ELUCIDATING THE INVOLVEMENT OF ETHYLENE AND OXIDATIVE STRESS DURING ON- AND OFF-TREE RIPENING OF TWO PEAR CULTIVARS WITH DIFFERENT RIPENING PATTERNS

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Abstract

Scarce information is available about the ripening process of European pears attached and detached from the tree. Accordingly, this study aimed to investigate the physiological and biochemical processes underlying both on- and off-tree fruit ripening in a summer ('Conference') vs. a winter ('Flor d'Hivern') pear cultivar. For each cultivar, a batch of fruit was harvested at the commercial harvest date and ripen at 20 °C and another batch was left to ripen on the tree. In both cultivars the inability of the fruit to soften on-tree, was related to a very limited ethylene metabolism but also associated to high content of H_2O_2 and low lipid peroxidation levels. In contrast, ripening in detached fruit was cultivar-dependent. In 'Conference' pears, the sharp firmness loss and colour changes observed during off-tree ripening were not strictly associated to an enhanced ethylene production but rather triggered by an oxidative related process preceding the climacteric rise. In contrast, 'Flor d'Hivern' pears experienced limited softening and degreening during off-tree ripening not being related to the action of ethylene or oxidative stress. Collectively our results showed that pear ripening was not exclusively dependent of ethylene production and that the fruit potential to limit oxidative damage may be involved with the inability of some European pear cultivars to ripen on-tree.

Keywords: 1-aminocyclopropane-1-carboxylic acid metabolism, H_2O_2, malondialdehyde, *Pyrus communis*, ripening

1. INTRODUCTION

Climacteric fruit are characterised by an increase in ethylene production and respiratory rate at the onset of ripening and the ability to ripen once detached from the plant (Lelièvre et al., 1997). European pears (*Pyrus communis* L.) are usually classified as climacteric fruit even though several cultivars are not capable to ripen normally after harvest unless receiving an ethylene or chilling treatment (Villalobos-Acuña and Mitcham, 2008). Thus, European pears are generally divided into summer or winter pears depending on their chilling requirements to achieve normal ripening (Saquet and Almeida, 2017). Summer pears require a minimum or no cold storage period to ripen normally and to produce ethylene after harvest. In contrast, winter pears need medium to long exposure to low temperatures to initiate the autocatalytic ethylene and thereby ripen (Villalobos-Acuña and Mitcham, 2008). Differences between summer and winter pear cultivars are also reflected during on-tree ripening, since the latter group would generally experience little or no firmness loss when left to ripen on the tree (Lindo-García et al., 2020a; Murayama et al., 1998).

A recent study on a typical summer pear cultivar ('Blanquilla') has shown that not only an enhanced ACC oxidase enzyme activity but higher sucrose content were likely modulating the capacity of this pear cultivar to ripen even on-tree (Lindo-García et al., 2019). Indeed, evidence exists suggesting that sucrose, in combination with other compounds, may be involved in the regulation of fruit development in both non-climacteric (i.e. strawberries) and climacteric fruit (Jia et al., 2013). Whether an impairment in ethylene biosynthesis or an altered sucrose metabolism may be responsible for the inability of winter pears to ripen on-tree is still unknown.

Strong evidences also suggest that not only ethylene but other hormones are involved in the regulation of fruit development and ripening (Kumar et al., 2014; Lindo-García et al.,

2020b; McAtee et al., 2013), and that the hormonal cross-talk may determine the capability of some fruit to ripen or not on-tree or once detached. In pears, it was proposed that gibberellins, and especially gibberellin 1, are likely acting as ripening inhibitors (Lindo-García et al., 2020b), thereby explaining the inability of the fruit to ripen and to produce ethylene when still attached to the tree. Other authors have shown that a decrease in auxin levels initiates the ripening process in 'Bartlett' pears and regulates the fruit's responsiveness to ethylene (Nham et al., 2015). Whether these specific hormones or other compounds may be considered as the 'tree factor' and account for the observed resistance to ripening on-tree, is still debatable but undoubtedly warrants further studies.

