1   **Interplay between hormones and assimilates during pear development and**

2   **ripening and its relationship with the fruit postharvest behaviour**

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Abstract

The ability of European pears (*Pyrus communis L.*) to ripen immediately after harvest is cultivar-dependent and relies on a range of physiological and biochemical events occurring during fruit growth and development that remain largely unknown. To gain further knowledge on these events, changes in the content of sugars, acids, major hormones and ethylene precursors or related enzymes were studied in two pear varieties ('Blanquilla' and 'Conference') with known differences in their postharvest ripening behaviour. In both cultivars, low contents of abscisic acid (ABA) seemed to be a prerequisite to initiate on-tree fruit ripening including sugar accumulation and softening. In 'Blanquilla' pears, the enhanced potential to produce ethylene and thereby to ripen upon harvest was associated to a late increase in ABA content paralleled by an accumulation of indole 3-acetic acid (IAA). In turn, the inability of 'Conference' fruit to produce ethylene upon harvest appeared to be related to a coordinated action of gibberellins (more specifically GA_1), salicylic acid (SA) and jasmonic acid (JA), which remained at high concentrations during the latest phases of fruit growth. Collectively, our results highlight that a complex hormonal cross-talk during the development and on-tree ripening of pear fruit may finally determine the ability of the fruit to ripen upon harvest.

Keywords: ACC, Chilling requirements, Fruit growth, Hormonal cross-talk, Sugar accumulation

1. INTRODUCTION

Pome fruit growth followed a single-sigmoid curve [1] that on a physiological basis could be divided in four phases: ovary development, cell division, rapid growth due to cell expansion and finally ripening [2]. Some authors, though, combine the first two phases into a single one, characterised by a limited increase in fruit weight [1]. The first step along fruit development, generally referred as 'fruit set', occurs just after pollination and may be defined as the passage from flower to fruit. Cell division begins soon after blooming and is maintained for few weeks depending on the fruit and cultivar. For instance, some apple varieties complete the cell division phase in three weeks while late pear cultivars may need at least six weeks [1]. After cell division, fruit increase their weight by 100-fold or more during the so-called cell expansion period [3]. Ripening, as the last phase during on-tree fruit development, is characterised by changes in colour, texture, aroma and nutritional quality leading to the final fruit appearance and flavour.

It is well known that changes at the hormonal level are responsible, to some extent, for the transition between the above-mentioned growth phases. It has been reported, for instance, that auxins and gibberellins (GAs) play their major role at early stages of development controlling cell division and cell expansion [4]. Later on during fruit growth, auxins seem to play a primary role in the control or initiation of fruit ripening [5], yet controversial results have been found depending on the fruit species suggesting that complex interaction among different hormones are involved in the initiation of fruit ripening [6]. For instance, an inverse relationship between auxin contents and ripening capacity was observed in achenes-free strawberries treated with exogenous auxins [7], but also in the tomato ripening inhibitor (rin) mutant [6] and rin-like apple mutant [8]. Similarly, GAs synthesized during the growth phase of tomato fruit contribute to a reduced expression of genes encoding the enzymes of ethylene synthesis and also to

reduced abscisic acid (ABA) synthesis [4]. ABA is one of the most important hormone in non-climacteric fruit which is involved in colour changes in grapes [9] and anthocyanins accumulation in cherries [10]. Although ethylene remained at low levels in grapes over the entire development, ABA levels gradually increased and reached their highest levels at the beginning of ripening [11]. In contrast, information regarding the involvement of ABA in the ripening process of climacteric fruit is rather scarce yet a putative role in promoting ripening has been found in tomato fruit [11].

Jasmonic acid (JA) and its methyl ester, methyl jasmonate, are other plant ubiquitous hormones that contribute to different morphogenetic events including cell division and adequate formation of tissues [12]. Jasmonates also act as growth inhibitors in root and shoots of *Arabidopsis* [13] and may promote climacteric fruit ripening by increasing ethylene production [14,15], chlorophyll degradation [16], the synthesis of aroma compounds [17] and the biosynthesis of several secondary metabolites and antioxidants [18] in a range of plant species. Salicylic acid (SA) is often considered as a signalling molecule that may trigger plant defence responses [19], acting both on gene expression and on the synthesis of defence compounds such as proline and JA [20]. Its putative role in fruit growth and the cross talk with others plant growth hormones is unknown although SA was found to inhibit ethylene production in pear cell suspension culture [21] as well as in canola plants [22].

Finally, ethylene is considered the major hormone involved in the ripening of climacteric fruit and numerous studies have investigated ethylene metabolism during ripening of pome fruit [23–25]. Although belonging to the climacteric class, pears are generally classified as summer or winter pears depending on the way ethylene is induced after harvest. Summer pears are able to ripen, hence to produce autocatalytic ethylene, just after harvest with a minimum or no chilling requirement. This group includes cultivars

93   such as 'Blanquilla' that may produce considerable amounts of ethylene after harvest and

94   even on-tree [23], but also 'Rocha' pears [26]. 'Conference' pears, on the other hand, may

95   be considered an intermediate between summer and winter pears, needing minimum

96   chilling requirements (about 15 d) to initiate their autocatalytic ethylene production yet

97   depending on the fruit maturity at harvest. On the other hand, winter pears such as

98   'Comice' and Beurré d'Anjou' need 30 d and 150 d of cold storage, respectively, to

99   induce ethylene production and reach their eating quality upon removal from cold storage

100   [27,28].

101   Although a large amount of studies have investigated the chilling requirements of winter

102   pears [28,29], only few studies exist on the physiological basis of this process and on the

103   biochemical events occurring on-tree that may determine the differences in the ripening

104   behaviour among cultivars. Even less information is actually available about the potential

105   role that phytohormones, or the complex cross-talk among them, can play in the

106   regulation of the pear ripening capacity. Accordingly, this study aimed to determine how

107   the hormonal and other biochemical changes occurring during fruit growth, may explain

108   the differential postharvest behaviour of different pear varieties.

109

110   **2. MATERIAL AND METHODS**

111   **2.1. Plant materials and experimental design**

112   'Blanquilla' and 'Conference' pears (*Pyrus communis* L.) were harvested on a commercial

113   orchard near Lleida (Catalonia, Spain) every 20 d from 10 d after full bloom (DAFB) until

114   the commercial harvest date (CHD, 122 DAFB in 'Blanquilla' and 132 DAFB in

115   'Conference'). Biochemical and physiological measurements were performed every 20 d

116   from 30 DAFB to the CHD whereas quality determinations were initiated at 70 DAFB.

117   In parallel, a batch of fruit (n=20) per variety was stored at 0 °C and 90 % RH to follow

118   the ethylene production after 15 d of cold storage.

119

120   **2.2.   Quality evaluations**

121   Flesh firmness was measured on 4 replicates of 10 fruit each with a penetrometer (TR

122   Turoni srl., Italy) equipped with an 8 mm probe as described by Chiriboga et al.[30]. Total

123   soluble solids (TSS; %) were measured on pear juice (blend of 10 fruit per replicate and 4

124   replicates) using a digital hand-held refractometer (Atago, Tokyo, Japan) whereas total

125   acidity (TA) was measured on the same juice samples by titration using NaOH 0.1N and

126   the results expressed as g malic L^{-1}. The index of absorbance difference ($I_{AD} = A_{670} - A_{720}$)

127   was measured on opposite sides of the equatorial parts of the fruit with a DA-Meter (TR

128   Turoni, Forli, Italy). The starch index was evaluated on 10 fruit samples as described by

129   Lindo-García et al.[23]. Weight was also monitored on 6 replicates of 10 fruit each during

130   all sampling dates. In parallel, from 30 DAFB, tissue from 5 fruit per replicate and 4

131   replicates (6 replicates for hormones assay) per sampling date was frozen in liquid nitrogen

132   and kept at -80 °C until further biochemical analysis.

