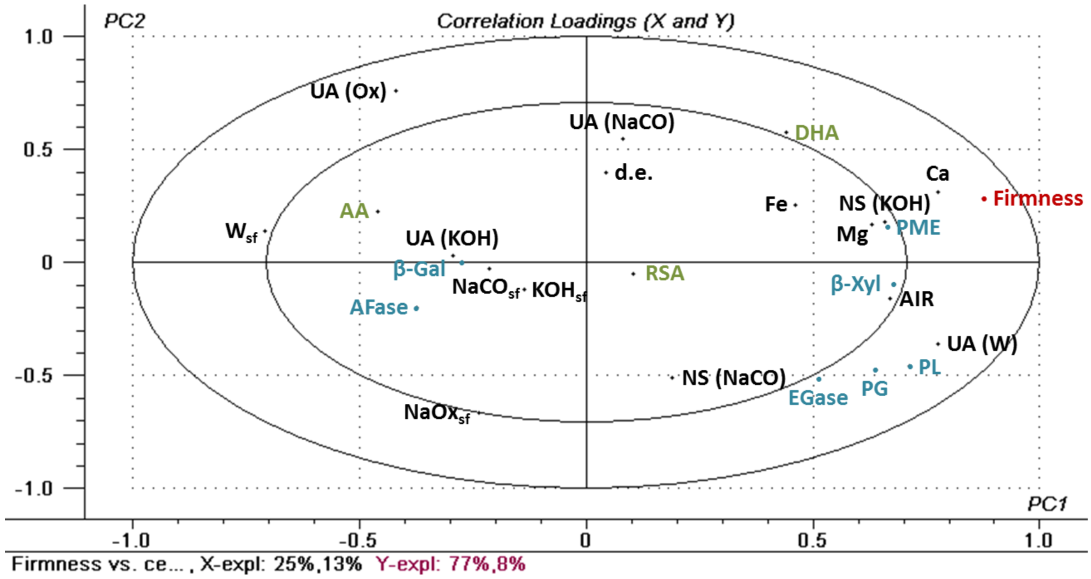
Abbreviations: AIR, alcohol-insoluble residue; W_{sf}, water-soluble fraction, $NaOx_{sf}$, oxalate-soluble fraction; Na_2CO_{3sf}, sodium carbonate-soluble fraction; KOH_{sf}, potassium hydroxide-soluble fraction.

Table 5. Specific activity (U mg^{-1} protein) of cell wall-related enzymes in acetone powders obtained from the pericarp of olive fruits at the green, turning and ripe stages.

		Non-pectolytic		Pectolytic				
				Backbone-acting			Side chain-acting	
Cultivar	**Maturity stage**	**β-Xyl**	**EGase**	**PG**	**PL**	**PME**	**AFase**	**β-Gal**
'Arbequina'	Green	0.032 a D	0.178 a E	0.681 a F	1.040 a D	60.373 a CD	0.049 b BC	0.109 c CD
	Turning	0.041 a B	0.131 b C	0.455 b E	0.613 b C	11.777 b C	0.071 a BC	0.159 b BC
	Ripe	0.033 a A	0.088 b D	0.201 c D	0.373 c E	9.871 b CD	0.068 a C	0.271 a C
'Argudell'	Green	0.088 a A	1.396 a BC	9.891 a C	5.073 a AB	167.170 a B	0.079 a A	0.395 ab AB
	Turning	0.053 b A	0.690 b A	2.432 b C	2.034 b C	22.981 b BC	0.087 a A	0.183 b B
	Ripe	0.024 c B	0.512 b C	2.356 b C	1.428 b CD	9.840 b CD	0.061 b CD	0.557 a B
'Empeltre'	Green	0.030 a D	0.262 ab E	1.142 ab F	0.829 ab D	24.344 a DE	0.040 b CD	0.244 a BCD
	Turning	0.032 a BC	0.423 a B	1.650 a D	2.048 a C	26.174 a B	0.043 b C	0.147 b BC
	Ripe	0.023 a B	0.119 b D	0.641 b D	1.100 b CD	3.480 b D	0.061 a CD	0.049 c D
'Farga'	Green	0.013 b E	0.159 a E	0.958 a F	2.190 a C	5.644 b E	0.026 c E	0.124 a CD
	Turning	0.018 b D	0.141 a C	0.640 b E	1.619 b C	23.704 a BC	0.043 b C	0.102 a CD
	Ripe	0.030 a A	0.071 b D	0.305 c D	0.970 c CDE	16.944 a B	0.105 a A	0.099 a CD
'Manzanilla'	Green	0.034 a D	1.441 a B	5.691 a D	2.807 a C	24.275 a DE	0.037 b DE	0.049 b D
	Ripe	0.009 b CD	0.101 b D	0.789 b D	1.547 b C	11.963 b BC	0.104 a A	2.158 a A
'Marfil'	Green	0.032 a D	0.828 b C	3.889 b E	2.940 a C	816.837 a A	0.033 a DE	0.085 b D
	Ripe	0.011 b CD	2.285 a A	9.700 a A	2.424 a B	45.134 b A	0.033 a E	0.132 a CD
'Morrut'	Green	0.049 a C	1.919 a A	12.562 a B	4.778 a B	182.640 a B	0.056 b B	0.605 a A
	Turning	0.029 b C	0.564 b AB	3.430 b B	2.649 b B	92.093 b A	0.070 a B	0.074 b D
	Ripe	0.007 c D	0.084 b D	0.628 c D	0.749 c DE	7.984 c CD	0.041 c E	0.523 a B
'Picual'	Green	0.061 a B	1.042 a CD	16.109 a A	5.843 a A	86.326 a C	0.049 a BC	0.329 ab BC
	Turning	0.034 b BC	0.660 b A	4.861 c A	3.436 b A	27.886 b B	0.048 a C	0.572 a A
	Ripe	0.029 b AB	0.735 b B	7.070 b B	3.370 b A	38.710 b A	0.055 a D	0.127 b CD
'Sevillenca'	Green	0.039 a CD	0.371 a E	3.408 a E	4.890 a AB	24.470 a DE	0.080 b A	0.168 a BCD
	Ripe	0.015 b C	0.129 b D	0.743 b D	2.554 b B	11.818 a BC	0.090 a B	0.129 a CD