Accordingly, the aim of this study was to investigate the major physiological and biochemical changes accompanying the ripening of a summer ('Conference') vs. a winter-type ('Flor d'Hivern') pear both on- and off-tree. Emphasis has been given on ACC metabolism but also on fruit oxidative behaviour and changes in assimilate levels to better understand the determining factors involved in the hypothesized 'tree factor' in pears.

2. MATERIALS AND METHODS

2.1. Plant materials and experimental design

'Conference' and 'Flor d'Hivern' pears (*Pyrus communis* L.) were harvested from a commercial orchard near Lleida (Catalonia, Spain). 'Conference' pear was selected as a model for summer pear while 'Flor d'Hivern' is a local cultivar, which does not produce ethylene even after long periods of cold storage, behaving like a winter pear type (Lindo-García et al., 2020a). At the commercial harvest date (CHD), fruit (n=174 per cultivar) were randomly harvested from 15 trees and stored in acclimatised chamber at 20 °C and 90 % relative humidity. Off-tree samples were evaluated at harvest and after 3, 7, 15, 21 and 28 d after commercial harvest (DACH). For the on-tree assay, fruit (n = 27 per sampling and cultivar) were randomly taken from 6 different trees and evaluated at the same sampling days than off-tree fruit. All fruit were taken from a similar position within the canopy and transported to the laboratory for immediate analysis as follows. Metereological data for the duration of the on-tree ripening period was retrieved from an agrometereological station located 2 Km away of the experimental orchard.

2.2. Quality evaluations

Flesh firmness (N) was measured on 3 replicates of 5 fruit each per ripening condition with a penetrometer (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 5 fruit per replicate and 3 replicates per sampling) using a digital hand-held refractometer (Atago, Tokyo, Japan) whereas titratable acidity (TA) was measured on the same juice samples by titration using NaOH 0.1N 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

112 equatorial parts of the fruit. In parallel, degreening was evaluated by visual inspection in
113 15 fruit in order to assess the colour turn during the ripening process.
114 The starch index (SI) was evaluated on 15 fruit samples as described by Almeida et al.
115 (2016) with some modifications. An equatorial slice of each fruit was cut and dipped in a
116 solution of 0.6 % (w/v) iodine in 1.5 % (w/v) potassium iodine for 10 min and then the
117 starch index was subjectively determined using the 10-point scale chart developed by the
118 CTIFL (France).
119 In parallel, two equatorial flesh slices covering all the fruit (avoiding the core fruit) from
120 four individual fruit per replicate and three replicates per ripening condition were frozen
121 in liquid nitrogen and kept at -80 °C until further biochemical analysis.

122 **2.3. Ethylene production**

123 Ethylene production (pmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al.
124 (2017) with some modifications. Three replicates of 4 fruit each were placed immediately
125 after harvest in 2 L flasks sealed with a silicon septum for sampling the gas of the
126 headspace after 2 h incubation in an acclimatized chamber at 20 °C. For the analysis of
127 ethylene production, gas samples (1 mL) were taken using a syringe and injected into a
128 gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a
129 FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona,
130 Spain) as previously described by Giné-Bordonaba et al. (2014).

131 **2.4. Enzymes and compounds related to ethylene metabolism**

132 1-aminocyclopropane-1-carboxylic acid synthase enzyme (ACS) and 1-
133 aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) were extracted from frozen
134 flesh tissue and analysed as described by Lindo-García et al. (2019). The results were
135 expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

1-aminocyclopropane-1-carboxylic acid (ACC) was extracted also from frozen flesh and analysed as described by Bulens et al. (2011) with some modifications. Briefly, 2 g of frozen tissue were homogenized with 4 mL of a 5 % (w/v) sulfosalicylic acid solution and vortexed until a homogenous mixture was obtained. The samples were then gently shaken for 30 min at 4 °C and centrifuged at 8,000 *g* for 10 min at 4 °C. Subsequently, the supernatant was stored at -80 °C until analysis. The extract reading was performed mixing 1.4 mL of the ACC extract with 400 µL of 10 mmol L^{-1} $HgCl_2$ and 200 µL of a solution of NaOCl saturated with NaOH (2:1 v/v). After 4 min, a 1 mL headspace gas sample was injected into a gas chromatograph and the results expressed as µmol C_2H_4 kg^{-1} on a fresh weight basis.