133   **2.3.   Ethylene production and respiration rate**

134   Ethylene production levels (nmol kg^{-1} s^{-1}) during growth were measured as described by

135   Giné-Bordonaba et al. [31]. Four replicates of 5 fruit each were placed in different flasks

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

137   an acclimatized chamber at 20°C. Gas samples (1 mL) were taken using a syringe and

138   injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington,

139   Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m $\times$ 1/8 $\times$ 2.1,

140   Tecknokroma, Barcelona, Spain). Fruit respiration was determined by quantifying the CO_2

141 concentration in the flask with an O_2/CO_2 gas analyser (CheckPoint O_2/CO_2, PBI

142 Dansensor, Ringsted, Denmark).

143 Kinetics of ethylene production upon harvest and after chilling were assessed using a flow-

144 through system according to Giné-Bordonaba et al. [32]. The ethylene production rate was

145 determined on four replicates of two pears each placed in 1500 mL flasks continuously

146 ventilated with humidified air at a flow rate of approximately 1.5 L h^{-1}. As previously,

147 ethylene production was measured by taking gas samples of effluent air from respiration

148 jars and injecting this sample into a gas chromatograph.

149 **2.4. Sugar and organic acid content**

150 Sugars (sucrose, glucose and fructose) and malic acid were extracted from frozen tissue

151 as described by Giné-Bordonaba et al. [31]. The supernatants of each sample extraction

152 were recovered and used for enzyme coupled spectrophotometric determination of

153 glucose and fructose (hexokinase/phosphoglucose isomerase), sucrose (β-fructosidase)

154 and malic acid (L-malate dehydrogenase) using commercial kits (BioSystems S.A.,

155 Barcelona, Spain) and following the manufacturer's instructions.

156 **2.5. Enzymes related to ethylene metabolism**

157 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-

158 carboxylic acid oxidase (ACO) enzymes were extracted and analysed as described by

159 Lindo-García et al. [23]. Enzyme activity was expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh

160 weight basis.

161 1-aminocyclopropane-1-carboxylic acid (ACC) was extracted and analysed as described

162 by Bulens et al. [33] with some modifications. Briefly, 2 g of frozen tissue were

163 homogenized with 4 mL of a 5% (w/v) sulfosalicylic acid solution and vortexed until a

164 homogenous mixture was obtained. The samples were gently shaken for 30 min at 4 °C

165 and then were centrifuged at 8,000 g for 10 min at 4 °C. Subsequently, the supernatant

166 was stored at -80 °C until analysis. The extract reading was performed mixing 1.4 mL of

167 the ACC extract with 400 µL of 10 mmol L^{-1} $HgCl_2$ and 200 µL of a solution of NaOCl

168 saturated with NaOH (2:1 v/v). After 4 min, a 1 mL headspace gas sample was injected

169 into a gas chromatograph and the results expressed as nmol C_2H_4 kg^{-1} on fresh weight

170 basis.

171 **2.6. Hormonal profile**

172 Phytohormones were extracted by mixing 100 mg of the fruit samples with 200 mL

173 methanol:isopropanol:acetic acid, 50:49:1 (v/v/v) and using ultrasonication and vortexing

174 (Branson 2510 ultrasonic cleaner, Bransonic, USA) for 30 min. Deuterium- labelled

175 internal standards, including d5-indole-3-acetic acid, gibberellins (d2-GA_1; OlChemim

176 Ltd. (Olomuc, Czech Republic)) and abscisic acid (d6-ABA; OlChemim) were added.

177 After centrifugation, the pellet was re-extracted using the same procedure and the

178 collected supernatants were merged and filtered through a 0.22 mm PTFE filter (Waters,

179 USA) before analyses. Phytohormones were analysed by UHPLC-ESI– MS/MS. The

180 system consisted of an Aquity $UPLC^{TM}$ System (Waters) quaternary pump equipped with

181 an autosampler. An $HALO^{TM}$ C18 (Advanced Materials Technology Inc., USA) column

182 (2.1 x 75 mm, 2.7 µm) was used. Solvent A was water with 0.05% glacial acetic acid

183 (Sigma-Aldrich, Steinheim, Germany) and solvent B was acetonitrile (Sigma-Aldrich)

184 with 0.05% glacial acetic acid. Flow rate was set at 0.6 mL min^{-1}. Quantification was

185 made considering recovery rates for each sample by using the deuterium-labelled internal

186 standards [34] and the results expressed on fresh weight basis (µg kg^{-1}) and per fruit basis

187 (µg $fruit^{-1}$) aiming to understand the accumulation of hormones without considering the

188 increase in fruit volume occurring during fruit growth.

189 **2.7. Statistical analysis**

190 All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS

191 Institute. Comparisons between varieties at specific time points were done by Student's

192 t-test at a significant level of $p \leq 0.05$. Correlations between experimental variables were

193 made by Spearman's Rank Correlations using RStudio® and p value based on a two-tailed

194 test (significant differences were $p \leq 0.05$).

195

196 **3. RESULTS**

197 **3.1. Different postharvest ripening behaviours**

198 'Blanquilla' pear was able to produce ethylene just after harvest, reaching values of

199 almost 0.1 nmol kg^{-1} s^{-1} after 11 d at 20 °C. In contrast, 'Conference' pears did not

200 produce ethylene after harvest, maintaining basal levels even after 15 d at 20 °C (Fig.

201 1A). After 15 d of cold storage, 'Conference' pears started to produce ethylene after 11

202 d at 20 °C reaching maximum levels of 0.4 nmol kg^{-1} s^{-1} after 15 d of storage at 20 °C.

203 On the other hand, ethylene production in cold-stored and then ripened 'Blanquilla' pear

204 was similar to that observed at harvest (Fig. 1B).

205 **3.2. Growth kinetics, morphological and quality changes**

206 The growth pattern in weight (g) of 'Blanquilla' and 'Conference' pears along the

207 different sampling dates is showed in Figure 2, highlighting two clearly differentiated

208 growth phases. The first one, occurring between 10 and 70 DAFB, showed a slow growth

209 rate (around 0.4 and 0.7 g per day for 'Blanquilla' and 'Conference', respectively)

210 followed by a second phase, from 70 DAFB to CHD, characterised by a faster growth

211 rate (around 1.6 and 2.8 g per day for 'Blanquilla' and 'Conference', respectively). A

212 marked decrease in the fruit firmness and titratable acidity along with an increase in the

213 TSS content and the starch index (Suppl. Fig. 1) was observed for both varieties parallel

214 to the second growth phase.