Values represent means of three replicates. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's *t* test).

Abbreviations: β-Xyl, β-xylosidase; EGase, endo-1,4-β-D-glucanase; PG, polygalacturonase; PL, pectate lyase; PME, pectin methylesterase; AFase, α-L-arabinofuranosidase; β-Gal, β-galactosidase.



PC2
Correlation Loadings (X and Y)
1.0
0.5
0
-0.5
-1.0
UA (Ox)
UA (NaCO)
DHA
d.e.
Ca
Firmness
AA
Fe
NS (KOH)
W$_{sf}$
Mg
PME
UA (KOH)
β-Gal
RSA
β-Xyl
NaCO$_{sf}$
KOH$_{sf}$
AIR
AFase
UA (W)
PL
NS (NaCO)
PG
EGase
NaOx$_{sf}$
PC1
-1.0
-0.5
0
0.5
1.0
Firmness vs. ce... , X-expl: 25%,13% Y-expl: 77%,8%

Figure 1

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Phenotypical data of olive fruits used in this study.

Cultivar	Maturity stage	Weight (g)	F:S ratio*	Water content (%)	Length (mm)	Diameter (mm)
'Arbequina'	Green	1.10	2.68	53.9	14.1 ab D	12.1 b CD
	Turning	1.27	3.18	55.2	13.4 b C	12.0 b B
	Ripe	1.59	4.24	58.2	14.7 a D	13.0 a DE
'Argudell'	Green	2.02	4.08	56.0	18.6 a C	13.9 b BC
	Turning	2.65	5.32	59.2	20.1 a B	15.3 ab A
	Ripe	2.81	5.57	59.6	20.0 a BC	15.9 a B
'Empeltre'	Green	3.18	4.05	56.1	23.7 a A	15.2 a B
	Turning	3.09	4.40	55.4	23.0 a A	15.0 a A
	Ripe	3.13	4.00	49.3	24.1 a A	15.0 a BCD
'Farga'	Green	1.28	2.47	54.8	16. 9 b CD	10.8 b C
	Turning	1.74	3.18	58.7	19.0 a B	12.5 a B
	Ripe	1.82	3.70	55.8	18.1 a C	12.0 a E
'Manzanilla'	Green	4.57	8.31	70.1	24.0 a A	18.6 a A
	Ripe	4.65	7.68	66.6	23.9 a A	19.4 a A
'Marfil'	Green	1.32	2.05	60.1	19.7 b BC	10.5 b C
	Ripe	1.98	3.95	53.6	21.6 a AB	13.3 a CDE
'Morrut'	Green	1.99	2.03	51.6	20.4 b BC	13.8 b BC
	Turning	2.34	2.52	51.1	20.4 b B	13.8 b A
	Ripe	2.08	2.74	37.6	21.8 a AB	15.5 a BC
'Picual'	Green	2.72	2.75	57.2	22.4 a AB	15.1 a B
	Turning	3.06	3.11	60.2	22.2 a AB	15.3 a A
	Ripe	4.30	4.35	49.6	24.1 a A	17.3 a AB
'Sevillenca'	Green	2.71	3.09	56.7	21.4 a ABC	14.1 a BC
	Ripe	3.32	4.97	52.0	22.1 a AB	15.5 a BC

