# Universitat de Lleida

1 **NEW INSIGHTS ON THE RIPENING PATTERN OF 'BLANQUILLA' PEARS:**

2 **A COMPARISON BETWEEN ON- AND OFF-TREE RIPENED FRUIT**

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22    **Abstract**

23    To better understand the key processes involved in the ripening of attached fruit, we have

24    investigated physico-chemical and biochemical changes occurring in 'Blanquilla' pear

25    during on-tree (attached fruit) and off-tree ripening (harvested fruit). Flesh firmness,

26    sugars, acids and the volatile profiles as well as ethylene metabolism, PG and PME

27    enzyme activities and oxidative damage were measured. Firmness loss in detached

28    'Blanquilla' pear (off-tree), was initially mediated by oxidative stress (higher

29    accumulation of malondialdehyde) and then by ethylene in a process in which 1-

30    aminocyclopropene 1-carboxylic acid (ACC) synthase was the limiting factor. In contrast

31    the progressive but slower softening observed during on-tree fruit ripening was not

32    associated to oxidative damage but rather to a delayed production of ethylene limited, in

33    turn, by the activity of ACC oxidase. An interesting association was found between the

34    initiation of the ethylene production and a concomitant increase of sucrose levels during

35    on-tree ripening also accompanied by a decline in hexanal. The putative role of these

36    compounds as a tree-associated factor modulating on-tree pear ripening is discussed.

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43    **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

68    on-tree (Larrigaudière et al., 2004). This specific behaviour makes this cultivar especially

69    suitable to study the biochemical events differentiating off- and on-tree pear ripening.

70    Earlier studies suggested that the inhibition or delay of on-tree fruit ripening was related

71    to the presence of an inhibiting substance called the 'tree factor'(Abeles, 1973) . The 'tree

72    factor' was then thought to be exported from the leaves to the fruit via the phloem, and to

73    affect the fruit ethylene production capacity (Sfakiotakis and Dilley, 1973).

74    Since then, different hypothesis have been proposed to explain the resistance to ripen on-

75    tree for numerous fruit. The initial hypothesis was based on the climacteric characteristics

76    of the fruit and on the existence of two systems of ethylene production, namely system 1

77    and system 2 (MCMurchie et al., 1972). System 1 is non-autocatalytic and operates in

78    immature fruit whereas System 2 operates during ripening to induce the autocatalytic

79    ethylene production (reviewed in Pech et al., 2008). According to this theory, on-tree

80    ripening impairment is associated to the maintenance of system 1. Klee (2004) suggested

81    that differences between cultivars and in the time of induction of system 2 on-tree,

82    depended on the basal levels of ethylene production by system 1 (even if the ethylene

83    levels were low). For others authors, differences between cultivars are related to

84    differences in the fruit sensitivity to ethylene (Biale and Young, 1981; McGlasson, 1985),

85    a sensitivity that is regulated at the receptor level (Kevany et al., 2007).

86    The regulation of fruit ripening in non-climacteric fruit and by extension, non-detached

87    fruit, is essentially hormonal. Abscisic acid (ABA) was found to play an essential role in

88    strawberry ripening by influencing softening, aroma development and anthocyanin

89    accumulation (Jia et al., 2011, 2013). Jasmonic acid is also involved in strawberry cell

90    wall metabolism (Mukkun and Singh, 2009) and anthocyanin accumulation in apples

91    (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)].

126 In parallel, flesh tissue from six individual fruit per replicate and three replicates per

127 ripening condition was frozen in liquid nitrogen and kept at -80 °C until further

128 biochemical analysis.

129 **2.3. Ethylene production**

130 Ethylene production (nmol kg$^{-1}$ s$^{-1}$) was measured as described by Giné-Bordonaba et al.

131 (2017) with some modifications. Four replicates of 3 fruit each were placed in 2 L flasks

132 sealed with a silicon septum for sampling the gas of the headspace after 3 h incubation in

133 an acclimatized chamber at 20 °C. For the analysis of ethylene production, gas samples (1

134 mL) were taken using a syringe and injected into a gas chromatograph (GC; Agilent

135 Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina

136 column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously

137 described by Giné-Bordonaba et al. (2014).