3.3. Sugar and organic accumulation during fruit growth

In this work, the glucose accumulation pattern was quite similar between the two varieties depicting higher glucose values in 'Conference' already from 70 DAFB (Fig. 3A) onwards. At the time of commercial harvest, glucose content was 10 and 7 g kg^{-1} for 'Conference' and 'Blanquilla' pears, respectively, being in average 4.6-fold higher than the values observed at 30 DAFB. Fructose was the predominant sugar in both varieties being maximum at 110 DAFB and slightly declining thereafter until the CHD (Fig. 3C). Sucrose content, on the other hand, remained stable until 70 DAFB and then increased by 4-fold until the CHD (Fig. 3B) parallel to the period of faster fruit growth. In turn, the minor changes in sucrose content until 70 DAFB were paralleled by the highest fruit CO_2 production and only start to rise when CO_2 production was relatively low (data not shown).

Malic acid is the predominant organic acid in pear fruit and its content both in 'Blanquilla' and 'Conference' pear was about 1.6 g kg^{-1} at 30 DAFB and then constantly increased through fruit development (14 mg per day) reaching values of 2.5 and 3 g kg^{-1} at 110 DAFB for 'Conference' and 'Blanquilla', respectively (Fig. 3D).

3.4. Changes in ethylene metabolism during growth and ripening

The ethylene production pattern during fruit growth was also similar between the two varieties (Fig. 4A) observing an increase of ethylene production rate from 30 to 50 DAFB and then a decreased, reaching basal levels at 70 DAFB. Significant differences in the ethylene production rate between cultivars were observed at the CHD, where 'Blanquilla' pears were capable of producing 6.5-fold higher ethylene levels than 'Conference' pears. Significant differences in ACC levels were found between cultivars with values of 500 nmol kg^{-1} in 'Blanquilla' at the time of commercial harvest date whereas 'Conference' exhibited 5-fold lower values (Fig. 4C). A massive increase in the ACC content of

240 'Blanquilla' fruit occurred from 110 DAFB to the CHD. Such differences in the ACC

241 levels between cultivars were not explained by an activation of ACC synthase (ACS), that

242 remained very low throughout the full growing period for both varieties (Fig. 4B), nor by

243 the increase in ACC oxidase activity near the commercial harvest date (Fig. 4D). Indeed,

244 the activity of this enzyme follow a similar pattern between the two varieties.

245 **3.5.   Hormonal changes during pear growth and ripening**

246 In our study, a differential accumulation pattern of ABA was observed between cultivars.

247 For both cultivars, ABA levels were high at earlier developmental stages (*ca*. 6 mg kg^{-1}

248 at 30 DAFB), and drastically declined thereafter until 70 DAFB (Fig. 5A). From this

249 moment until the CHD, ABA levels continue to decline in 'Conference' pears, whereas

250 these levels steadily increased in 'Blanquilla' pears reaching values of 1.5 mg kg^{-1}. The

251 results expressed on a fruit basis, hence showing the net accumulation of this hormone

252 within the fruit, showed that ABA content in 'Blanquilla' pear increased from fruit set

253 until the commercial harvest date whereas ABA levels in 'Conference' remained steady

254 during most of the fruit development process (Fig. 5A, insert). It is interesting to note that

255 in both cultivars the ABA peak during growth (observed at 30 DAFB; Fig. 5A) precede

256 that of ethylene (50 DAFB; Fig. 4A).

257 The changes in the content of indole 3-acetic acid (IAA) also differed between cultivars

258 and especially at earlier developmental stages (Fig. 5B). 'Conference' pears showed its

259 highest value of IAA (around 40 µg kg^{-1}) at 50 DAFB whereas, at the same time, IAA

260 levels in 'Blanquilla' were the lowest (around 20 µg kg^{-1}). During the period of maximum

261 growth (second growth phase from 70 DAFB until the CHD), IAA levels in 'Blanquilla'

262 pears remained stable whereas a clear decrease and significantly lower values ($p \leq 0.05$)

263 for this hormone wereobserved in 'Conference' pears, yet with a transient peak at 90

264   DAFB. A similar behaviour was observed considering the data in fruit basis (Fig. 5B,

265   insert).

266   As described for the other phytohormones, changes in the content of the most active

267   gibberellin, GA_1, were also notably different between the two cultivars. At 30 DAFB,

268   GA_1 levels in 'Blanquilla' pears were relatively high (12 µg kg^{-1}) but decreased later up

269   to 5 µg kg^{-1} at 70 DAFB. A completely different accumulation pattern was found during

270   this first growth phase in 'Conference' pears for which GA_1 levels increased from 5 µg

271   kg^{-1} to 10 µg kg^{-1} from 30 to 70 DAFB. After 70 DAFB, GA_1 levels remained stable in

272   'Blanquilla' pears but slightly increased up to harvest date in 'Conference' pears (both on

273   a concentration or fruit basis). At CHD, GA_1 levels in 'Conference' pears were 2-fold

274   higher than in 'Blanquilla' (Fig. 5C).

275   JA levels remained stable (80 µg kg^{-1}) in 'Blanquilla' pears during all the growing period.

276   However, in 'Conference' pear two peaks in JA levels were observed at 50 and 110 DAFB

277   (Fig. 5D), and values during this second growth phase were generally higher than those

278   observed in 'Blanquilla', and especially at 110 DAFB where significant differences ($p \leq$

279   0.05) were observed between varieties.. On fruit basis, a similar tendency was observed

280   for both varieties until 70 DAFB, yet from this date onwards, 'Blanquilla' fruit also

281   showed constantly lower JA levels than 'Conference' pear (Fig. 5D, insert). Similarly,

282   significant differences were found in the kinetics of accumulation SA between the two

283   cultivars during growth. 'Blanquilla' pear showed a peak of SA at 50 DAFB (around 25

284   µg kg^{-1}) whereas 'Conference' reached the same values at later developmental stages (90

285   DAFB) (Fig. 5E).

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287

288

4. DISCUSSION

Notable differences exist in the ripening capacity and the fruit sensitivity to cold stress among different pear varieties. Our data further confirm that 'Blanquilla' pear is a typical summer pear variety able to produce ethylene just after harvest whereas 'Conference' pear needed at least 15 d of cold storage following harvest to initiate the production of ethylene. Similar results have been showed in previous studies of 'Blanquilla' pear where ethylene production increased to 0.08 nmol kg^{-1} s^{-1} after 15 d at 20 °C [23] as well as in 'Conference' [35] or other 'winter' pear varieties [36] which needed variable chilling periods to produce detectable amounts of ethylene. Even though the chilling-requirements to initiate ripening in many winter pear varieties are well documented [28,29], scarce information exists on the physiological basis of this process and on the biochemical events occurring on-tree that may determine the differences in the ripening behaviour among cultivars.

4.1. Growth rate or changes at the assimilate level do not differ between cultivars with different ripening capacity.

In spite of the notable differences in the final fruit size and postharvest behaviour both varieties showed similar growth patterns. The number of phases involved in pear growth remains controversial [1–3,24], but our results show for both varieties two clear differentiated phases, and hence agreed with a previous study in Japanese pear [37]. The first phase is likely associated to ovary development [3] and rapid cell divisions [2] whereas the second growth phase is probably related to an increase of the cell volume due to cell enlargement.