Values represent means of 50 fruits for weight, F:S ratio and water content, and of 10 fruits for length and diameter. Fruit weight, F:S ratio and water content were determined jointly for 50 fruits, and values reported represent the average of the 50 olives assessed. For length and diameter data, different capital letters denote significant differences among the cultivars for a given maturation stage, and different lower-case letters stand for significant differences among maturation stages for a given cultivar, at $P \leq 0.05$ (Student's t-test).

* F:S ratio, flesh to stone ratio.

Supplementary Table 2. Some chemical characteristics of olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	Anthocyanins (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	RSA (%)	AA (nmol g^{-1} DW)	DHA (nmol g^{-1} DW)
'Arbequina'	Green	0.4 b CD	27.6 a EF	94.4 a A	0.16 a A	0.11 a B
	Turning	0.7 b C	20.8 c D	88.2 b AB	0.13 b B	0.06 b D
	Ripe	2.5 a C	23.0 b CD	89.3 ab BC	0.16 a D	0.06 b AB
'Argudell'	Green	0.4 c CDE	37.8 a C	88.8 a BC	0.14 a B	0.02 b G
	Turning	0.6 b C	33.6 ab B	90.7 a AB	0.11 c C	0.04 a E
	Ripe	0.8 a D	33.1 b B	89.7 a BC	0.12 b E	0.04 ab CD
'Empeltre'	Green	0.3 c DE	26.8 a EF	88.7 a BC	0.08 b E	0.11 a B
	Turning	1.0 b A	21.0 b CD	81.2 b C	0.09 b D	0.11 a A
	Ripe	3.6 a B	20.7 b D	91.3 a AB	0.17 a CD	0.07 b A
'Farga'	Green	0.6 c A	25.2 b FG	82.4 c D	0.11 b CD	0.09 a CD
	Turning	0.8 b BC	19.6 c D	86.2 b BC	0.14 b AB	0.08 a C
	Ripe	3.7 a B	31.2 a B	90.5 a B	0.29 a B	0.06 b AB
'Manzanilla'	Green	0.6 b A	42.3 a B	93.5 a AB	0.11 b CD	0.06 a EF
	Ripe	8.0 a A	41.9 a A	91.1 a AB	0.33 a A	0.06 a AB
'Marfil'	Green	0.4 a CDE	22.3 a G	92.8 b AB	0.16 a A	0.20 a A
	Ripe	0.3 b D	22.7 a CD	98.9 a A	0.08 b F	0.05 b BCD
'Morrut'	Green	0.4 b DC	49.9 a A	93.2 a AB	0.10 b D	0.07 b DE
	Turning	0.7 b C	38.5 b A	91.7 a A	0.11 b C	0.09 a B
	Ripe	2.2 a C	24.3 c C	91.6 a AB	0.18 a C	0.05 c BC
'Picual'	Green	0.3 c E	34.1 a D	88.3 a BC	0.12 c C	0.09 a BC
	Turning	0.9 b AB	24.1 b C	87.1 a AB	0.15 b A	0.08 ab C
	Ripe	2.4 a C	24.7 b C	79.2 a D	0.17 a CD	0.06 b AB
'Sevillenca'	Green	0.5 b AB	28.1 a E	84.3 a CD	0.14 a B	0.05 a F
	Ripe	1.9 a C	12.3 b E	81.8 a CD	0.10 b F	0.03 b D

Values represent means of three replicates. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's t test).

Abbreviations: RSA, radical-scavenging capacity; AA, reduced ascorbic acid; DHA, dehydroascorbic acid.

