NEW INSIGHTS ON THE RIPENING PATTERN OF 'BLANQUILLA' PEARS: A COMPARISON BETWEEN ON- AND OFF-TREE RIPENED FRUIT

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Abstract

To better understand the key processes involved in the ripening of attached fruit, we have investigated physico-chemical and biochemical changes occurring in 'Blanquilla' pear during on-tree (attached fruit) and off-tree ripening (harvested fruit). Flesh firmness, sugars, acids and the volatile profiles as well as ethylene metabolism, PG and PME enzyme activities and oxidative damage were measured. Firmness loss in detached 'Blanquilla' pear (off-tree), was initially mediated by oxidative stress (higher accumulation of malondialdehyde) and then by ethylene in a process in which 1-aminocyclopropene 1-carboxylic acid (ACC) synthase was the limiting factor. In contrast the progressive but slower softening observed during on-tree fruit ripening was not associated to oxidative damage but rather to a delayed production of ethylene limited, in turn, by the activity of ACC oxidase. An interesting association was found between the initiation of the ethylene production and a concomitant increase of sucrose levels during on-tree ripening also accompanied by a decline in hexanal. The putative role of these compounds as a tree-associated factor modulating on-tree pear ripening is discussed.

Keywords: ethylene, hexanal, oxidative stress, softening, sucrose, tree-factor.

1. INTRODUCTION

Fruit have been classified in climacteric and non-climacteric depending on their respiratory and ethylene production patterns during ripening (Paul et al., 2012). In climacteric fruit, an increase in the respiration rate and the ethylene production is observed at the onset of ripening, a phenomenon that is not observed in non-climacteric fruit (Lelievre et al., 1997). The autocatalytic ethylene production typically allows climacteric fruit to ripen once detached from the tree, whereas non-climacteric fruit do not have this capacity (Van de Poel et al., 2014).

Generally, it is accepted that most European pears, albeit classified as climacteric, are not able to completely ripen on-tree or at the time of commercial harvest unless they received a chilling or ethylene treatment (Villalobos-Acuña and Mitcham, 2008). Depending on the necessity of this chilling period, pears may be grouped into two classes: winter and summer pears. The first ones, include cultivars such as 'Comice' and 'Beurré d'Anjou', require long chilling periods after harvest to produce ethylene and therefore to start the ripening process (Villalobos-Acuña and Mitcham, 2008). That said, the length of the chilling period required to initiate the ripening of winter pears largely differ among cultivars. For instance, 'Beurré d'Anjou' pears may need up to 150 d of cold storage to induce ethylene production whereas 'Packham Triumph' pears may require no longer than 15 d of cold storage (Larrigaudière et al., 2016).

In contrast, summer pears such as 'Rocha' (Saquet and Almeida, 2017), 'Blanquilla' (Larrigaudière et al., 2004) or 'Conference' (Chiriboga et al., 2011) pears require minimum or no exposure to low temperatures to induce this process, as soon as they are harvested at the appropriate maturity. 'Blanquilla' pears also known as 'Spadona di Salermo' in Italy and 'Krystalli' in Greece, is a cultivar capable of ripening both off- and

on-tree (Larrigaudière et al., 2004). This specific behaviour makes this cultivar especially suitable to study the biochemical events differentiating off- and on-tree pear ripening.

Earlier studies suggested that the inhibition or delay of on-tree fruit ripening was related to the presence of an inhibiting substance called the 'tree factor'(Abeles, 1973) . The 'tree factor' was then thought to be exported from the leaves to the fruit via the phloem, and to affect the fruit ethylene production capacity (Sfakiotakis and Dilley, 1973).

Since then, different hypothesis have been proposed to explain the resistance to ripen on-tree for numerous fruit. The initial hypothesis was based on the climacteric characteristics of the fruit and on the existence of two systems of ethylene production, namely system 1 and system 2 (MCMurchie et al., 1972). System 1 is non-autocatalytic and operates in immature fruit whereas System 2 operates during ripening to induce the autocatalytic ethylene production (reviewed in Pech et al., 2008). According to this theory, on-tree ripening impairment is associated to the maintenance of system 1. Klee (2004) suggested that differences between cultivars and in the time of induction of system 2 on-tree, depended on the basal levels of ethylene production by system 1 (even if the ethylene levels were low). For others authors, differences between cultivars are related to differences in the fruit sensitivity to ethylene (Biale and Young, 1981; McGlasson, 1985), a sensitivity that is regulated at the receptor level (Kevany et al., 2007).

The regulation of fruit ripening in non-climacteric fruit and by extension, non-detached fruit, is essentially hormonal. Abscisic acid (ABA) was found to play an essential role in strawberry ripening by influencing softening, aroma development and anthocyanin accumulation (Jia et al., 2011, 2013). Jasmonic acid is also involved in strawberry cell wall metabolism (Mukkun and Singh, 2009) and anthocyanin accumulation in apples (Rudell et al., 2002). More recently, Jia et al. (2013) have shown that certain fruit

92 biochemical constituents, such as sucrose also play a key role as a signal involved in
93 strawberry and tomato fruit ripening.
94 Further studies are still needed to better understand the physiological basis of non-
95 climacteric and on-tree fruit ripening, especially for pears that may ripen or not on-tree.
96 Accordingly, the objective of this study was to compare the ripening behaviour of
97 'Blanquilla' pears ripening off- and on- the tree. Emphasis was given to monitor changes
98 in global quality traits but also, and especially, on the biochemical and physiological
99 processes explaining these quality changes.
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2. MATERIALS AND METHODS

2.1. Plant materials and experimental design

'Blanquilla' pear (*Pyrus communis* L.) were harvested on a commercial orchard near Lleida (Catalonia, Spain) at the optimum commercial harvest date (CH; about 125 d after full bloom, DAFB) for the off-tree trial, and 3, 6, 10, 15, 20, 25 and 30 d after commercial harvest (DACH) for the on-tree assay. Off-tree fruit were stored at 20 °C and 85 % of relative humidity and samples were evaluated at 3, 6, 10, 15 and 20 d. On-tree fruit were harvested and transported to the laboratory each sampling day for immediate analysis.

2.2. Quality evaluations

Flesh firmness was measured on 3 replicates of 6 fruit each per ripening condition with a hand held penetrometer (53200, T.R.Turoni srl., Italy) equipped with an 8 mm probe as described by Chiriboga et al. (2011). Total soluble solids (TSS; %) were measured on pear juice (blend of 6 fruit per replicate and 3 replicates per sampling) using a digital hand-held refractometer (PAL-1 , Atago, Tokyo, Japan) whereas titratable acid (TA) concentrations were measured on the same juice samples by titration using 0.1 N NaOH and the results expressed as g malic acid L^{-1}.

The index of absorbance difference ($I_{AD} = A_{670} - A_{720}$) as an indicator of the fruit maturity was measured with a DA-Meter (TR Turoni, Forli, Italy) on opposite sides of the equatorial parts of the fruit.

The starch index was evaluated on 18 fruit samples as described by Almeida et al. (2016) with some modifications. An equatorial slice of each fruit was cut and dipped in a solution of 0.6 % (w/v) iodine in 1.5 % (w/v) potassium iodine for 10 min and then the starch index was subjectively determined using the 10-point scale chart developed by the CTIFL (France). The Streif Index was calculated as [firmness / (SSC * starch index)].

In parallel, flesh tissue from six individual fruit per replicate and three replicates per ripening condition was frozen in liquid nitrogen and kept at -80 °C until further biochemical analysis.

2.3. Ethylene production

Ethylene production (nmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al. (2017) with some modifications. Four replicates of 3 fruit each were placed in 2 L flasks sealed with a silicon septum for sampling the gas of the headspace after 3 h incubation in an acclimatized chamber at 20 °C. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously described by Giné-Bordonaba et al. (2014).