138 **2.4. Enzymes related to the ethylene metabolism and fruit softening**

139 1-Aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) was extracted as

140 described by Chiriboga et al. (2012) with some modifications. The sample (0.5 g of frozen

141 tissue) was homogenized in 1 mL of buffer containing 400 mmol L$^{-1}$ MOPS at pH 7.2, 10

142 % glycerol, 30 mmol L$^{-1}$ ascorbic acid sodium salt and PVP 40000 2 %. The homogenate

143 was gently shaken for 10 min at 1 $^{o}$C and centrifuged at 17,000 $g$ for 30 min at 4 °C.

144 Subsequently, the supernatant was stored at -80 °C until analysis.

145 Enzyme activity was analysed as described in Giné-Bordonaba et al. (2017). The mixture

146 was aired and incubated for 60 min at 30 °C, after which a 1 mL headspace gas sample

147 was injected into a gas chromatograph and the results were expressed as nmol C$_2$H$_4$ kg$^{-1}$

148 s$^{-1}$ on fresh weight basis.

149 The extraction and activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS)

150 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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249 **3.    RESULTS AND DISCUSSION**

250 **3.1.    Changes in overall quality during on-tree and off-tree ripening**

251 The flesh firmness of 'Blanquilla' pear at harvest was 60 N, which was inside the

252 commercial harvest range for this pear cultivar (Gamrasni et al., 2010). After harvest,

253 firmness from off-tree fruit decreased rapidly from days 3 to 6 (*ca.* -11.2 N/d) and then

254 more gradually until reaching 5 N at 20 d. In contrast, the loss of firmness in attached

255 fruit started 10 d later than that observed in detached fruit and was more progressive

256 (-1.65 N/d), reaching 5 N only after more than 30 d (Fig. 1A).

257 TSS of the pears at harvest was 13 % (Fig. 1B), thereby similar to the results obtained in

258 previous studies on other European pear cultivars like 'Jules d'Airolles', 'Abate Fetel'

259 and 'Spadona'(Gamrasni et al., 2010; Yim and Nam, 2016). No differences in TSS were

260 observed between off-tree and on-tree fruit until 5 d. Then, TSS was higher in off-tree

261 samples than in those fruit ripened on-tree.

262 No significant differences were found for TA between off-tree and on-tree fruit (Fig.1C).

263 TA at harvest was $4.10 \pm 0.10$ g malic $L^{-1}$ and then steadily decreased (-0.07 g malic $L^{-1}$)

264 until reaching values of *ca.* 2 g malic $L^{-1}$ both for off-tree and on-tree fruit. TA values in

265 this work were slightly higher than those observed in previous studies with 'Blanquilla'

266 (Larrigaudière et al., 2004)  for which TA values were always lower than 3 g malic $L^{-1}$.

267 Different orchards or agroclimatic conditions may explain the differences in the TA

268 values between both studies.

269 The SI at harvest was almost 6 (Fig. 1D) and then gradually increased until reaching

270 values of completely mature fruit (SI = 10). The highest SI in off-tree fruit was reached

271 10 d after storage at 20 °C, whereas on-tree ripened fruit needed 25 d to reach the same

272 value. The slower starch degradation during on-tree ripening may be explained by: (1)

273 the differences in the ethylene production kinetics (Fig. 2A), since it is well documented

274   that starch degradation is for some pome fruit cultivars an ethylene-related phenomena

275   (Thammawong and Arakawa, 2007) and a good indicator of the fruit maturity stage (Peirs

276   et al., 2002); but also by (2) the continuous supply of carbohydrates from source-to-sink

277   occurring in attached fruit and satisfying the fruit needs for respiration and other catabolic

278   process.

279   Chlorophyll degradation measured by the index of absorbance difference $I_{AD}$ (Fig.1E), as

280   well as the Streif index (Fig.1F), also reflected the slower ripening pattern of attached

281   fruit compared with detached fruit and highlighted the suitability of the former non-

282   destructive measurement as a potential tool to determine the optimal harvest date in

283   'Blanquilla' pears. However, this tool less accurately represented quantitative differences,

284   not only in firmness loss but in other quality attributes, and was less useful to follow the

285   ripening process during off-tree ripened fruit.