Along the changes in fruit size, sugars (glucose, fructose and sucrose) and acids tended to accumulate similarly between both varieties throughout fruit growth. A similar accumulation pattern has been described for other species such as cherry [31], loquat [38]

314 but also apple [24]. While glucose and fructose constantly increase through fruit

315 development, sucrose started to peak at later developmental stages coinciding with the

316 period of low fruit respiration (data not shown). This result suggests that sucrose may be

317 a major substrate for respiration in pear fruit, as it has been described in other plant models

318 [39]. Higher sucrose content in 'Blanquilla' than in 'Conference' at the CHD further

319 confirm the association between the accumulation of this compound with the initiation of

320 the autocatalytic ethylene production on-tree [23]. In turn, the constant accumulation of

321 malate throughout fruit development also reinforce the results from recent studies

322 suggesting that this compound may account little or nothing as a respiratory substrate in

323 a range of species [40,41].

324 **4.2.   Ethylene plays a key role at different stages during pear growth**

325 Numerous studies have investigated ethylene metabolism during ripening of pome fruit

326 [23–25] yet most of them have focused at later fruit developmental stages. The peak in

327 ethylene production observed at 50 DAFB is likely involved in the regulation of cell size

328 promoting the passage from cell division to cell elongation, as suggested by Small et al.

329 [42]. Similar patterns of ethylene production during growth have been reported in apples

330 [24,43], observing high ethylene levels at early stages of development, followed by a

331 notable decrease prior the commercial harvest and a final increase until the CHD. The

332 observed differential activation of ACC levels and ACO activity clearly show that

333 'Blanquilla' pear was physiologically more mature at the CHD and explain why this

334 variety does not require chilling to initiate its autocatalytic ethylene production (Fig. 1).

335 Indeed, the higher ethylene production at harvest in 'Blanquilla' is in agreement to

336 previous studies that demonstrate the ability of this pear variety to produce ethylene even

337 on-tree [23]. However, in 'Conference' pears, cold storage is needed to increase the levels

338 of ACC [44] and hence to produce significant amounts of ethylene upon chilling. Whether

339 the differences in ACC among varieties are due to a differential capacity of each cultivar

340 to conjugate ACC in malonyl ACC (MACC) remains to be further confirmed yet similar

341 MACC levels are found in both varieties after cold storage (ranging from 2 to 5 nmol g⁻

342 ¹) [44]. Based on these results, and available literature [45–48], we further investigated

343 how other phytohormones may be involved in inhibiting or triggering ACC metabolism

344 on-tree, and hence accounting for the different ripening capacity among pear varieties.

345 .

346 **4.3.   The involvement of the hormonal cross-talk in the pear ripening behaviour**

347 4.3.1. <u>ABA, a key hormone involved in sugar accumulation and more indirectly in the</u>

348 <u>initiation of pear ripening.</u>

349 The low levels of ABA in unripe 'Blanquilla' fruit followed by the steady increase prior

350 to commercial harvest is in agreement to the behaviour described in other climacteric fruit

351 such as apples (cv. 'Red winesap') [49]. The high levels of ABA at 30 DAFB suggest

352 that this hormone may own a prominent role during the first stages of pear development,

353 and that lower levels of this hormone may be required for the transition from fruit

354 expansion to ripening (Fig. 2). Furthermore, the peak of ABA preceding that of ethylene

355 agrees with the data of Zhang et al. [11] showing that a cross-talk between ABA and

356 ethylene may be required to trigger certain stages of pear fruit growth. Although evidence

357 suggests that ABA plays an important role as an inducer of ripening along with ethylene

358 in the late stage of fruit development [11], very few reports are actually available on their

359 role during the entire fruit growth. It is generally recognized that ABA levels increase

360 from maturation to harvest in climacteric fruit, while in non-climacteric fruit such as

361 sweet cherries, the levels of ABA increase before maturation but decrease thereafter until

362 the time of harvest [50]. Accordingly, our data (Fig. 5A) suggest that 'Blanquilla' pears

363 followed a typical climacteric pattern with a slight increase in ABA content at the end of

364   the fruit growth , hence in agreement to that described in apples [50], while 'Conference'

365   pears rather resemble a non-climacteric fruit [51]. Although both cultivars are recognized

366   as climacteric, such differences in ABA accumulation during growth is of particular

367   interest and may explain, at least in part, the differences in the postharvest ripening

368   behaviour and chilling requirements of these cultivars. Based on available data, we may

369   speculate that cold storage in 'Conference' pear can trigger the accumulation of

370   endogenous ABA, as observed in sweet cherries [10], and thereby enable the fruit to ripen

371   thereafter.

372   Besides the role of ABA in fruit ripening and its cross-talk with ethylene, several

373   evidences exist about the involvement of ABA in sugar accumulation and starch

374   hydrolysis, most of them related to non-climacteric fruit such citrus fruit, cherries but also

375   in melon [6,12,49]. A recent study demonstrated that endogenous concentration of ABA

376   increased during fruit development in sweet cherries, along with the corresponding

377   increase in sugars such as glucose and fructose [53]. Our results confirm the relationship

378   between sugar accumulation and ABA levels and further support recent hypothesis in

379   which the accumulation of specific sugars was needed for the initiation of the

380   autocatalytic ethylene production on-tree in 'Blanquilla' pears [23] through its cross-talk

381   with ABA [54].

382   4.3.2. <u>IAA influences cell division and expansion and may act as a ripening inducer</u>

383   IAA is considered to play a key role in fruit development and ripening [2,48,54]. In our

384   study, the changes in IAA varied depending on the variety. For instance, the temporal

385   changes of IAA in the flesh of 'Conference' pears support previous studies [55], reporting

386   that two peaks of auxin occured during tomato fruit development. At later fruit

387   developmental stages, and especially during the period of maximum sugar accumulation

388   and initiation of ethylene production, IAA levels were 2.1-fold higher in 'Blanquilla'

389 than in 'Conference' pears confirming a putative role for auxins as essential elements in

390 the induction of the fruit responsiveness to ethylene [56]. In other plant models, it was

391 hypothesised that auxin-induced ethylene synthesis is actually connected to ABA and

392 leads to growth inhibition [57]. This hypothesis may explain not only the ability of

393 'Blanquilla' pears to ripen immediately after harvest but also its slower size if compare

394 to 'Conference'.

395 4.3.3. Gibberellin 1 (GA$_1$) promotes fruit set but inhibit**s** ripening

396 Several authors have elucidated the importance of GAs, along with auxins, during fruit

397 growth and development by promoting fruit set [6,58]. At early stages of development,

398 GA$_1$ has been related to the promotion of fruit set and growth in cherry [52], tomato [55]

399 and Japanese pear [59]. Although auxins are known to regulate cell division and

400 expansion, GAs have also been described to promote cell expansion in tomato fruit [60].

401 Along with the data regarding IAA, differences in GA$_1$ between both varieties may further

402 explain why 'Conference' pears are larger than 'Blanquilla' pears at harvest. In the latest

403 stages of fruit development, near to the CHD, GAs may act as a ripening inhibitor

404 delaying the ripening process as it has been observed in tomato fruit [61]. Indeed, an

405 important GA-ethylene-ABA cross-talk was found in tomatoes, where GAs at fruit set

406 decrease the expression of ETR6, ACS and ACO genes as well as those genes related to

407 ABA degradation [45]. In these lines, our data shows that GA$_1$, although undoubtedly

408 involved in fruit growth, may also contributes to the ripening inhibition observed in

409 'Conference' pears by affecting the accumulation of both ethylene and ABA as reported

410 by others [45]. Nevertheless, further studies are needed to better understand the relation

411 of GA$_1$ with the accumulation of specific compound or other phytohormones as well as

412 its putative implication in fruit ripening.