2.4. Enzymes related to the ethylene metabolism and fruit softening

1-Aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) was extracted as described by Chiriboga et al. (2012) with some modifications. The sample (0.5 g of frozen tissue) was homogenized in 1 mL of buffer containing 400 mmol L^{-1} MOPS at pH 7.2, 10 % glycerol, 30 mmol L^{-1} ascorbic acid sodium salt and PVP 40000 2 %. The homogenate was gently shaken for 10 min at 1 °C and centrifuged at 17,000 *g* for 30 min at 4 °C. Subsequently, the supernatant was stored at -80 °C until analysis.

Enzyme activity was analysed as described in Giné-Bordonaba et al. (2017). The mixture was aired and incubated for 60 min at 30 °C, after which a 1 mL headspace gas sample was injected into a gas chromatograph and the results were expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

The extraction and activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) enzyme was determined as previously described by Chiriboga et al. (2013). Briefly, 5 g of

151 frozen tissue were homogenized with 10 mL of extraction buffer containing 200 mmol
152 L^{-1} tricine buffer at pH 8.5, 10 mmol L^{-1} dithiothreitol (DTT), 20 µmol L^{-1} pyridoxal
153 phosphate and 2 % (w/v) PVP. The homogenized was centrifuged at 18,000 *g* for 20 min
154 at 4 °C. Subsequently, 2.5 mL aliquot was loaded into a Sephadex G-25 column (PD 10,
155 GE Healthcare, Buckinghamshire, UK), previously equilibrated with 5 mmol L^{-1} tricine
156 buffer pH 8, 1 mmol L^{-1} DTT and 2 µmol L^{-1} pyridoxal 5-phosphate. The enzyme was
157 eluted with 3.5 mL of the same buffer and 1.5 mL was incubated for 2 h at 25 °C with 200
158 mmol L^{-1} tricine buffer pH 8 and 100 µmol L^{-1} of S-adenosyl-L-methionine (SAM). The
159 reaction was then stopped with 100 mmol L^{-1} $HgCl_2$, and 1mL of the product was mixed
160 and stirred with 100 µL of NaOCl and saturated with NaOH (2:1 v/v). After 2 min, a 1 mL
161 headspace gas sample was injected into a gas chromatograph and the results were
162 expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.
163 Pectin methyl esterase (PME; EC 3.1.1.11) enzyme was extracted using the method
164 described by Plaza et al. (2003). PME was extracted by homogenisation of 2 g of frozen
165 ground sample with 6 mL of an extraction solution (1 mol L^{-1} NaCl in 0.2 mol L^{-1} sodium
166 phosphate buffer pH 7.5). The resulting mixture was shaken for 10 min at 4 °C,
167 centrifuged at 16,000 *g* for 20 min at 4 °C and then the supernatant filtered through six
168 cheesecloth layers. Finally, PME activity from the resulting extract was quantified by
169 titration as described elsewhere (Yeom et al., 2000).
170 Polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15) extraction and
171 determination was conducted by following the methods described by Van linden et al.
172 (2008). PG activity was calculated as the release of reducing groups per unit of time and
173 per fresh weight (µmol kg^{-1} s^{-1}) based on the two reaction periods as described in Giné-
174 Bordonaba et al. (2017).
175 **2.5. Sugar and organic acid content**

176 Sugars (sucrose, glucose and fructose) and malic acid were extracted from frozen tissue
177 as described by Giné-Bordonaba et al. (2017). The supernatants of each sample extraction
178 were recovered and used for enzyme coupled spectrophotometric determination of glucose
179 and fructose (hexokinase/phosphoglucose isomerase) and sucrose (β-fructosidase) using
180 commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's
181 instructions.

182 Malic acid was extracted dissolving 2 g of frozen tissue in 5 mL of distillate water. The
183 resulting supernatant from malic extraction was recovered and used for enzyme coupled
184 spectrophotometric determination (L-malate dehydrogenase) of malic acid using
185 commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's
186 instructions.

187 **2.6. Determination of malondialdehyde content**

188 Malondialdehyde (MDA) was analysed as an index of lipid peroxidation using the
189 thiobarbituric acid reactive substrates (TBARS) and according to Martinez-Solano et al.
190 (2005) with some modifications. Briefly, 0.5 g of frozen tissue was homogenized in 4 mL
191 of 0.1 % trichloroacetic acid (TCA) solution. Then, the samples were centrifuged at
192 24,000 *g* for 20 min at 20 °C and 0.5 mL of the supernatant was added to 1.5 mL of a 0.5
193 % thiobarbituric acid (TBA) in 20 % TCA solution. Another aliquot (0.5 mL) of the
194 supernatant was added to a solution containing only 20 % TCA as a control. The mixture
195 was incubated at 90 °C for 30 min until stopped by placing the reaction tubes in an ice-
196 water bath. Then, the samples were centrifuged at 24,000 *g* for 10 min at 4 °C and the
197 absorbance of the supernatant was read at 532 nm. The value for non-specific absorption
198 at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was
199 calculated using the extinction coefficient 155 L $mmol^{-1}$ cm^{-1} and the results expressed as
200 μmol kg^{-1} on a fresh weight basis.

2.7. Volatile determination by SPME-GC-FID

The extraction and concentration of volatile compounds was done as described by Qin et al. (2012) with some modifications. A SPME fibre with 65-µm layer of polydimethylsiloxane–divinylbenzene (65 µm PDMS/DVB; Supelco Co., Bellefonte, PA, USA) was used and activated before sampling according to the manufacturer's instructions. For each extraction, 10 g of frozen tissue was placed into a 20-mL vial containing 3.6 g of NaCl to facilitate the release of volatile compounds. Before sealing the vial, 2 µL of 32 µL L^{-1} 3-nonanone was added as internal standard. A magnetic follower was added to each vial, which was placed into a constant-temperature water bath at 40 °C with stirring. Samples were equilibrated for 20 min and then the SPME fibres were exposed to the headspace of the sample for 30 min to adsorb the volatiles. The volatile compounds were subsequently desorbed over 10 min at 240 ºC into the splitless injection port of the chromatograph. The volatile constituents were identified and quantified with an HP 6890A gas chromatograph with a flame ionization detector equipped with a capillary column with cross-linked free fatty acids as the stationary phase (FFAP; 50m×0.2mm×0.33 mm). Helium was used as the carrier gas at a constant flow of 1.0 mL min^{-1}. The injector and detector temperatures were 240 ºC. The oven temperature programme was 35 ºC for 8 min, increasing at 2 ºC min^{-1} to 140 ºC and holding for 2 min, then increasing at 10 ºC min^{-1} to 240 ºC and holding for 5 min. Compounds were identified by comparing their respective retention index with those of standards. All of the standards for the volatile compounds studied in this work were analytical grade or the highest quality available. Quantification was performed using individual calibration curves for each compound. The concentrations of volatile compounds were expressed as µg kg^{-1} on a fresh weight basis.

225 Compound identification was performed on an Agilent 6890N gas chromatograph/mass
226 spectrometer (Agilent Technologies, Inc.) using the same capillary column as used in the
227 GC analyses. Mass spectra were obtained by electron impact ionization at 70 eV. Helium
228 was used as the carrier gas, and the same temperature gradient programme described
229 previously was used for MS acquisition. Spectrometric data were recorded (Hewlett-
230 Packard 3398 GC Chemstation) and compared with those from the original NIST
231 HP59943C library mass spectra.

232 **2.8. Statistical Analysis**

233 All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS
234 Institute Inc. Mean comparisons for the interaction ripening condition * day was
235 evaluated using Tukey's test at a significance level of $p \leq 0.05$, while comparisons
236 between ripening conditions at specific days was done by least significant difference
237 values (LSD; $p \leq 0.05$) using critical values of t for two-tailed tests for the rest of
238 parameters.