286   **3.2.  Ethylene metabolism and its regulation on- and off-tree**

287   Differences in the kinetics of ethylene production were found between off-tree and on-

288   tree ripened fruit (Fig. 2A). Harvested fruit (off-tree) exhibited a typical pre-climacteric

289   behaviour with a delay (5-6 d) in the initiation of ethylene production and then a sharp

290   increase up to 0.12 nmol kg$^{-1}$ s$^{-1}$ at 20 DACH. The kinetic of ethylene production for on-

291   tree fruit was much more progressive, with an extension of the lag period up to 10 d and

292   a slower ethylene production rate, being nearly half of that observed in fruit ripened off-

293   tree (Fig. 2A). Temperature conditions among on- and off-tree ripened fruit were

294   relatively similar (Supplementary Figure 1) and did not explain the differences in ethylene

295   production. However, it is also likely that warmer temperatures during on-tree ripening

296   may lead to higher ethylene production, yet not reaching similar values to those observed

297   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 $\mu$mol kg$^{-1}$. In off-tree samples, this

394 concentration increased up to 30 $\mu$mol 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

398     stress. This process may also be related to the 'water-stress' phenomena experienced by

399     detached fruit, which in turn may be linked to ABA. A strong negative correlation was

400     observed between the MDA content and firmness (Fig. 4B). A similar behaviour was also

401     observed in Japanese pear (Li and Wang, 2009) for which an 8-fold increase in MDA

402     content was described after 6 d at room temperature if compared to the values at harvest.

403     In contrast to off-tree fruit, MDA levels in on-tree ripened fruit only significantly

404     increased after 25 d and regardless of the changes in ethylene production and firmness

405     loss. Overall, these results suggest that oxidative stress was unlikely involved in the

406     firmness loss observed after 10 d and in the induction of the ripening process in fruit

407     ripened on-tree. As described earlier, and in contrast to off-tree ripened fruit, on-tree fruit

408     softening appeared to be exclusively ethylene dependent.

409     Collectively these results are of interest and highlight clear differences in the

410     susceptibility of off- and on-tree fruit to oxidative stress that likely determine the initiation

411     of fruit ripening.

412     **3.5.   Changes in the volatile profile during on and off-tree ripening**

413     Pears are highly appreciated by consumers due in part to their unique and complex aroma

414     profiles associated to each each specific cultivar (Chen et al., 2018) but also to its

415     characteristic ripening process. Accordingly, we investigated whether ripening off- tree

416     impaired or enhanced the development of the 'Blanquilla' pear volatile profile. Fifteen

417     compounds belonging to different chemical classes: esters (8), aldehydes (2), alcohols (4)

418     and terpene (1) were identified (Table 2). The predominant compounds in all the samples

419     with concentrations higher than 1500 µg kg$^{-1}$ were hexanal, butyl butanoate, and α-

420     farnesene, as found for 'Yali' pear (Chen et al., 2006). The presence and abundance of

421     volatile compounds on fruit ripened off-tree for 6 d was similar to that observed on fruit

422     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.

472

473

474  ## 3  CONCLUSIONS

475  The results from this study provide new information on the biochemical events

476  differentiating on-tree and off-tree 'Blanquilla' pear ripening. In both samples, ACC

477  metabolism plays a key role yet under different regulatory mechanisms. In off-tree pears,

478  ripening (ethylene production and softening) is initially regulated by oxidative stress that

479  likely promotes the further autocatalytic burst of ethylene production through an

480  activation of ACS. In contrast, no oxidative stress was detected in on-tree fruit, where

481  ripening seems to be regulated by ACO and initially inhibited by hexanal, but also, in the

482  later stages of ripening, by the accumulation of sucrose possibly triggering the initiation

483  of ethylene production.

484  Future studies are required to better understand the role that ethylene, volatiles and

485  sucrose or its interplay with other crucial hormones such as ABA may have in the ripening

486  process of pear fruit, and especially in other pear cultivars that do not have the capacity

487  to ripen on-tree. The results from this study may provide a better understanding of the

488  ripening process in attached pears hence making easier the decisions for optimal harvest

489  in terms of fruit quality.

490

491

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690

691 **Table 1:** PG (nmol kg$^{-1}$ s$^{-1}$) and PME (µmol kg$^{-1}$ s$^{-1}$) levels in Blanquilla pear during off-

692 tree and on-tree ripening. Means ± standard deviation followed by the same letter are not

693 significant different at $p \leq 0.05$ (n=3).