413

414   4.3.4. <u>JA and SA may inhibit on-tree ripening and determine pear postharvest behaviour</u>

415   JA and SA have been reported to play a crucial role in abiotic and biotic plant stress

416   [15,20]. However, their involvement during fruit growth and ripening is elusive. In

417   contrast to the results reported herein, endogenous JA concentrations decreased during

418   apple fruit growth [17]. Thus said, in the same study, the authors found that JA inhibited

419   to some extent a range of ripening-related processes [17] which is consistent with our data

420   for 'Conference' pear. In this sense, the higher net JA content in 'Conference' during the

421   second growth phase can explain the inability of this cultivar to ripen. Kondo et al. [46]

422   also showed that application of a JA analogue decreased the activity of ACC synthase

423   and delayed fruit ripening in 'La France' pears. Our results are also consistent with the

424   works of Nahm et al. [62] who showed that JA may modulate the ethylene pathway

425   through a negative regulation of EIN3 gene expression in 'Bartlett' pear.

426   In a similar way, SA has been described as an effective ripening inhibitor [22], affecting

427   several ripening related processes (respiratory rate, softening, cell wall degrading

428   enzymes, sugars accumulation, etc.) in fruit such as banana [47]. The higher levels of SA,

429   alone or in combination with other hormones (GA_1 and JA), observed in the late stages

430   of the 'Conference' pear growth, may explain the lower or non-detectable ethylene

431   production produced by this cultivar at harvest, since this compound has been described

432   to inhibit ACO enzyme activity [22,47].

433

434

435   **CONCLUSIONS**

436   The present work demonstrates that, although ethylene is considered the major hormone

437   involved in the ripening of climacteric fruit such as pears, other hormones play a decisive

438   role during fruit growth and development through a complex but coordinated cross-talk

439   that may finally determine the pear ripening behaviour both on-tree and during

440 postharvest. Accordingly, two different models are presented in figure 6 describing the

441 hormonal and assimilate regulation on pear fruit ripening.

442 In fruit capable to ripen on-tree such as 'Blanquilla', once on-tree ripening was initiated,

443 (at 70 DAFB), ABA progressively accumulates (2-fold higher at CHD that at 70 DAFB)

444 in parallel to the fruit capacity to produce ethylene (ACC accumulation). These sustained

445 higher levels of ABA or ethylene also likely promote the action of IAA that remained at

446 high levels at the end of fruit growth and development for this variety.

447 On the other hand, the inability of 'Conference' fruit to produce ethylene, and thereby to

448 normally ripen upon harvest, may be due to a coordinated action of GA_1, SA and JA

449 (already known as ripening inhibitors for other species), which remained at high

450 concentrations during the latest phases of fruit growth leading to an inhibition of the

451 ethylene metabolism.

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667

692   'Blanquilla' (●) and 'Conference' (○) along fruit growth and development (represented

693   as days after full bloom; DAFB). Error bars represent the standard error of the means

694   (n=6). Inserts describe for each graph the temporal changes on a fruit basis. * indicate

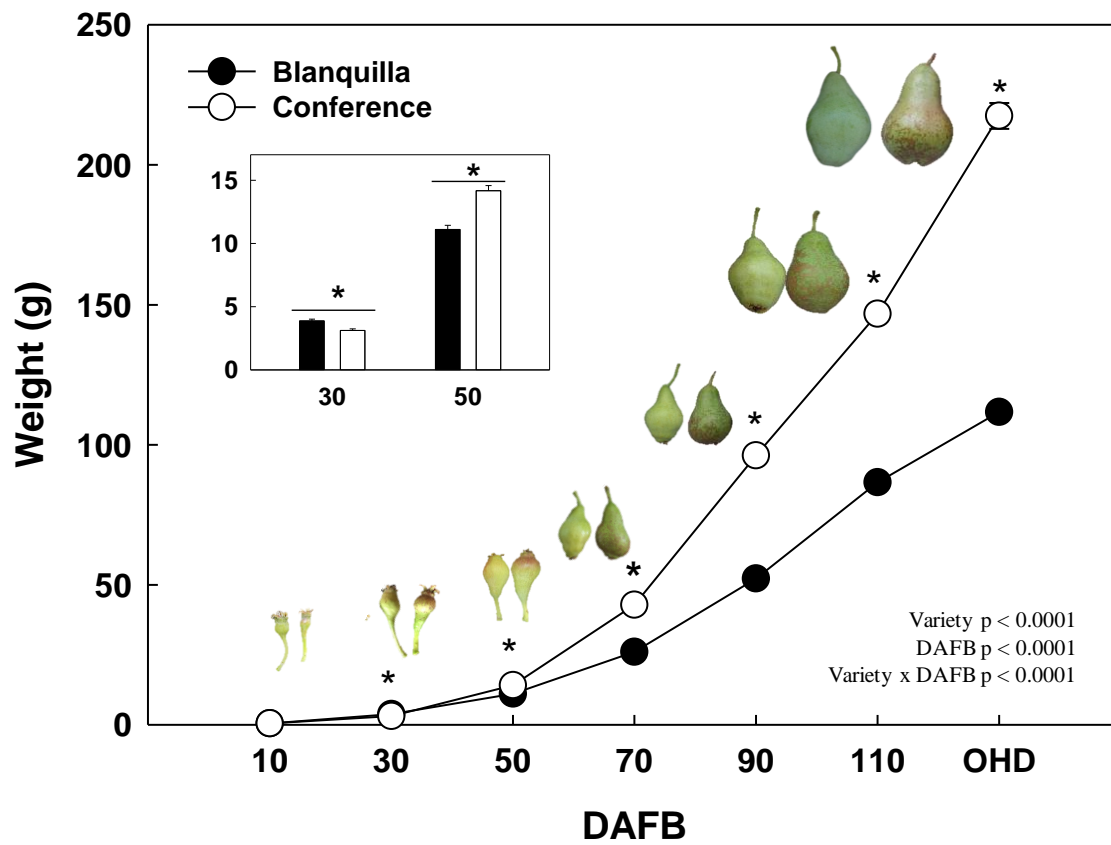695   significant differences at $p \leq 0.05$ between cultivars at a specific sampling.