239 A principal component analysis (PCA) was also performed to characterize the samples
240 according to their volatile profile, quality parameters and biochemical traits. The samples
241 included in the PCA were: On-tree 0 d, On-tree 6 d, On-tree 25 d and Off-tree 6 d. A total
242 of 33 variables (26 volatile compounds, 3 quality parameters (firmness, titratable acidity
243 and total soluble solids) and 4 biochemical traits (sucrose, glucose, fructose and malic
244 acid)) were used to perform the data matrix. Data were centered and weighted using the
245 inverse of the standard deviation of each variable in order to avoid the influence of the
246 different scales used for the variables. All analyses were carried out using the PCA
247 platform of JMP® 13.1.0 SAS Institute Inc.

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3. RESULTS AND DISCUSSION

3.1. Changes in overall quality during on-tree and off-tree ripening

The flesh firmness of 'Blanquilla' pear at harvest was 60 N, which was inside the commercial harvest range for this pear cultivar (Gamrasni et al., 2010). After harvest, firmness from off-tree fruit decreased rapidly from days 3 to 6 (*ca.* -11.2 N/d) and then more gradually until reaching 5 N at 20 d. In contrast, the loss of firmness in attached fruit started 10 d later than that observed in detached fruit and was more progressive (-1.65 N/d), reaching 5 N only after more than 30 d (Fig. 1A).

TSS of the pears at harvest was 13 % (Fig. 1B), thereby similar to the results obtained in previous studies on other European pear cultivars like 'Jules d'Airolles', 'Abate Fetel' and 'Spadona'(Gamrasni et al., 2010; Yim and Nam, 2016). No differences in TSS were observed between off-tree and on-tree fruit until 5 d. Then, TSS was higher in off-tree samples than in those fruit ripened on-tree.

No significant differences were found for TA between off-tree and on-tree fruit (Fig.1C). TA at harvest was 4.10 ± 0.10 g malic L^{-1} and then steadily decreased (-0.07 g malic L^{-1}) until reaching values of *ca.* 2 g malic L^{-1} both for off-tree and on-tree fruit. TA values in this work were slightly higher than those observed in previous studies with 'Blanquilla' (Larrigaudière et al., 2004) for which TA values were always lower than 3 g malic L^{-1}. Different orchards or agroclimatic conditions may explain the differences in the TA values between both studies.

The SI at harvest was almost 6 (Fig. 1D) and then gradually increased until reaching values of completely mature fruit (SI = 10). The highest SI in off-tree fruit was reached 10 d after storage at 20 °C, whereas on-tree ripened fruit needed 25 d to reach the same value. The slower starch degradation during on-tree ripening may be explained by: (1) the differences in the ethylene production kinetics (Fig. 2A), since it is well documented

that starch degradation is for some pome fruit cultivars an ethylene-related phenomena (Thammawong and Arakawa, 2007) and a good indicator of the fruit maturity stage (Peirs et al., 2002); but also by (2) the continuous supply of carbohydrates from source-to-sink occurring in attached fruit and satisfying the fruit needs for respiration and other catabolic process.

Chlorophyll degradation measured by the index of absorbance difference I_{AD} (Fig.1E), as well as the Streif index (Fig.1F), also reflected the slower ripening pattern of attached fruit compared with detached fruit and highlighted the suitability of the former non-destructive measurement as a potential tool to determine the optimal harvest date in 'Blanquilla' pears. However, this tool less accurately represented quantitative differences, not only in firmness loss but in other quality attributes, and was less useful to follow the ripening process during off-tree ripened fruit.

3.2. Ethylene metabolism and its regulation on- and off-tree

Differences in the kinetics of ethylene production were found between off-tree and on-tree ripened fruit (Fig. 2A). Harvested fruit (off-tree) exhibited a typical pre-climacteric behaviour with a delay (5-6 d) in the initiation of ethylene production and then a sharp increase up to 0.12 nmol kg^{-1} s^{-1} at 20 DACH. The kinetic of ethylene production for on-tree fruit was much more progressive, with an extension of the lag period up to 10 d and a slower ethylene production rate, being nearly half of that observed in fruit ripened off-tree (Fig. 2A). Temperature conditions among on- and off-tree ripened fruit were relatively similar (Supplementary Figure 1) and did not explain the differences in ethylene production. However, it is also likely that warmer temperatures during on-tree ripening may lead to higher ethylene production, yet not reaching similar values to those observed in detached fruit.

298 The ability of ‘Blanquilla’ pear to produce ethylene at relatively high levels on-tree is
299 atypical in pears. Only one study have shown a similar tendency in ‘La France’ pears
300 which needed up to 14 d on-tree to produce 1 μL of ethylene kg^{-1} h^{-1} (equivalent to 0.01
301 nmol kg^{-1} s^{-1}, 10-fold lower values; Murayama et al., 1998). However, similar trends have
302 been reported in ‘Gala’ (Lin and Walsh, 2008) and others apple cultivars. Others summer
303 pear cultivars, such as ‘Conference’, are more resistant to production of ethylene and are
304 more difficult to ripen, even off-tree, if harvested when slightly immature (Chiriboga et
305 al., 2011).To better understand the specific behaviour of ‘Blanquilla’ pear regarding
306 ethylene production, we analysed the changes in ACC metabolism and more specifically
307 the changes in the activity of the enzymes ACS and ACO both off- and on-tree.
308 The differences of ethylene production between on- and off-tree samples (Fig. 2A) were
309 not exclusively explained by differences in ACS enzyme activity (Fig. 2B). In off-tree
310 samples, ACS activity remained inactive for 3 d and sharply increased thereafter until day
311 6. In on-tree fruit, a steady increase in the ACS activity was observed throughout the
312 different samplings. ACO activity in off-tree fruit was higher than on-tree (Fig. 2C),
313 increasing immediately after harvest and reaching a value of 0.17 nmol kg^{-1} s^{-1} at 3
314 DACH. Overall, our results suggest that ACO and ACS act differentially as limiting
315 factors for ethylene production during on- and off-tree ripening, respectively. ACO in on-
316 tree ripened fruit was activated only after 10 d, parallel to the increase in the ethylene
317 production rate (Fig. 2A and 2C). These findings support the theory of the ‘tree factor’
318 (Abeles, 1973) where it was hypothesized that the ‘tree factor’ is an inhibitor of ethylene
319 production exported from the leaves to the fruit via the phloem (Sfakiotakis and Dilley,
320 1973). This inhibitor is thought to affect System 2 ethylene production (Lin and Walsh,
321 2008) and its action may be inhibited by defoliation and girdling techniques (Sfakiotakis
322 and Dilley, 1973). Our results are in accordance with the ‘tree factor’ theory and with the

323 putative presence on an inhibitor on-tree. With this in mind, we analysed the differences
324 in assimilates accumulation (sugars and acids) and changes in the fruit volatiles during
325 off- and on-tree fruit ripening.

326 **3.3. Are assimilates involved in the regulation of on-tree pear ripening?**

327 Malic acid content did not differ between the fruit ripened on-tree and off-tree and
328 generally decreased from *ca*. 2 to 1.5 g kg^{-1} through storage at 20 °C or ripening on the
329 tree (Fig. 3A). Faster utilisation of malate in off-tree ripened fruit was observed from day
330 10 onwards if compared to fruit ripened on-tree. The decreased in malic acid off-tree was
331 paralleled by changes in DA-values, maximum starch index and opposite to the rise of
332 the ethylene. This pattern may be easily explained by the fact that malic acid is a
333 respiratory substrate and it is probably used by pear fruit as the carbon source in the
334 tricarboxylic acid cycle (Ma and Chen, 2003). Albeit no information is readily available
335 for pears, in grapes, malic acid is thought to be an important respiratory substrates
336 (Famiani et al., 2014) and postharvest studies on apples also point out the importance of
337 this compound in fruit respiration (Liu et al., 2016).