694

| | 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 |

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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 ≤ 0.05; n=3). nd = non detected.

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| 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**

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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.

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743    **Figure 1**:

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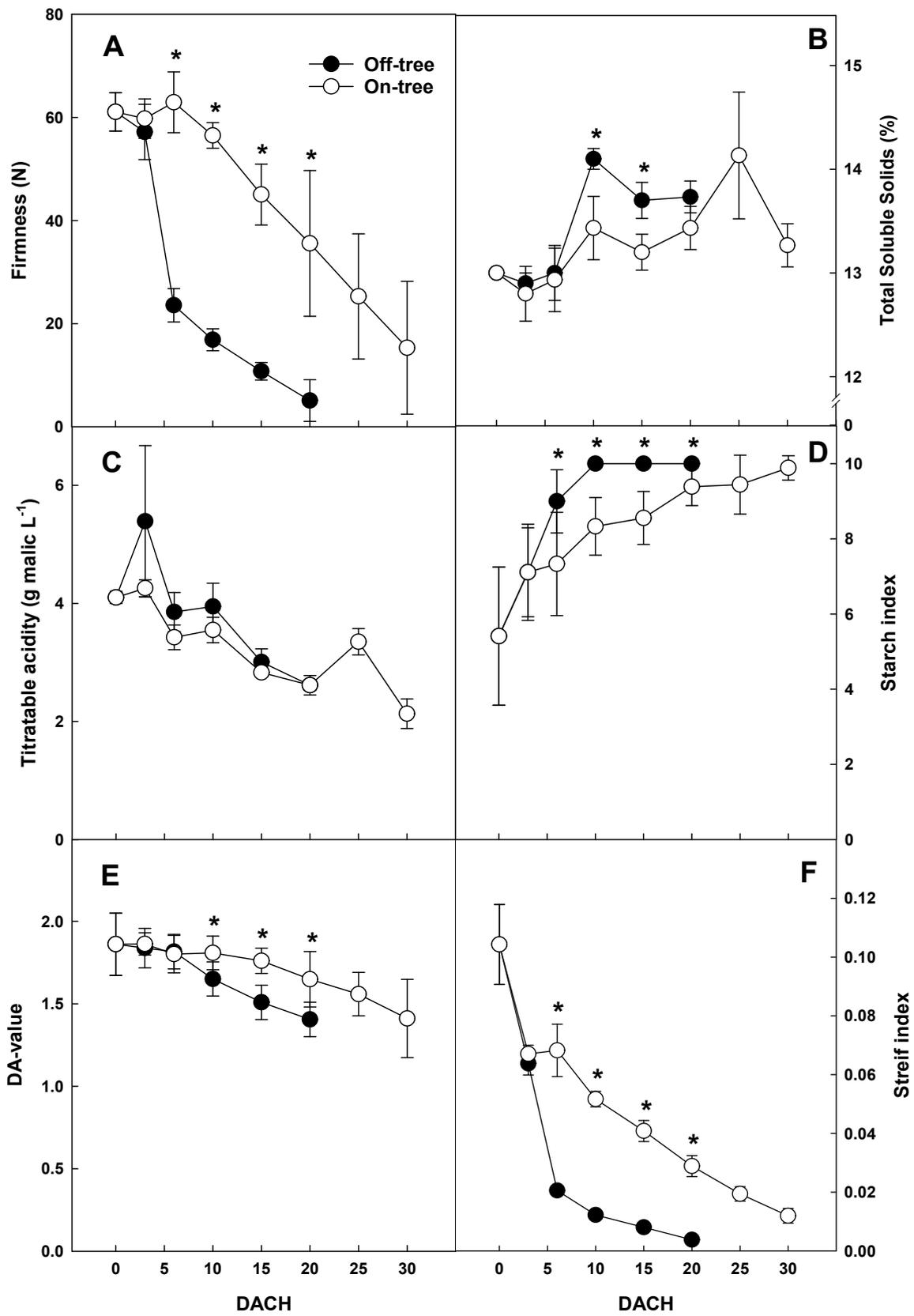
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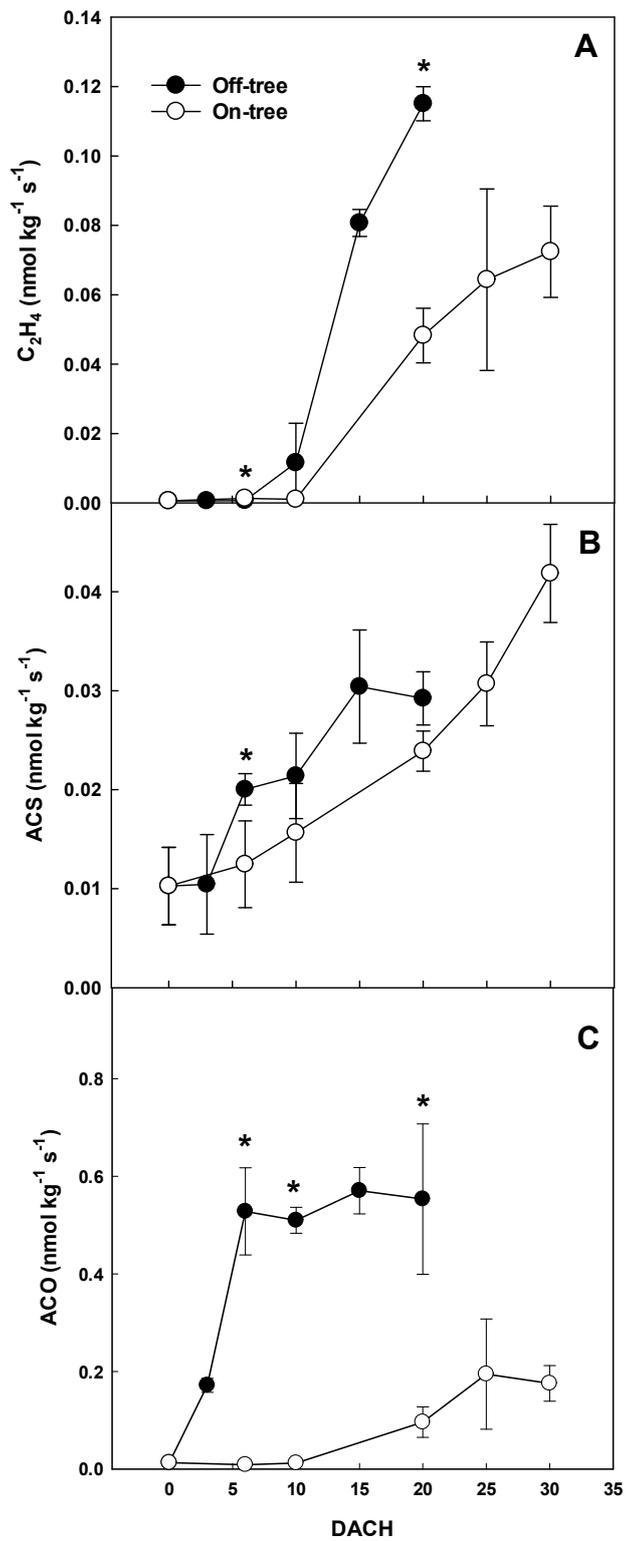759