696   **Figure 6:** Proposed model for the hormonal cross-talk and its interaction with specific

697   biochemical compounds or related physiological events in 'Blanquilla' (Summer pear

698   capable of ripening after harvest) and 'Conference' (Winter-like variety requiring a short-

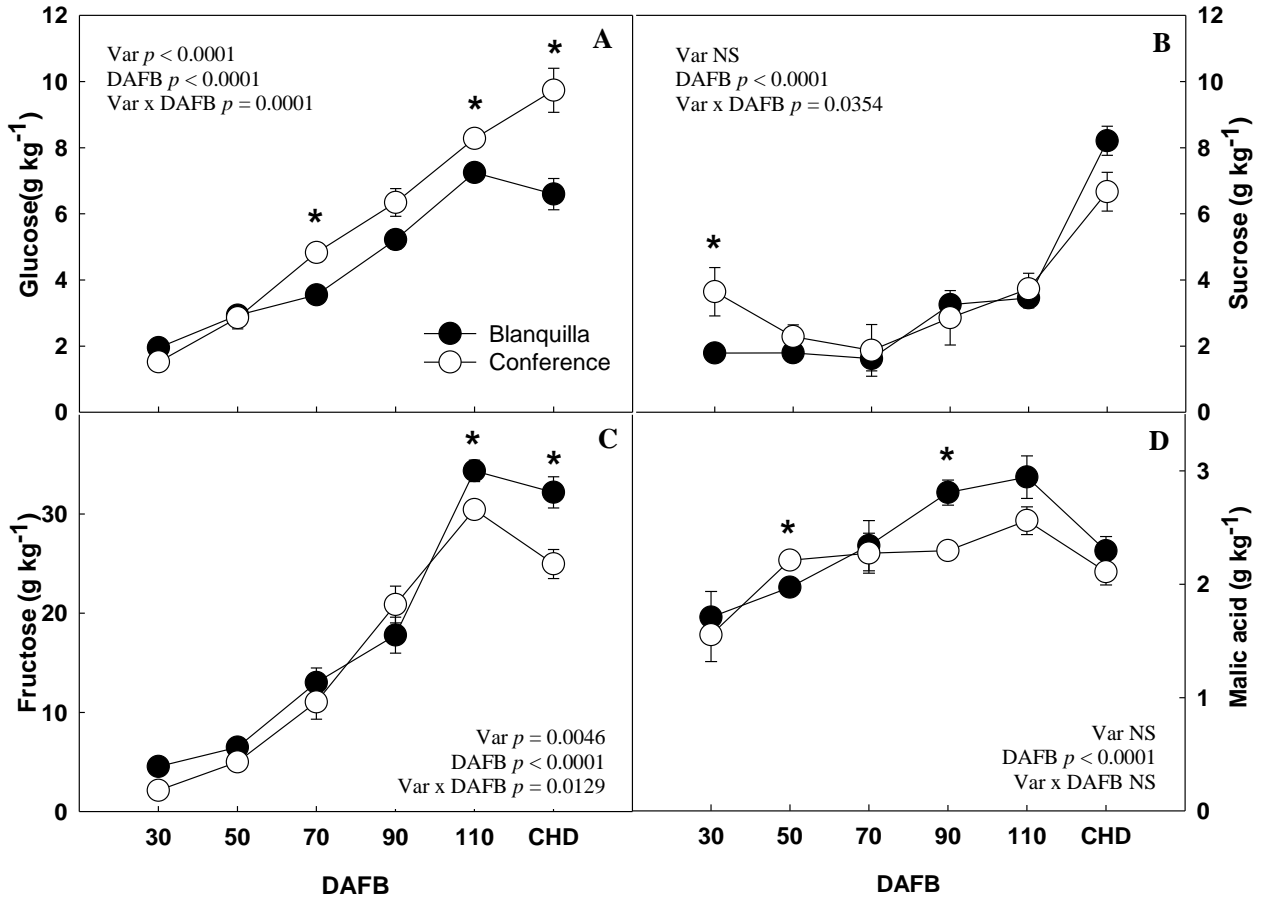699   chilling period to initiate ripening).

700

701   **Figure 1:**

702

250

Weight (g)