2.5. Antioxidant capacity, hydrogen peroxide and malondialdehyde contents

Malondialdehyde (MDA), as an index of lipid peroxidation, was analysed as described by Martínez-Solano et al. (2005) using the thiobarbituric acid reactive substrates (TBARS) and the results expressed as nmol kg^{-1} s^{-1}. Antioxidant capacity was analysed using the Ferric Reducing Antioxidant Power (FRAP) assay as previously described by Giné-Bordonaba and Terry (2016). Results were expressed as g $FeCl_3$ kg^{-1} of fresh weight. H_2O_2 levels were determined as described by Giné-Bordonaba et al. (2017) using the Bioxytech H_2O_2-560 (OXIS International Inc., Portland, OR USA) colorimetric assay following the manufacturer's instructions. The content was expressed as mmol kg^{-1} of fresh weight.

2.6. Sugar and organic acid content

Malic acid and sugars (sucrose, glucose and fructose) were extracted from flesh frozen tissue as described by Giné-Bordonaba et al. (2017). Malic acid was extracted dissolving 2 g of frozen tissue in 5 mL of distillate water. The samples were slightly shaken for 10 min at room temperature and then centrifuged at 24,000 *g* for 7 min at 20 °C. The resulting

supernatant was recovered and used for enzyme coupled spectrophotometric determination (L-malate dehydrogenase) of malic acid using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

For sugars determination, 2 g of frozen flesh tissue were diluted in 5 mL of 62.5 % (v/v) aqueous methanol solvent and placed in a thermostatic bath at 55 °C for 15 min, mixing the solution with a vortex every 5 min to prevent layering. Then, the samples were centrifuged at 24,000 *g* for 15 min at 20 °C. The supernatants of each sample were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase) and sucrose (β-fructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

2.7. Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute Inc. Mean comparisons between ripening conditions at specific days for each cultivar was done by Student's *t*- test ($p \leq 0.05$) using critical values of *t* for two-tailed tests. Least significant difference values (LSD; $p = 0.05$) for the interaction ripening condition*DACH were calculated for mean separation using critical values of *t* for two-tailed tests.

3. RESULTS

3.1. Fruit quality changes during on- and off-tree ripening

Significant differences were observed when comparing the rate of firmness loss (softening) between on- and off-tree ripened fruit for both cultivars. 'Conference' pears (harvested at 62 N) experienced a sharp firmness loss from day 7 to day 21 (2.7 N d^{-1}) during off-tree ripening, reaching final firmness values of 5 N at day 21 (Fig. 1A). In contrast, the rate of firmness loss was much slower on-tree (0.2 N d^{-1}) and fruit never reached the optimal firmness for consumption (20-30 N; Torregrosa et al., 2019). In 'Flor d'Hivern' pears, firmness values were similar for both ripening scenarios until day 7 (*ca.* 48 N). Later, firmness values off-tree constantly decreased until reaching values of 26 N at day 28 whereas no firmness changes were observed on-tree (Fig. 1B).

Changes in I_{AD} values for both cultivars generally paralleled the changes observed in fruit firmness (Fig. 1; r^2 = 0.94 and 0.95 at $p \leq 0.05$ for 'Conference' and 'Flor d'Hivern', respectively). Briefly, in off-tree 'Conference' pears, I_{AD} value at harvest was 2.1 and then decreased by 5-fold at day 21 whereas relatively stable I_{AD} values (*ca.* 2) were observed in on-tree ripened fruit (Fig, 1C). A similar pattern was observed in 'Flor d'Hivern' pears but with lower values than those observed in 'Conference' pear (Fig. 1D). TSS content increased both on- and off-tree in 'Conference', yet a faster increase was observed in off-tree ripened fruit, reaching similar values (13.8 %) at day 21 and day 28, respectively (Table 1). The pattern observed in 'Flor d'Hivern' was however completely different. TSS content in off-tree ripened fruit was about 14 % and remained relatively unchanged along the storage period, while TSS content in on-tree ripened fruit decreased by 1.2-fold from day 7 to day 28 reaching final values of 11.6 % (Table 1).