338 Only slight differences in glucose accumulation were observed between off- and on-tree
339 fruit until 10 d (Fig. 3B). Afterwards, the glucose levels slightly increased in off-tree
340 ripened fruit (up to 10 g kg^{-1}), whereas remained at a constant value of 5 g kg^{-1} in samples
341 ripened on-tree. In both on- and off-tree, fructose was the predominant sugar with
342 concentrations ranging from 40 to 50 g kg^{-1} (Fig. 3B). Accordingly, fructose, sorbitol,
343 sucrose and, in lower amount, glucose are known to be the major sugars in pears (Barroca
344 et al., 2006). In off-tree samples, sucrose levels decreased between 15 and 20 DACH (Fig.
345 3C) in parallel to a slight increases in glucose and fructose levels. This behaviour has also
346 been described by Itai et al. (2015) in 'Gold Nijisseiki' pear. In contrast, the sharp increase
347 in sucrose content observed in attached fruit after 10 d (Fig. 3C) was not related to

348 changes in those of glucose and fructose but coincident with the induction of the ethylene
349 burst and to the initiation of the ripening process. This last result is of interest and shows,
350 in agreement with the results described by Murayama et al. (2015) or Kim et al., (1987)
351 that sucrose or galactose may act as a signal molecule for on-tree fruit ripening. Indeed,
352 previous studies have also shown that galactosyl compounds stimulate C_2H_4 production
353 in tomato (Kim et al., 1987).
354 There is clear evidence that sucrose may play a pivotal role in different processes of plant
355 biology such as for instance the signalling of assimilates partitioning (Chiou and Bush,
356 1998) or the induction of anthocyanin biosynthesis (Teng et al., 2005). It is also
357 recognized that sucrose plays an important role in the regulation of tomato (climacteric)
358 and strawberry (non-climacteric) fruit ripening (Jia et al., 2016, 2013). Our results further
359 suggest a pivotal role of this sugar in pear ripening and especially when understanding
360 the capacity to ripening on-tree. Although on-tree pear fruit cannot be considered as non-
361 climacteric fruit model, the overall physiological changes observed in attached pears, and
362 especially regarding ACO, let us to hypothesize that on-tree pear ripening may be
363 regulated by similar effectors than those controlling non-climacteric fruit ripening.
364 Accordingly, sucrose may act as an important regulatory factor of 'Blanquilla' pear
365 ripening on-tree. Further studies are needed to confirm these results and especially to
366 determine the role that sucrose or its interplay with other key hormones (i.e. abscisic acid)
367 may have on regulating on-tree ripening.

368 **3.4. The physiological basis of firmness loss both on- and off-tree in relation to cell-**
369 **wall degrading enzymes and oxidative stress**

370 It is interesting to note the lack of a relationship observed between the ethylene production
371 and softening for off-tree ripened fruit. Loss of firmness was observed after 3 d of
372 ripening at 20 °C (Fig. 1A), while detectable ethylene production started only after 5 d

373 (Fig. 2A). Since firmness loss is assumed to be an ethylene dependent process, such
374 atypical behaviour in 'Blanquilla' pear remains to be clarified.
375 Hence, in an attempt to further understand the softening pattern of 'Blanquilla' pear we
376 investigated the activity of some cell-wall degrading enzymes including PME and PG
377 which are thought to be ethylene-dependent (Pech et al., 2008). No differences in PG
378 activity were observed between off-tree and on-tree ripened fruit (Table 1). PG activity
379 remained at a constant levels both on- and off-tree indicating that this enzyme is likely
380 ethylene-independent in 'Blanquilla' pear. In other studies, it has also been demonstrated
381 that initial fruit softening (i.e. in tomato) is associated with a decline in some cell wall
382 components without increased PG activity (Gross and Wallner, 1979). Only slight
383 differences between on- and off-tree samples were found for PME (Table 1), suggesting
384 also that this enzyme may not have a pivotal role in 'Blanquilla' pear softening. These
385 results are consistent with those observed in 'Golden Reinders' apples (Ortiz et al., 2011)
386 and also in tomato (Tieman and Handa, 1994) where PME activity did not play a key role
387 on fruit softening.
388 Since differences in the softening pattern between on-tree and off-tree samples were not
389 explained by cell-wall degrading enzymes nor by ethylene metabolism, we hypothesized
390 that such differential pattern could be mediated by oxidative stress. Accordingly, we
391 measured the MDA contents (Fig. 4A), a typical marker of oxidative stress resulting from
392 lipid peroxidation.
393 At harvest, the concentration of MDA was about 17 μmol kg^{-1}. In off-tree samples, this
394 concentration increased up to 30 μmol kg^{-1}during the first week of storage. At the same
395 time, ethylene production remained low and the fruit lost more than half of their initial
396 firmness. These results clearly suggest that initial firmness loss (from 3 to 6 DACH) in
397 off-tree 'Blanquilla' pear was not ethylene dependent but rather associated to oxidative

stress. This process may also be related to the 'water-stress' phenomena experienced by detached fruit, which in turn may be linked to ABA. A strong negative correlation was observed between the MDA content and firmness (Fig. 4B). A similar behaviour was also observed in Japanese pear (Li and Wang, 2009) for which an 8-fold increase in MDA content was described after 6 d at room temperature if compared to the values at harvest. In contrast to off-tree fruit, MDA levels in on-tree ripened fruit only significantly increased after 25 d and regardless of the changes in ethylene production and firmness loss. Overall, these results suggest that oxidative stress was unlikely involved in the firmness loss observed after 10 d and in the induction of the ripening process in fruit ripened on-tree. As described earlier, and in contrast to off-tree ripened fruit, on-tree fruit softening appeared to be exclusively ethylene dependent.

Collectively these results are of interest and highlight clear differences in the susceptibility of off- and on-tree fruit to oxidative stress that likely determine the initiation of fruit ripening.

3.5. Changes in the volatile profile during on and off-tree ripening

Pears are highly appreciated by consumers due in part to their unique and complex aroma profiles associated to each each specific cultivar (Chen et al., 2018) but also to its characteristic ripening process. Accordingly, we investigated whether ripening off- tree impaired or enhanced the development of the 'Blanquilla' pear volatile profile. Fifteen compounds belonging to different chemical classes: esters (8), aldehydes (2), alcohols (4) and terpene (1) were identified (Table 2). The predominant compounds in all the samples with concentrations higher than 1500 μg kg^{-1} were hexanal, butyl butanoate, and α-farnesene, as found for 'Yali' pear (Chen et al., 2006). The presence and abundance of volatile compounds on fruit ripened off-tree for 6 d was similar to that observed on fruit ripened on-tree for 25 d, both of them characterised with similar firmness values. No