● Blanquilla
○ Conference

*

15

10

5

0

30   50

*

*

*

*

*

*

*

Variety p < 0.0001
DAFB p < 0.0001
Variety x DAFB p < 0.0001

200

150

100

50

0

10   30   50   70   90   110   OHD
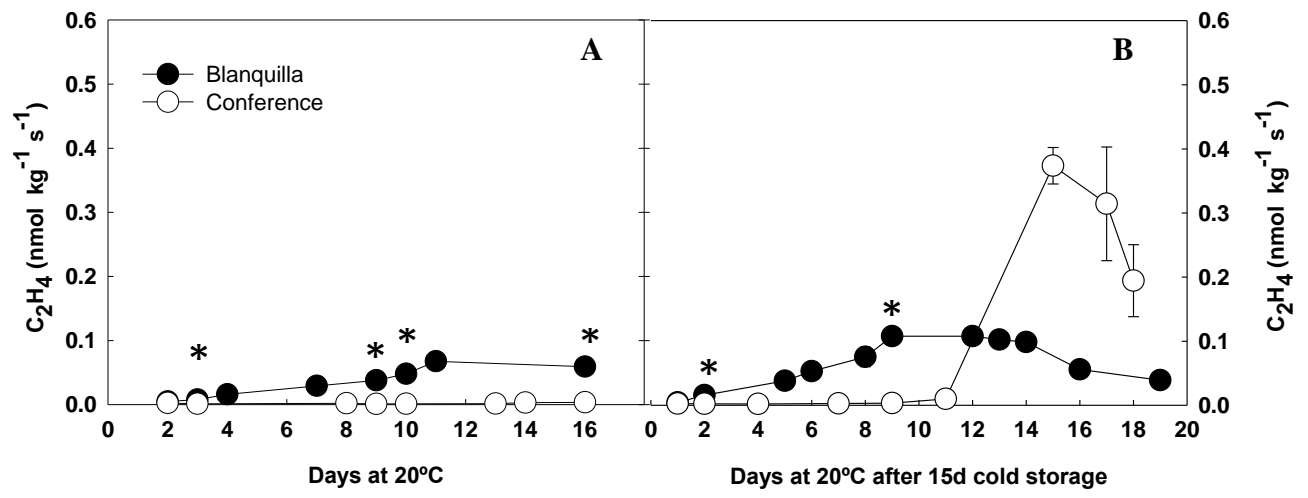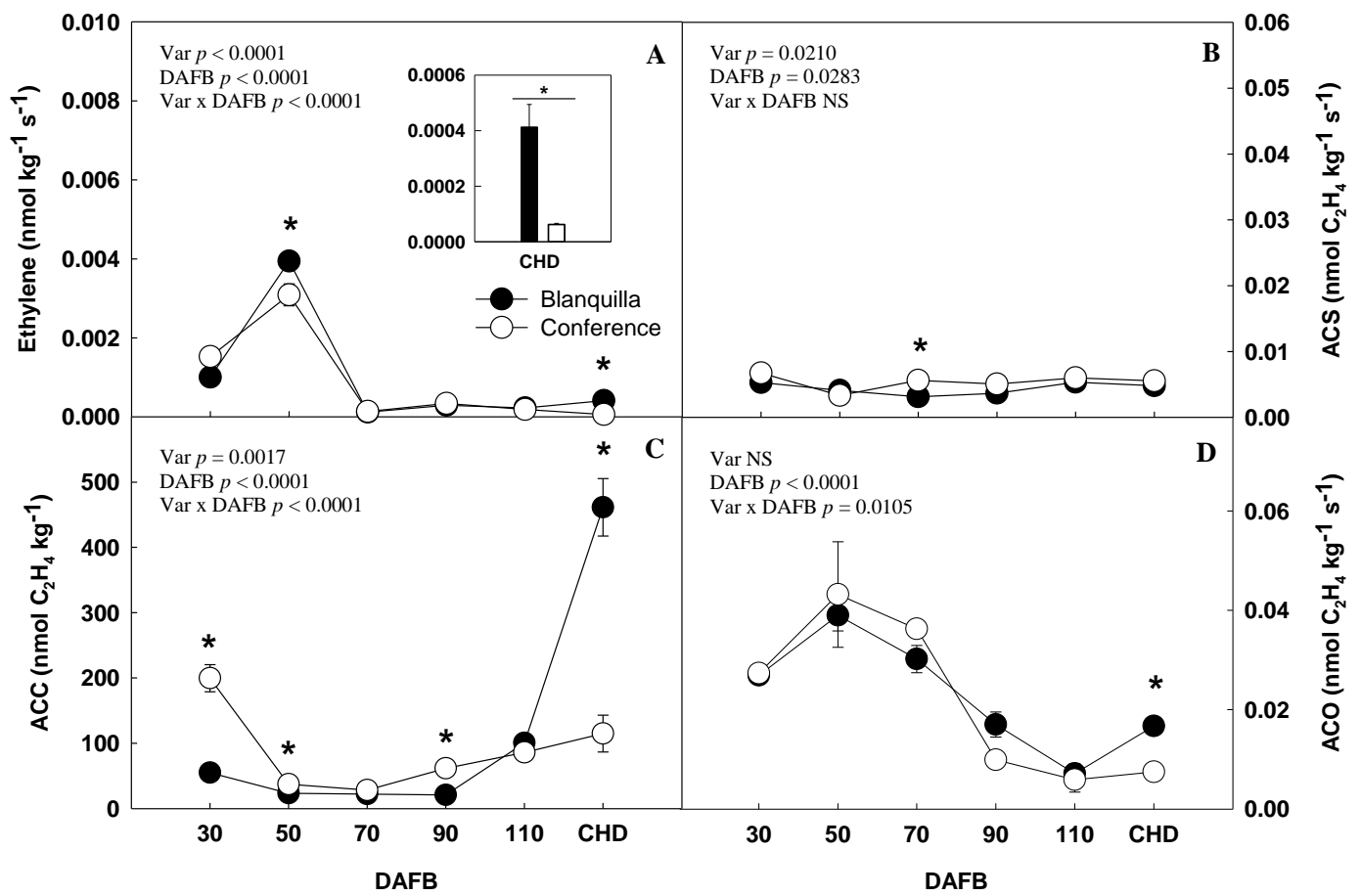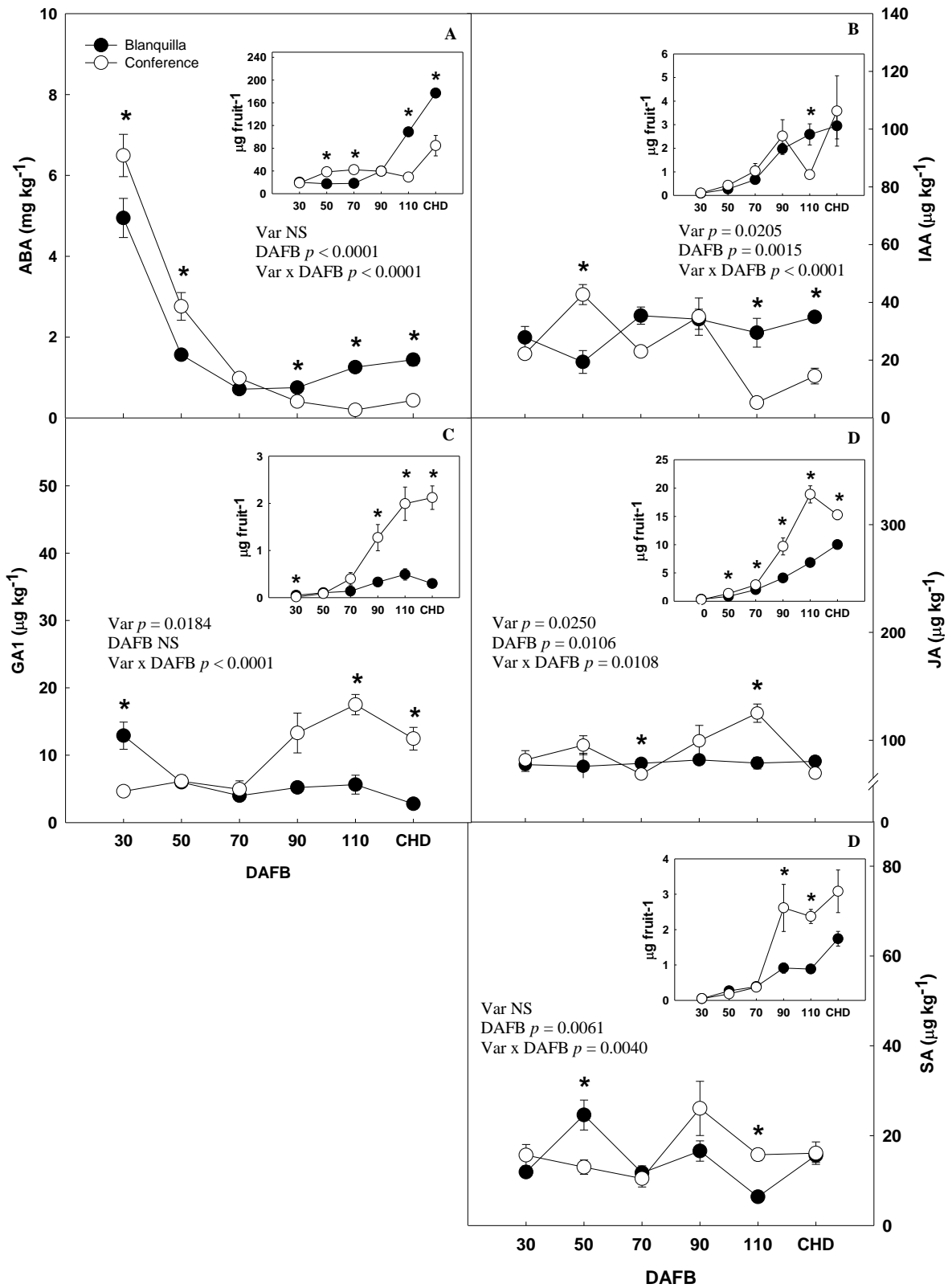
DAFB