Completely different patterns were also observed for the fruit acidity changes between cultivars. In 'Conference' pear, titratable acidity (TA) decreased both on- and off-tree but

with values consistently higher in off-tree ripening (Table 1). TA values in 'Flor d'Hivern' remained similar until day 7 in both ripening scenarios. After this day, TA was maintained at 2.3 g malic L^{-1} in on-tree ripened fruit whereas it decreased by 1.3-fold in fruit ripened off-tree at 20 ºC (Table 1).

Starch Index (SI) in 'Conference' pears ripened off-tree reached the maximum value (10) after 15 d at 20 ºC, whereas on-tree ripened fruit showed a constant SI value until day 21. Later, SI increased by 1.4-fold reaching a value of 7.5 at day 28 (Fig. 1E). On the other hand, SI of 'Flor d'Hivern' pears at harvest almost doubled the value observed in 'Conference' pears, and reached the maximum value off-tree at day 7, whereas on-tree fruit did not reach this maximum value until day 28 (Fig. 1F).

The different patterns observed in off- and on-tree ripened fruit in these quality parameters may help to define the optimum harvest date for the different pear cultivars investigated herein improving then their final quality and storability.

3.2. Ethylene production and ACC metabolism

In 'Conference' pear, both on- and off-tree ripened fruit showed a similar pattern of ethylene production until day 15. Later, ethylene production off-tree increased about 6-fold, reaching a value of 0.7 pmol kg^{-1} s^{-1} whereas ethylene production remained low on-tree until day 21 and increased later to 2 pmol kg^{-1} s^{-1} at day 28. Ethylene production in 'Flor d'Hivern' was very low (values ranging from 0.08 to 0.24 pmol kg^{-1} s^{-1}) and did not show any clear pattern when comparing on- and off-tree ripened fruit (Fig. 2).

ACS activity in off-tree ripened 'Conference' pear increased from day 7 by 2.3-fold to reach values of 0.07 nmol $kg^{-1}s^{-1}$ at day 21 whereas no changes were noticed in on-tree ripened fruit (stable values around 0.03 nmol kg^{-1} s^{-1} during the ripening process; Fig. 2). However, in 'Flor d'Hivern' pears, no clear pattern was observed regarding ACS activity. (Fig. 2).

Significant differences in the ACC content were found between ripening conditions in 'Conference' pears. ACC levels in on-tree fruit were constant along all the ripening process, however, off-tree fruit showed a peak of ACC content at day 15 (0.25 μmol kg^{-1}; 14.5-fold higher than values observed from harvest to day 7) and ACC levels slightly declined thereafter until day 21. In 'Flor d'Hivern' pears, no clear peaks of ACC could be detected and ACC values were generally higher in on- than in off-tree ripened fruit (Fig. 2).

In contrast to the sound differences between cultivars detailed earlier, both cultivars showed a sharp increase of ACO activity in off-tree ripened fruit. ACO activity increased from day 3, reaching maximum values of 2 nmol kg^{-1} s^{-1} at day 15 and 1.5 nmol kg^{-1} s^{-1} at day 28 for 'Conference' and 'Flor d'Hivern', respectively (Fig. 2). On-tree ripened fruit maintained stable values during all the ripening process, although 'Conference' showed a slight increase from day 21 to day 28, paralleled to the slight increase observed in ethylene production (Fig. 2).

3.3. Oxidative and peroxidative changes

MDA levels in 'Conference' pears ripened off-tree gradually increased from harvest until day 21. On-tree ripened fruit did not show significant variations along the ripening process, maintaining values of 4 nmol kg^{-1} s^{-1} (Fig. 3A). A similar trend was observed in on- and off-tree ripened 'Flor d'Hivern' pears that exhibited unchanged MDA values for both ripening conditions (Fig. 3B).