423 significant differences in the concentration of any volatile compound were found between
424 these samples, except hexyl acetate, which was almost 3-fold higher in fruit ripened off-
425 tree for 6 d. In the case of ripe fruit (On-tree 25d and Off-tree 6d), 4 straight esters (propyl
426 acetate, butyl acetate, pentyl acetate and hexyl acetate) and one alcohol (1-butanol) were
427 the majority compounds. 1-Butanol was present in all the samples analysed being *ca.* 20-
428 fold higher in ripe than in unripen fruit (0 DACH). These results are in accordance with
429 previous studies in 'Bartlett' pears, where 1-butanol concentration drastically increased
430 during ripening (Zlatić et al., 2016). In addition, it has been shown that completely ripe
431 pear have a higher concentration of esters than firmer ones (Makkumrai et al., 2014), so
432 this may explain the fact that the majority of esters in our samples were present in fruit
433 ripened on-tree for 25 d and in off-tree for 6 d yet not at the time of commercial harvest.
434 Hexanal was the principal aldehyde and there were no significant differences neither
435 between the content in immature fruit (on-tree 0 d and on-tree 6 d), nor between ripe fruit
436 samples (off-tree 6 d and on-tree 25 d). In contrast, there were significant differences for
437 this compound when comparing different DACH, the hexanal content declining as fruit
438 ripened off-tree but also on-tree. In on-tree ripened fruit (25 DACH), hexanal contents
439 were nearly half than that observed in fruit at the time of commercial harvest. Similar
440 results were obtained by Makkumrai et al. (2014) in 'Bartlett' pear, where hexanal also
441 decreased as the fruit ripened. Interestingly, hexanal contents remained high during the
442 first days of on-tree fruit ripening, when no softening occurs, pointing out a potential role
443 of this compound to modulate on-tree ripening process of 'Blanquilla' pear. Exogenous
444 application of this compound are known to inhibit fruit ripening (Pak Dek et al., 2018) by
445 decreasing transcript levels of phospholipase D and other ripening-related genes.
446

3.6. Exploring the organoleptic changes occurring during on- and off-tree pear ripening

To further explore the relationship between the taste-related (individual sugars, malic acid, TSS and TA) and the volatile composition of fruit ripened on- and off-tree, we performed a multivariate analysis. A principal component analysis (PCA) was carried out to assess differences between on-tree and off-tree samples or among the different days on-tree. Two principal components 1 (PC1) and 2 (PC2) were sufficient to explain 75.7 % of total variability of the samples (Fig. 5). There were three well-separated groups: one to the left of the plot corresponding to the samples on-tree 0 d and on-tree 6 d, a second group on the top right of the plot corresponding to samples ripened on-tree for 25 d, and the third group located in the middle down corresponding to the samples ripened off-tree for 6 d. The highest emissions of α-farnesene, 2-methyl-1-butanol, octyl acetate, hexanal and butyl hexanoate, together with high firmness and high glucose concentration, were found for fruit harvested at the optimum commercial date (0 DACH) but also for fruit ripened on-tree for 6 d (more immature fruit). Conversely, fruit let attached on the tree for 25 d, showed the lowest emissions of these variables along with the highest emission of ethanol, hexyl propanoate, propyl acetate, butanol, benzyl alcohol, together with high concentrations of sucrose, fructose and total soluble solids (TSS). The last group was related to the samples ripened off-tree for 6 d which was characterised by including those samples with higher amounts of butyl, hexyl and pentyl acetate, all of these compounds being previously identified as primary contributors to pear aroma (Suwanagul et al., 1998). Overall, and despite the lack of significant differences when considering absolute values, on-tree ripened fruit have a distinct volatile blend and physicochemical characteristics than fruit ripened off-tree. Future studies should address if such differences can result in different consumer preferences.

3 CONCLUSIONS

The results from this study provide new information on the biochemical events differentiating on-tree and off-tree 'Blanquilla' pear ripening. In both samples, ACC metabolism plays a key role yet under different regulatory mechanisms. In off-tree pears, ripening (ethylene production and softening) is initially regulated by oxidative stress that likely promotes the further autocatalytic burst of ethylene production through an activation of ACS. In contrast, no oxidative stress was detected in on-tree fruit, where ripening seems to be regulated by ACO and initially inhibited by hexanal, but also, in the later stages of ripening, by the accumulation of sucrose possibly triggering the initiation of ethylene production.

Future studies are required to better understand the role that ethylene, volatiles and sucrose or its interplay with other crucial hormones such as ABA may have in the ripening process of pear fruit, and especially in other pear cultivars that do not have the capacity to ripen on-tree. The results from this study may provide a better understanding of the ripening process in attached pears hence making easier the decisions for optimal harvest in terms of fruit quality.

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Table 1: PG (nmol kg^{-1} s^{-1}) and PME (µmol kg^{-1} s^{-1}) levels in Blanquilla pear during off-tree and on-tree ripening. Means ± standard deviation followed by the same letter are not significant different at $p \leq 0.05$ (n=3).

PG (nmol kg^{-1} s^{-1})			PME (µmol kg^{-1} s^{-1})		
DACH	Off-tree	On-tree	DACH	Off-tree	On-tree
0	19.38±2.052 a	19.38±2.052 a	0	6.64±1.838 bc	6.64±1.838 bc
3	18.15±2.613	-	3	5.20±0.797	-
6	16.47±1.385 b	20.18±1.871 ab	6	4.52±1.019 cd	10.36±0.207 a
10	18.06±2.937 ab	17.91±0.418 ab	10	5.23±0.759 bcd	11.07±1.639 a
15	17.84±1.182	-	15	5.29±0.906	-
20	18.08±2.167 ab	23.09±1.708 a	20	8.84±0.323 ab	3.60±0.738 cd
25	-	18.30±1.632	25	-	5.67±1.108
30	-	17.44±0.764	30	-	2.72±0.955

707 **Table 2:** Average concentrations of volatile compounds. Means ± standard deviation
708 followed by the same small letter indicate no significant differences among the control
709 and on-tree samples for each compound. Means ± standard deviation followed by the
710 same capital letter indicate no significant differences between the samples on-tree 25d
711 and off-tree 6d for each compound ($p \leq 0.05$; n=3). nd = non detected.

712

Volatile compounds (µg kg^{-1})	**On-tree**			**Off-tree**
	0d (61.1 N)	6d (62.9 N)	25d (25.3 N)	6d (23.6 N)
Ethanol	nd	nd	0.46±0.158 A	0.22±0.025 A
Propyl acetate	nd	nd	543.36±332.428 A	372.10±108.541 A
Butyl acetate	nd	nd	8620.90±2668.309 A	16953.53±5685.289 A
Hexanal	18080.67±1344.572 a	19547.21±2445.170 a	10635.48±4289.035 bA	7897.73±2053.258 A
Butanol	23.77±10.561 b	17.17±2.011 b	375.36±123.527 aA	415.84±103.391 A
Pentyl acetate	nd	nd	844.64±158.209 A	1363.10±582.786 A
2-Methyl-1-Butanol	177.12±19.420 ab	200.60±54.486 a	95.51±25.378 bA	68.82±10.577 A
Butyl butanoate	3921.15±1387.091 a	2653.79±998.638 a	3041.76±1068.421 aA	4194.76±501.560 A
Hexyl acetate	nd	nd	9435.01±112.496 B	24294.35±3825.698 A
Hexyl propanoate	nd	nd	50.00±10.156	nd
Butyl hexanoate	36.33±1.810	nd	nd	nd
Octyl acetate	262.58±40.925 a	371.63±90.566 a	223.14±34.882 aA	175.28±33.557 A
Benzaldehyde	nd	nd	27.91±5.930	nd
α-farnesene	3864.85±565.301 a	3626.88±261.110 ab	2098.00±717.585 bA	1595.90±343.353 A
Benzyl alcohol	nd	nd	67.19±4.980	nd

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720 **LIST OF FIGURES**

721

722 **Figure 1**. Changes in fruit firmness (A), total soluble solids (B), titratable acidity (C),
723 starch index (D), DA-value (E) and Streif index (F) during off-tree (●) and on-tree (○)
724 ripening. DACH stand for Days After Commercial Harvest. Error bars represent the
725 standard deviations of the means (n=3). Stars indicate significant differences at $p \leq 0.05$.