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704   **Figure 2:**

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706   **Figure 3:**
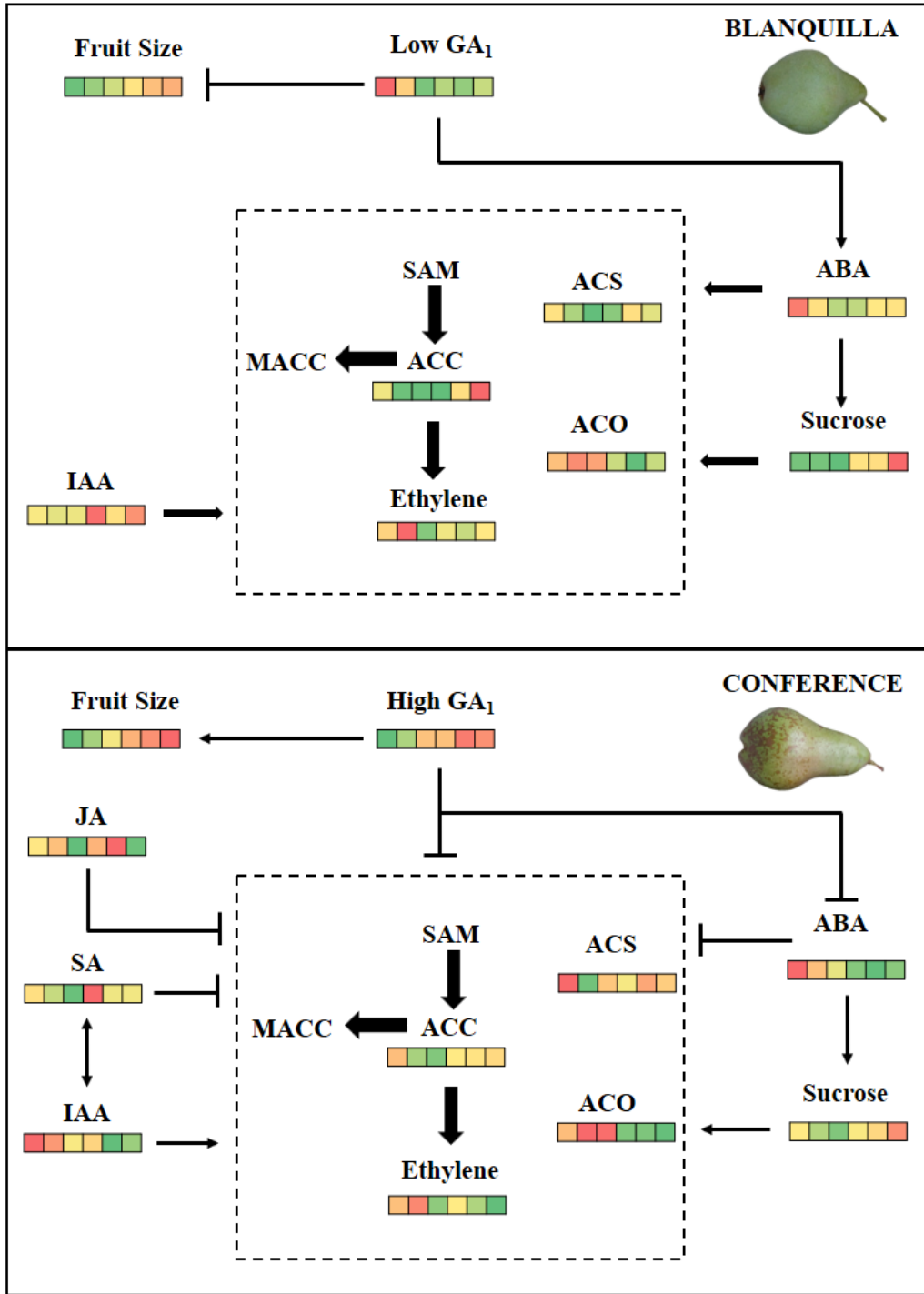
707

708 **Figure 4:**
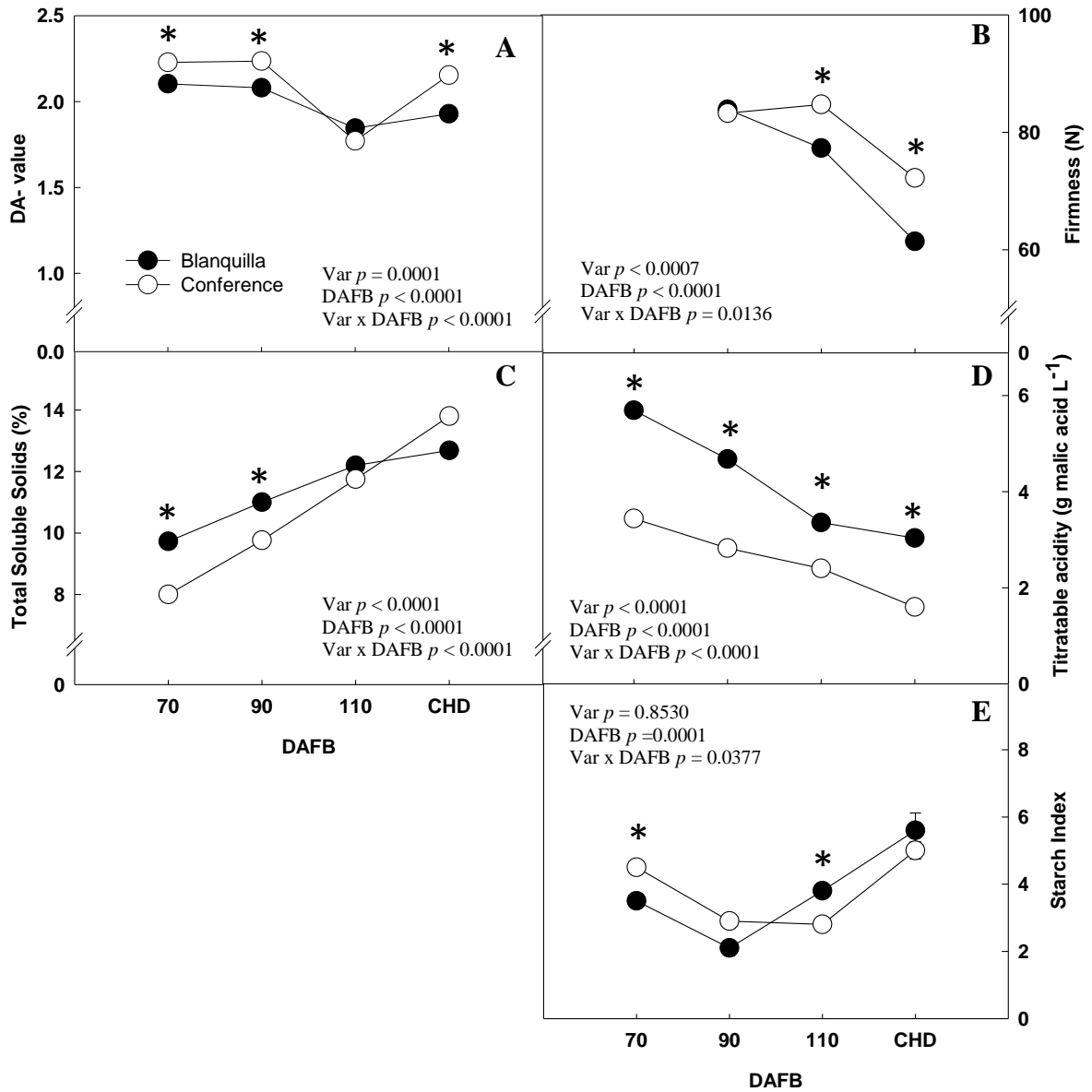
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711

712 **Figure 5:**

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714

715   ■ Low concentration   ■ High concentration

716   **Figure 6 :**

717

718   **Figure 6: Supplementary Figure 1:** Changes in DA-value (A), firmness (B), total

719   soluble solids (C), titratable acidity (D) and star index (D) in 'Blanquilla' (●) and

720   'Conference' (○) during different days after full bloom (DAFB). Error bars represent the

721   standard error of the means (n=4). Stars indicate significant differences at $p \leq 0.05$.

722

733   **Supplementary Figure 2:** Visualization of Spearman's rank correlation matrix between

734   quality and biochemical traits for both varieties (A) and for 'Conference' and 'Blanquilla'

735   separately (B; above and below, respectively). Numbers in the diagonal represent the

736   correlations between the same traits among the studied varieties. Circles above and below

737   the diagonal reported the correlation coefficients between traits. Colour intensity of each

738   circle is proportional to the correlation coefficients while the circle size is proportional to

739   the significance level. White squares denote non-significant correlations ($p > 0.05$).