Likewise, no clear differences were observed in the fruit antioxidant capacity when comparing on- and off-tree ripened fruit for any of the two cultivars (Fig. 3C and D).

Changes in H_2O_2 content were significantly different between on- and off-tree ripening for both cultivars. In 'Conference' pears off-tree, H_2O_2 content sharply decreased after a transient increase at day 3 to reach values of 15 mmol kg^{-1} at day 15 and thereafter. In

contrast, H_2O_2 content on-tree remained fairly unchanged until day 21 to decline thereafter (1.4-fold lower at day 28 than at day 21; Fig. 3E). On-tree ripened 'Flor d'Hivern' pears showed two transient peaks of H_2O_2 at days 3 and 21 whereas H_2O_2 levels off-tree steadily decreased along the ripening process showing values at day 28 *ca.* 2-fold lower than those observed at harvest (Fig. 3F).

3.4. Sugar and malic acid accumulation during off- and on-tree ripening

Slight differences in malic acid content were observed in 'Conference' pears when comparing on- and off-tree ripening. In this pear cultivar, malic acid content decreased in fruit ripened off-tree, and especially from day 7 onwards, but remained unchanged in fruit ripened on-tree (Fig. 4A). Similarly, malic acid content did not significantly differ between on- and off-tree ripened 'Flor d'Hivern' pears (Fig. 4B).

Main differences between on- and off-tree ripened fruit for 'Conference' pear were found in glucose content. Despite the decrease observed in both ripening conditions from harvest to day 3, glucose content increased later by 1.3-fold until day 21 off-tree but remained constant on-tree (Fig. 4C). However, glucose content in 'Flor d'Hivern' remained constant except a transient peak at day 3in on-tree ripened fruit (Fig. 4D).

In 'Conference' pears, off-tree ripened fruit showed a gradual decrease in fructose content from 65 g kg^{-1} at harvest to 53 g kg^{-1} at day 15 followed by an important increase (1.5-fold) thereafter. In contrast, fructose values slightly increased on-tree with a transient peak observed at day 7 (Fig. 4E). Fructose content in 'Flor d'Hivern' pears remained relatively unchanged along ripening (*ca.* 80 g kg^{-1}) regardless of the ripening conditions (Fig. 4F).

As for glucose, sucrose changes were different between on- and off-tree ripened fruit as well as between cultivars. Sucrose levels in 'Conference' pears doubled during the ripening process both on- and off-tree with values generally higher off-tree (Fig. 4G). In

279 contrast, in ‘Flor d’Hivern’ pears, sucrose content decreased in attached fruit while the
280 opposite pattern was observed in fruit detached to the tree (Fig. 4H).

4. DISCUSSION

4.1. On- and off-tree pear ripening is not strictly mediated by ethylene

In contrast to that described earlier for other summer pears cultivars (Lindo-García et al., 2019), on-tree ripening in 'Conference' pears was not accompanied by noticeable changes in fruit firmness or starch degradation. Generally, significant differences existed in most ripening related traits when comparing on- and off-tree ripening for this pear cultivar. This said, such differences were not strictly related to the fruit capacity to produce ethylene that remained at low levels in both conditions (Fig. 2). Firmness loss and degreening (Fig. 1A and Suppl. Fig. 2) observed in off-tree ripened fruit may be also related to an increase in ethylene sensitivity and to the low levels of ethylene produced by this cultivar. Accordingly, Johnston et al. (2009) reported that sensitivity to ethylene in apple increased as fruit ripen and that changes of some ripening traits may have different sensitivities to ethylene. Likewise, the increase in starch index in off-tree ripened 'Conference' pear may also be related to increased sensitivity to ethylene even though the relationship between starch degradation and ethylene is still controversial (Johnston et al., 2009; Singh et al., 2017).