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769 **Figure 2:**

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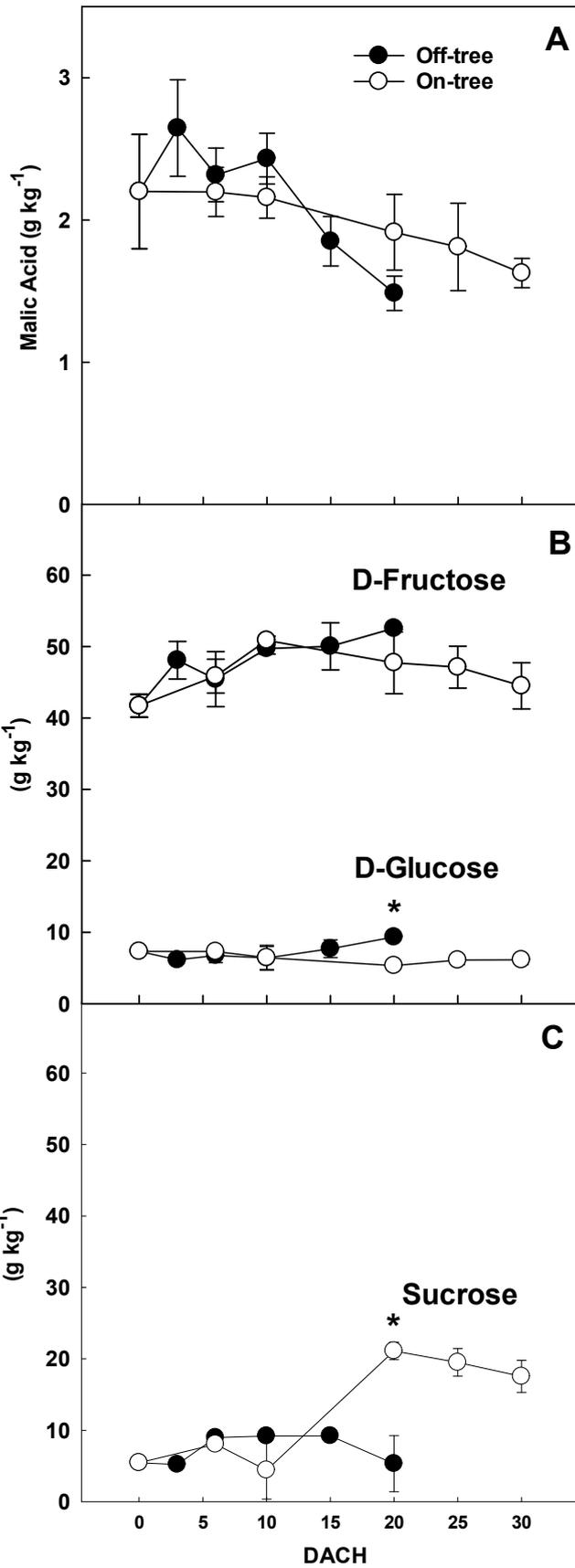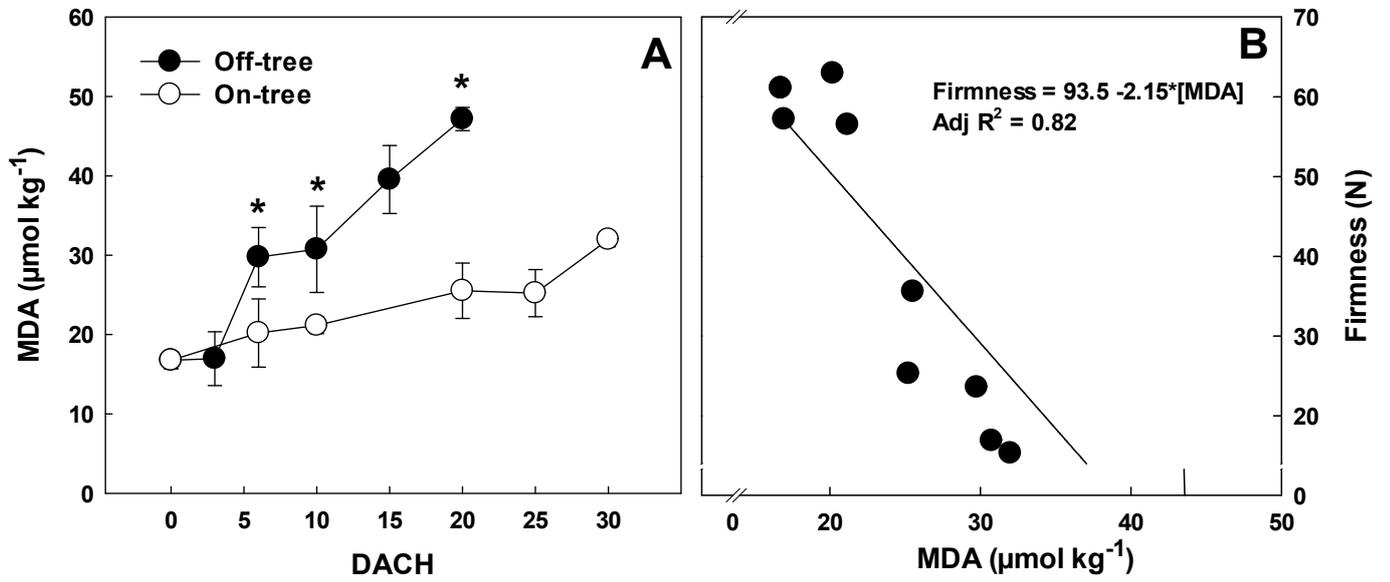786