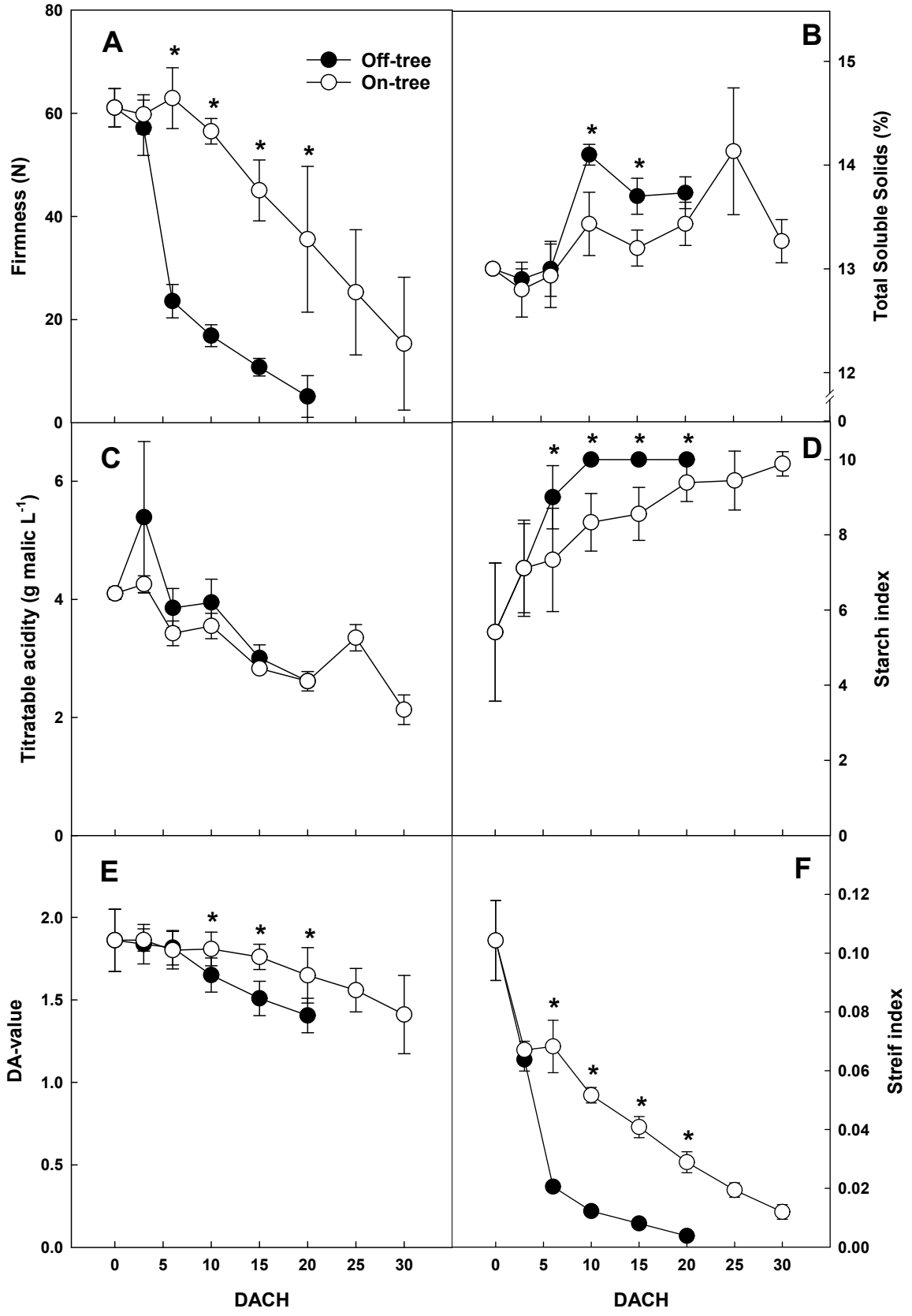
726 **Figure 2**. Changes in ethylene production (A), ACC synthase activity (B) and ACC
727 oxidase activity (C) in off-tree (●) and on-tree (○) ripening. DACH stand for Days After
728 Commercial Harvest. Error bars represent the standard deviations of the means (n=4 for
729 ethylene production and n=3 for ACC and ACS activity). Stars indicate significant
730 differences at $p \leq 0.05$.

731 **Figure 3.** Changes in malic acid content (A), D-Glucose and D-fructose levels (B) and
732 sucrose levels (C) during off-tree (●) and on-tree (○) ripening. DACH stand for Days
733 After Commercial Harvest. Error bars represent the standard deviations of the means
734 (n=3). Stars indicate significant differences at $p \leq 0.05$.

735 **Figure 4**. Changes in the concentration of malondialdehyde (A) and the correlation
736 between firmness and MDA content (B) during off-tree (●) and on-tree (○) ripening.
737 DACH stand for Days After Commercial Harvest. Error bars represent the standard
738 deviations of the means (n=3). Stars indicate significant differences at $p \leq 0.05$.

739 **Figure 5.** Principal components analysis of volatile profile, quality parameters and
740 biochemical traits in control fruit (OHD = 0d on-tree), fruit ripened 6d on-tree, fruit
741 ripened 25d on-tree and fruit ripened 6d off-tree.

742



743 **Figure 1**:

744

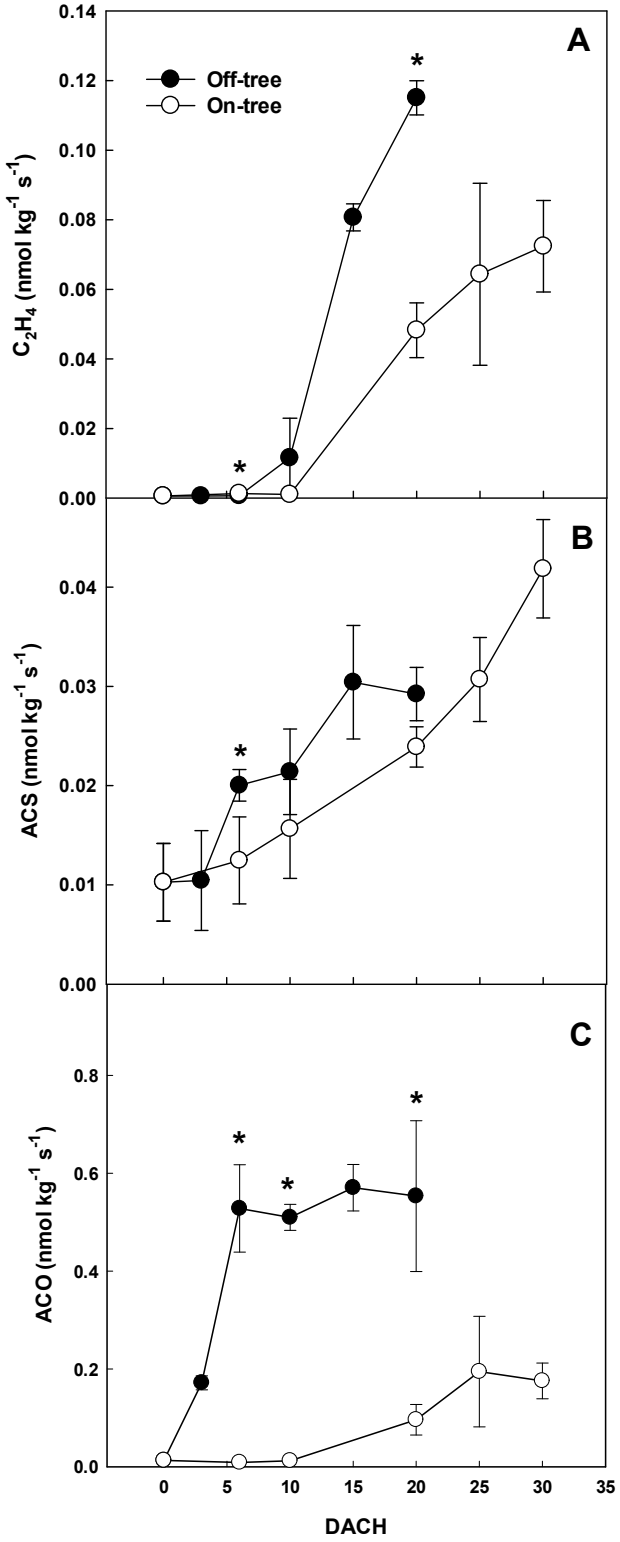

A
B
C
Off-tree
On-tree
C_2H_4 (nmol kg^{-1} s^{-1})
ACS (nmol kg^{-1} s^{-1})
ACO (nmol kg^{-1} s^{-1})
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.04
0.03
0.02
0.01
0.00
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
DACH
*