The inhibition of ethylene production in 'Conference' pears during on-tree ripening was paralleled by a general inactivation of ACC metabolism (ACS and ACO). During off-tree ripening, the lack of ethylene production was not due to limited ACS activity as previously reported in 'Blanquilla' pears (Lindo-García et al., 2019) nor to a lack of ACC or inhibition of ACO activity. In this pear cultivar, temperature conditions when comparing on- and off-tree ripened fruit were fairly similar (Suppl. Fig. 1) thereby suggesting that differences in ethylene metabolism between on- and off-tree ripened fruit may not be linked to weather conditions. In this sense, other compounds such as hormones, sucrose or some molecules still unknown (Jia et al., 2013; Meyer et al., 2017)

306 likely produced by the mother plant may be responsible for inhibiting the fruit ethylene
307 production in 'Conference' pears (Lindo-García et al., 2020b; Nham et al., 2015).
308 A complex hormonal cross-talk leading to inhibited or enhanced ethylene production has
309 been detailed in several species (Jiang et al., 2000; Trainotti et al., 2007; Zhang et al.,
310 2009). High gibberellin 1 content in 'Conference' pears when still attached to the tree
311 may explain why this cultivar is not able to produce ethylene on-tree or immediately after
312 harvest (Lindo-García et al., 2020b). This hypothesis is further supported by the fact that
313 'Conference' pears, like other pears, need a short chilling period to ripen properly
314 (Hansen and Mellenthin, 1979; Villalobos-Acuña and Mitcham, 2008) and that low
315 temperatures are indeed known to decrease the content of gibberellins in other plants
316 (Pinthus et al., 1989; Reid et al., 1974).
317 During on-tree ripening, the very low ethylene production in 'Conference' may be also
318 attributed to a restricted ACC metabolism since ACS and ACO activity as well as ACC
319 levels remained low and unchanged. These findings are consistent with the theory of the
320 'tree factor' (Abeles et al., 1992) in which an ethylene inhibitor is exported from the
321 leaves to the fruit via the phloem (Sfakiotakis and Dilley, 1973) limiting on-tree ripening.
322 Such theory has been extensively investigated in avocado fruit (Liu et al., 2002; Pedreschi
323 et al., 2014; Tingwa and Young, 1975), a climacteric fruit unable to ripen unless detached
324 from the tree. As said, gibberellins as well as other hormones (such as jasmonic or
325 salicylic acids) may account for the observed inhibition of ACC metabolism (Kondo et
326 al., 2007; Lindo-García et al., 2020b; Zhang et al., 2003).
327 Similarly to that observed for 'Conference' pears, the observed changes in fruit softening
328 or degreening (Fig. 1B and Suppl. Fig. 2) in detached 'Flor d'Hivern' fruit were neither
329 explained by the fruit ethylene production capacity. This pear cultivar did not produce
330 ethylene during either on- or off-tree ripening behaving like a non-climacteric fruit and

showing an unusual ACC metabolism. In fact, even the low temperatures observed during on-tree ripening if compared to those of off-tree ripened fruit (Supplementary Fig. 1) were not sufficient to induce the ethylene production in this cultivar. Whether ethylene sensitivity is different when comparing on- and off-tree ripened fruit is still unknown and warrants further investigations.

Even though ethylene did not increase in detached fruit, ACO activity was clearly enhanced during off-tree ripening, reaching levels similar to those observed in 'Conference'. Since ACC is commonly conjugated to an inactive form, the malonyl ACC (MACC; (de Poel and Van Der Straeten, 2014)), inhibition of ethylene production and ACC deficiency during off-tree ripening in 'Flor d'Hivern' might be due to an increase in MACC. Our results for this specific pear cultivar suggest, for the first time, the existence of a non-climacteric-like cultivar among the *Pyrus communis*. Previous studies carried out in other Pyrus spp. have already identified the existence of non-climacteric like cultivars (i.e. cv. 'Nijisseiki'; *Pyrus pyrifolia*) nor producing or responding to propylene treatments (Downs et al., 1991). This said, further works should determine the molecular regulation of ripening impairment in 'Flor d'Hivern' to further confirm this hypothesis.