Supplementary Table 3. Incidence of defects and disorders in olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	Fly [a] (%)	Fungus [b] (%)	Bruised (%)	Wrinkled (%)
'Arbequina'	**Green**	0	0	0	0
	Turning	14	0	0	0
	Ripe	10	0	6	0
'Argudell'	**Green**	12	100	0	0
	Turning	0	34	8	0
	Ripe	12	34	0	0
'Empeltre'	**Green**	60	0	0	0
	Turning	76	0	0	0
	Ripe	24	0	6	22
'Farga'	**Green**	4	0	0	100
	Turning	10	0	0	100
	Ripe	6	0	2	6
'Manzanilla'	**Green**	56	100	0	0
	Ripe	20	8	6	44
'Marfil'	**Green**	0	0	4	0
	Ripe	2	0	78	0
'Morrut'	**Green**	6	0	0	100
	Turning	0	0	18	6
	Ripe	4	2	18	0
'Picual'	Green	6	0	0	0
	Turning	6	0	4	0
	Ripe	8	0	0	2
'Sevillenca'	Green	24	0	0	0
	Ripe	0	0	16	28

Values represent the percentage of affected fruits within a sample of 50 olives.
[a] Infestation by *Bactrocera oleae*.
[b] Infection by *Camarosporium dalmaticum*.

Supplementary Table 4. Mineral content (mg kg^{-1} DW) in olive fruits at the green, turning and ripe stages.

Cultivar	Maturity stage	Boron (B)		Calcium (Ca)		Iron (Fe)		Magnesium (Mg)		Manganese (Mn)		Potassium (K)	
'Arbequina'	Green	20.85	a A	1167.93	a C	5.24	a BC	397.27	a EF	4.96	b B	18039.28	a B
	Turning	16.34	c A	775.41	c A	5.36	a BC	356.30	a C	5.37	a A	18445.57	a A
	Ripe	17.50	b A	864.24	b A	4.58	a C	372.63	a CD	5.40	a A	20171.60	b A
'Argudell'	Green	12.55	ab BCD	846.94	a D	6.37	a B	511.41	a ABC	4.57	a BC	16831.24	a BCD
	Turning	11.36	b B	750.94	b AB	5.87	a B	526.42	a A	4.70	a B	17629.81	a A
	Ripe	13.01	a B	737.58	b B	5.61	a B	509.93	a A	4.50	a C	16767.58	a B
'Empeltre'	Green	15.73	a B	521.16	a F	6.13	a B	383.96	a EF	3.75	a D	17497.64	a BC
	Turning	15.36	a A	506.48	a C	4.88	a C	383.15	a BC	3.81	a C	16362.33	a A
	Ripe	9.93	b CD	381.36	b DEF	5.55	a B	379.88	a CDE	3.11	b F	17379.98	a B
'Farga'	Green	8.48	ab D	689.16	a E	6.55	a B	523.13	a AB	5.64	a A	25893.75	a A
	Turning	9.99	a B	541.46	b C	7.33	a A	432.41	b B	4.71	b B	18340.79	b A
	Ripe	7.65	b D	458.40	b CD	6.81	a A	385.64	b CD	3.96	c D	17169.67	b B
'Manzanilla'	Green	9.65	a CD	685.86	a E	4.56	a C	350.84	a F	3.24	b E	14031.45	b CD
	Ripe	13.21	a B	711.18	a B	5.13	a BC	434.71	a B	4.87	a B	22449.74	a A
'Marfil'	Green	9.68	a CD	1837.64	a A	12.81	a A	552.95	a A	4.45	a C	25367.38	a A
	Ripe	12.70	a BC	413.00	b DE	4.61	b C	400.51	b BC	3.42	a EF	12680.52	b C
'Morrut'	Green	13.61	a BC	903.23	a D	4.44	b C	432.33	a DE	3.32	a DE	13389.29	a D
	Turning	17.13	a A	573.03	b C	3.90	c D	394.22	a BC	3.67	a C	15603.07	a A
	Ripe	13.03	a B	306.01	c F	5.49	a B	345.77	a D	3.32	a F	16528.14	a B
'Picual'	Green	16.82	a AB	1299.97	a B	6.30	a B	475.72	a BCD	4.70	a BC	18206.73	a B
	Turning	15.06	b A	691.40	b B	3.96	a D	332.13	b C	3.41	b C	16966.15	a A
	Ripe	13.94	b B	367.07	c EF	4.44	a C	299.38	b E	3.28	b F	16444.52	a B
'Sevillenca'	Green	14.08	a B	827.86	a D	5.09	b BC	439.66	a CDE	3.57	a DE	19181.58	a B
	Ripe	15.41	a AB	530.41	b C	7.06	a A	401.15	b BC	3.75	a DE	20046.42	a A

Values represent the means of two replicates per cultivar and maturity stage. Different capital letters denote significant differences among the cultivars for a given maturity stage, and different lower-case letters stand for significant differences among maturity stages for a given cultivar, at $P \leq 0.05$ (Student's *t* test).