Figure 2:

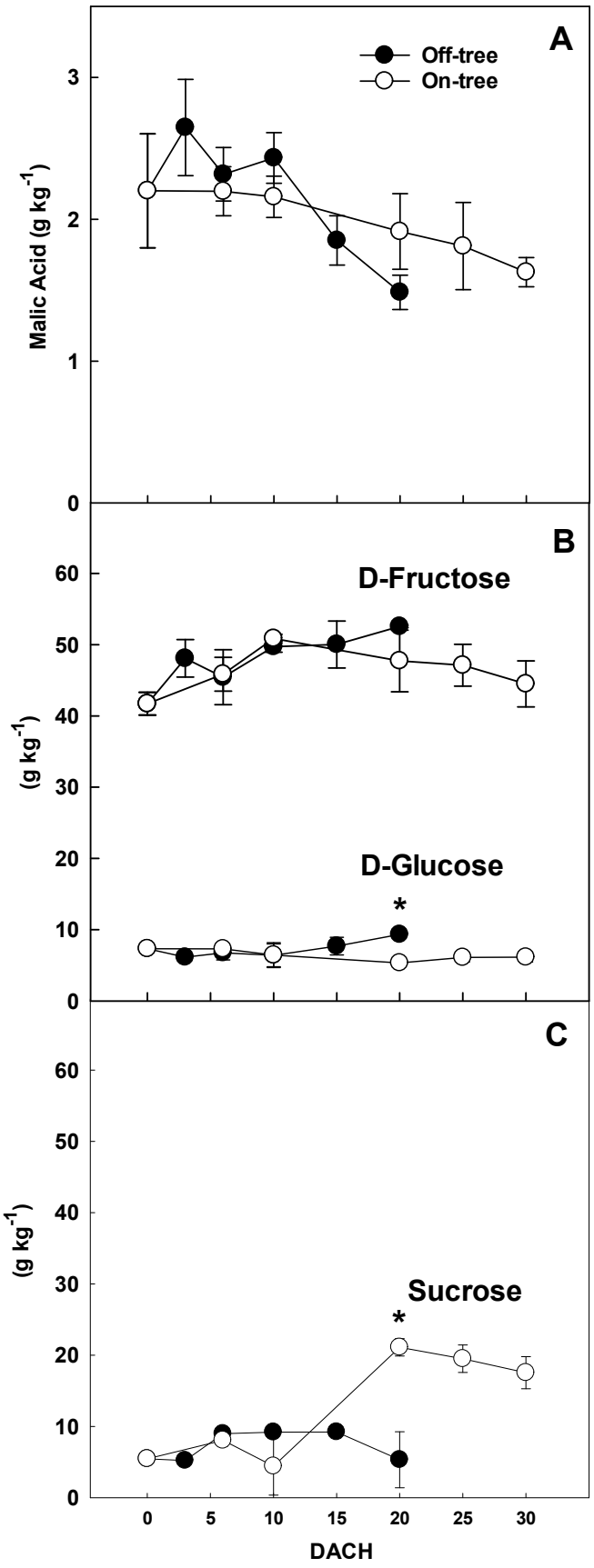

A
Off-tree
On-tree
Malic Acid (g kg-1)
B
D-Fructose
D-Glucose
(g kg-1)
*
C
Sucrose
(g kg-1)
*
DACH

Figure 3:

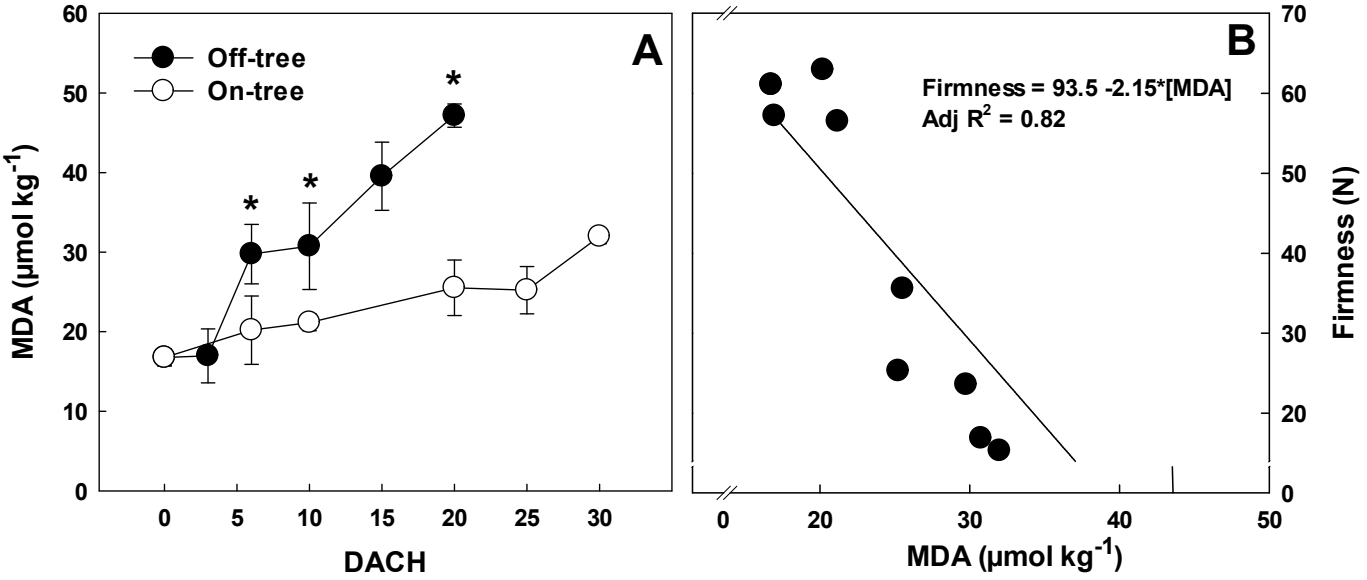

A
Off-tree
On-tree
MDA (µmol kg-1)
DACH
B
Firmness = 93.5 -2.15*[MDA]
Adj R2 = 0.82
MDA (µmol kg-1)
Firmness (N)

Figure 4:

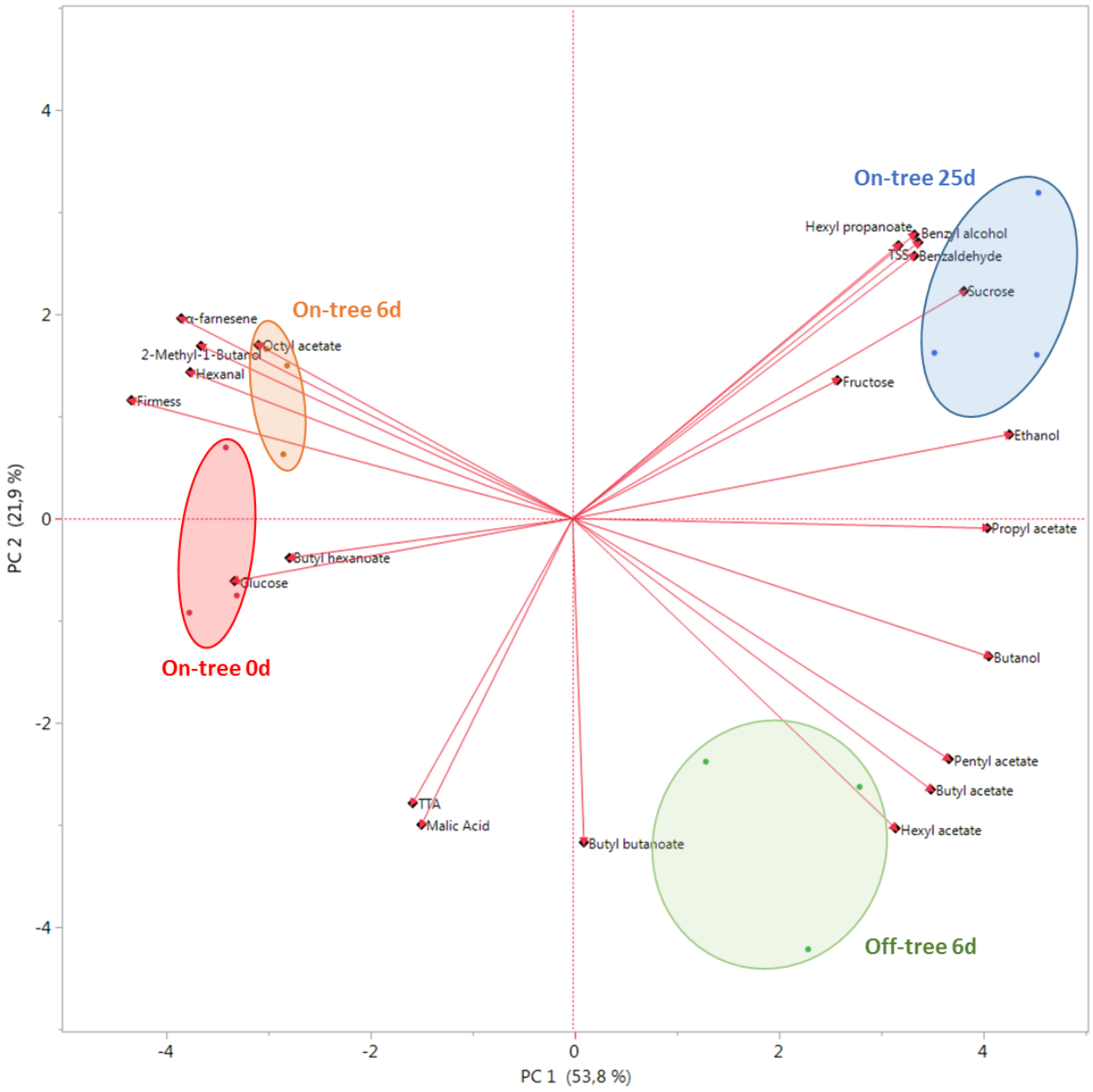
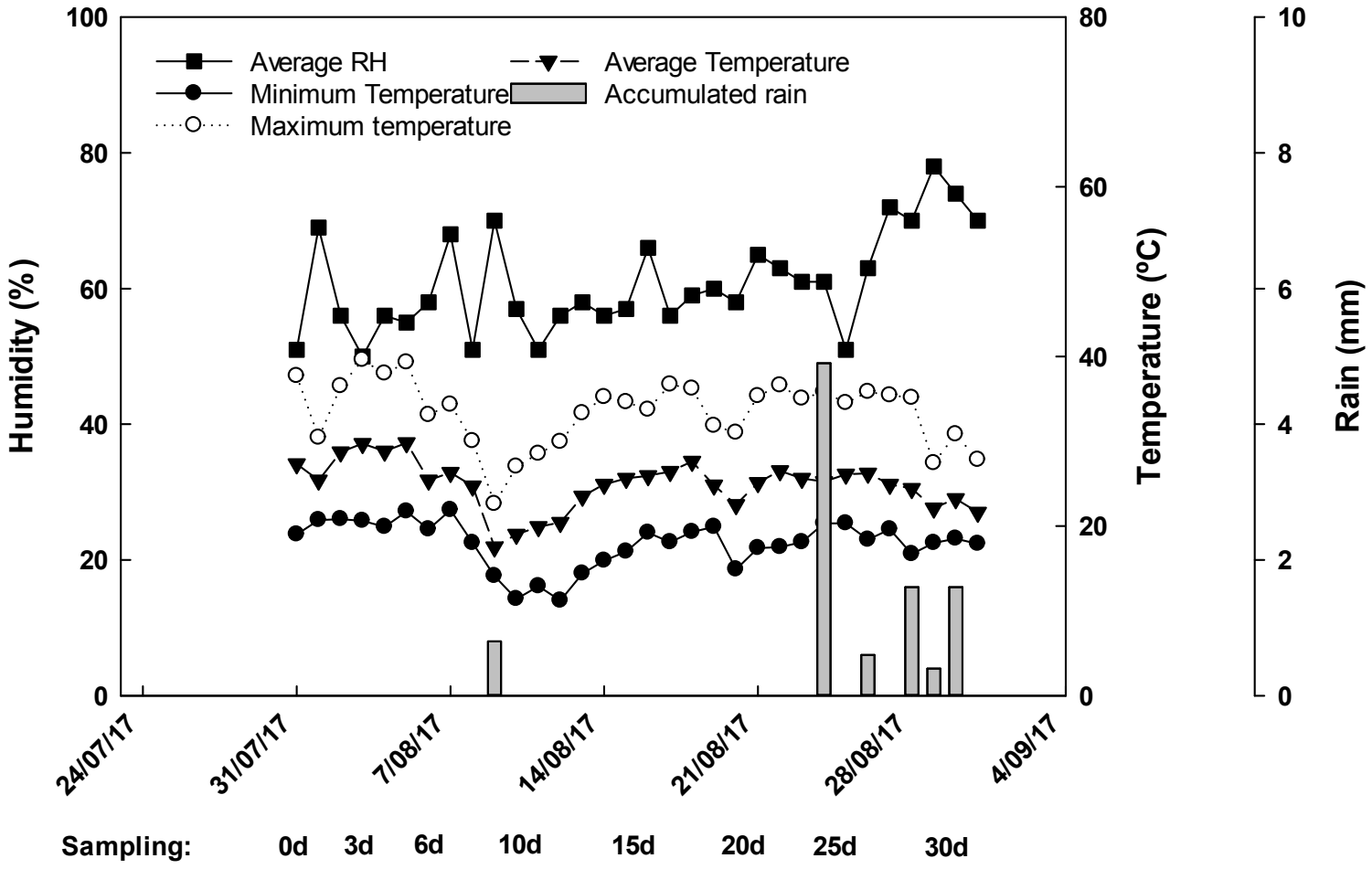

On-tree 25d
On-tree 6d
On-tree 0d
Off-tree 6d
Hexyl propanoate
Benzyl alcohol
TSS
Benzaldehyde
Sucrose
Fructose
Ethanol
Propyl acetate
Butanol
Pentyl acetate
Butyl acetate
Hexyl acetate
Butyl butanoate
TTA
Malic Acid
Butyl hexanoate
Glucose
α-farnesene
Octyl acetate
2-Methyl-1-Butanol
Hexanal
Firmess
PC 2 (21,9 %)
PC 1 (53,8 %)
4
2
0
-2
-4
-4
-2
0
2
4

Figure 5:



821

822 **Supplementary Figure 1:** Temperature, relative humidity and rainfall during the period between samplings for on-tree ripened fruit. Off-tree
823 ripened fruit were stored at 20±0.5 ºC and 85% RH.