4.2. An oxidative process may be responsible for triggering off-tree ripening in some pear cultivars

In a previous study, the initiation of fruit softening and ripening in 'Blanquilla' pears was associated to an oxidative stress leading to higher MDA content and preceding the climacteric rise (Lindo-García et al., 2019). Similar results were found in this work in 'Conference' pears in which softening and increase in lipid peroxidation (MDA content) clearly precede the initiation of ethylene production in off-tree ripened fruit. Although these changes were much more limited in 'Conference' than in 'Blanquilla' pears (Lindo-

García et al., 2019), and to some extent the MDA content measured by the methodology described herein may be overestimated, our results indicate that oxidative processes may be key factors that trigger the ripening capability in summer pears.

Since the levels of H_2O_2 decreased during off-tree ripening (Fig. 3E), the oxidative processes leading to higher MDA content were not likely mediated by H_2O_2, nor accompanied by a decline in the fruit antioxidant capacity (Fig. 3C). These findings differ from previous studies carried out in tomato (Kumar et al., 2016) and cherry fruit (Giné-Bordonaba et al., 2017) in which H_2O_2 levels were reported to increase along the ripening process. Nonetheless, they are in accordance with those observed in 'Blanquilla' (Larrigaudière et al., 2004) and 'Conference' pears (Larrigaudière et al., 2001) during postharvest cold storage.

During on-tree ripening, and despite of the higher H_2O_2 levels if compared to off-tree ripening, MDA content in 'Conference' pears only increased slightly in accordance with the observed low softening rate. A similar behaviour was found for 'Flor d'Hivern' pears that exhibited constant levels in lipid peroxidation markers despite significant differences in H_2O_2 levels between these two ripening scenarios. Collectively, these results clearly indicate that pears, when attached on-tree, very effectively impaired the action of H_2O_2 and oxidative damage. This behaviour may explain the differences in ripening behaviour observed on-tree and is in accordance with an idea that the 'tree factor' is not only associated with an inhibition of ACC metabolism but also with the endogenous capacity of pears to prevent oxidative damage on-tree. Further studies that may consider the putative roles of lipoxygenases and antioxidant enzymes, among others, are needed to better determine the real nature of the 'tree factor'.

4.3. The role of assimilates during the ripening process in summer vs. winter pears

With this in mind, we further analysed the putative role that photo-assimilates may play during on- and off-tree pear ripening. Among them, sucrose has been reported to act as an important signal molecule regulating fruit development and ripening in both climacteric and non-climacteric species (Jia et al., 2013). In 'Blanquilla' pear, the increase in sucrose content was concomitant with the increase in the fruit ethylene production and thereby the ability to ripen on-tree (Lindo-García et al., 2019). In this work, sucrose levels also increased in 'Conference' pears but remained unchanged in 'Flor d'Hivern' on-tree, being always at levels significantly lower than in 'Blanquilla' pears (i.e. maximum of about 8 g kg^{-1} in 'Conference' and 'Flor d'Hivern' vs. 20 g kg^{-1} in 'Blanquilla'; Lindo-García et al., 2019). In 'Blanquilla', the ethylene production was initiated only when sucrose levels were higher than 10 g kg^{-1}. Collectively these results suggest then that this value might be a threshold value that has to be reached to initiate ethylene production among different pear cultivars.

On the other hand, the unexpected decrease from day 7 (Fig. 4H) in sucrose content during on-tree ripening of 'Flor d'Hivern' pears was in accordance with the TSS changes (Table 1). Such a decrease may be triggered by the lower field temperatures likely causing sucrose breakdown as commonly observed during cold storage of apples and pears (Drake and Eisele, 1999; Itai and Tanahashi, 2008).

In addition to sucrose, other sugars as well as malic acid play an essential role in many processes during fruit development and ripening (Ciereszko, 2018; Fernie and Martinoia, 2009). The decrease in malic acid along off-tree ripening for both cultivars may be explained by its function as a respiratory substrate (Famiani et al., 2014) as previously described in apple (Liu et al., 2016) and 'Blanquilla' pears (Lindo-García et al., 2019).