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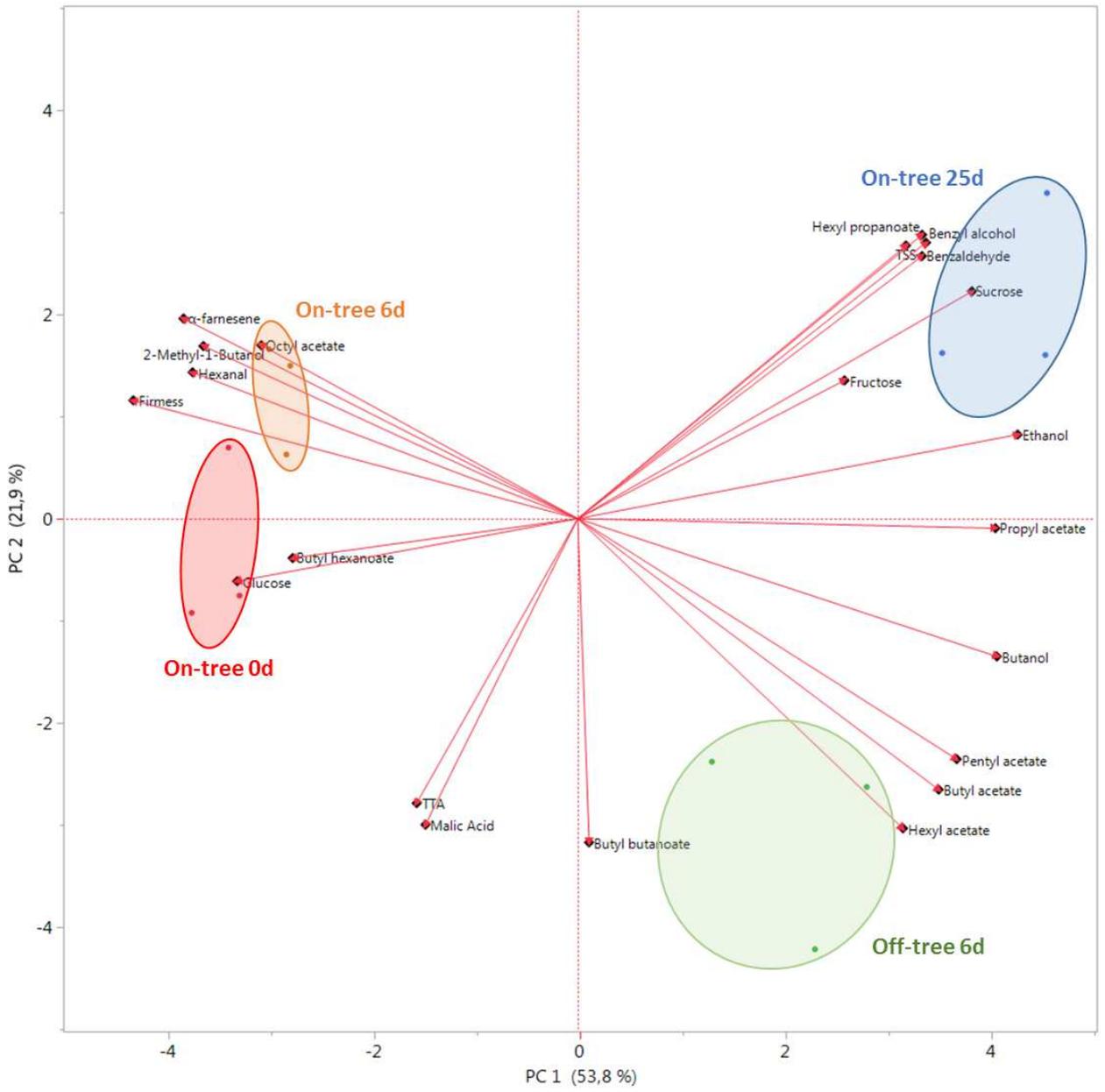
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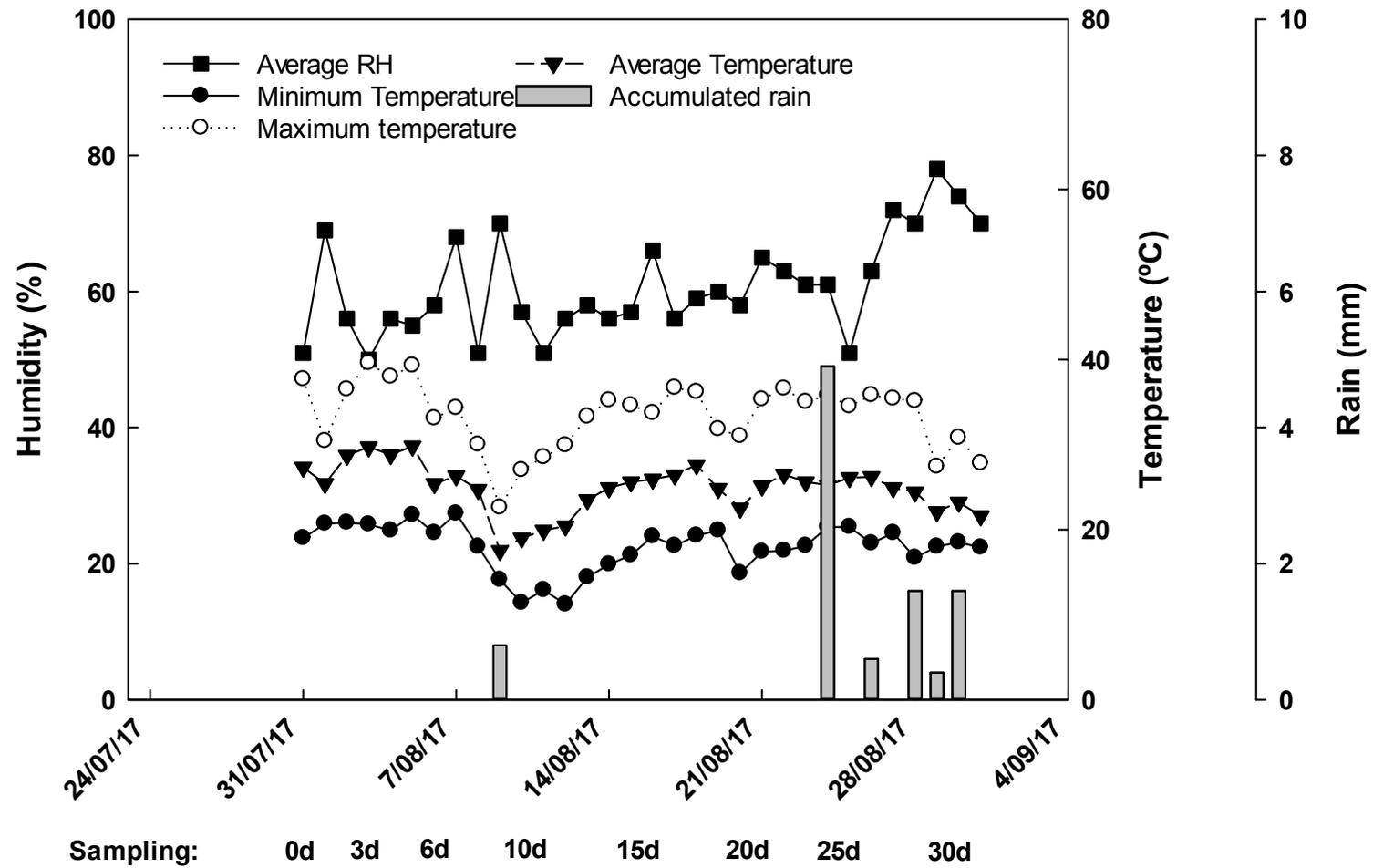
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814 **Figure 5**:

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**Supplementary Figure 1:** Temperature, relative humidity and rainfall during the period between samplings for on-tree ripened fruit. Off-tree

ripened fruit were stored at 20±0.5 ºC and 85% RH.