405 On an another hand, the sharp increase in glucose content observed in ‘Conference’ off-
406 tree but not in ‘Flor d’Hivern’ pears, is likely the reflection of the ripening-related events
407 resulting from the degradation of complex sugars (i.e. starch) to glucose. In this sense,
408 further studies investigating the influence of exogenous sugar applications on pear
409 ripening, both on- and off-tree, are warrant.
410

5. CONCLUSIONS

The results from this study provide new information about the complex ripening process of fruit attached and detached to the tree among different European pears. They may be useful to understand the ripening physiology of pear or even to decide the optimum harvest date among the studied cultivars. Unlike other summer pear cultivars, our results showed that 'Conference' pear was not able to completely ripen (soften) on-tree. In this cultivar the ripening impairment observed on-tree was a consequence of low ACC metabolism. In contrast, during off-tree ripening, softening and colour changes seemed to be triggered by an oxidative process and later by an enhanced ACC metabolism, yet not being accompanied by higher fruit ethylene production. On the other hand, the winter pear 'Flor d'Hivern' owns an unusual ACC metabolism and high resistance to oxidative damage, behaving to some extent like a non-climacteric fruit. This said this pear cultivar also experienced some softening and degreening during off-tree ripening which are triggered so far by unknown causes.

Author's contribution

JGB, CL and VLG conceived and designed the experiment. VLG and ED performed all field and storage samplings including quality measurements and sample preparation for biochemical analysis. VLG, GE and MLL performed the analysis of ethylene and ethylene-related enzymes or precursors. VLG, CL and JGB wrote the manuscript and all remaining authors contributed in improving and revising the final version.

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594

595 **Table 1:** Changes in total soluble solids (TSS; %) and titratable acidity (TA; g malic L^{-1})
596 during off- and on-tree ripening for 'Conference' and 'Flor d'Hivern' cultivars. Means ±
597 standard error followed by the same letter for each cultivar and at specific sampling day
598 are not significantly different at $p \leq 0.05$ (n=3). LSD values ($p \leq 0.05$) for the interaction
599 ripening condition*days in 'Conference' cultivar were 0.73 and 0.27 for TSS and TA,
600 respectively; and in 'Flor d'Hivern', 0.70 and 0.29, respectively.
601

	TSS (%)			
	'Conference'		**'Flor d'Hivern'**	
Days	Off-tree	On-tree	Off-tree	On-tree
0	12.8 ± 0.09 a	12.8 ± 0.09 a	13.6 ± 0.12 a	13.6 ± 0.12 a
3	12.6 ± 0.13 a	12.2 ± 0.19 a	13.6 ± 0.03 a	13.7 ± 0.25 a
7	13.0 ± 0.23 a	12.0 ± 0.23 b	13.7 ± 0.27 a	13.6 ± 0.03 a
15	13.5 ± 0.12 a	12.2 ± 0.29 b	13.8 ± 0.18 a	12.9 ± 0.12 b
21	13.9 ± 0.30 a	12.9 ± 0.28 a	13.3 ± 0.09 a	12.7 ± 0.09 b
28		13.7 ± 0.42	13.8 ± 0.58 a	11.6 ± 0.26 b

	TA (g malic L^{-1})			
	'Conference'		**'Flor d'Hivern'**	
Days	Off-tree	On-tree	Off-tree	On-tree
0	2.1 ± 0.11 a	2.1 ± 0.11 a	2.2 ± 0.04 a	2.2 ± 0.04 a
3	1.6 ± 0.12 a	1.7 ± 0.05 a	2.1 ± 0.05 a	2.2 ± 0.06 a
7	1.7 ± 0.14 a	1.6 ± 0.04 a	2.3 ± 0.05 a	2.3 ± 0.18 a
15	1.6 ± 0.12 a	1.2 ± 0.10 a	1.8 ± 0.11 b	2.5 ± 0.03 a
21	1.5 ± 0.05 a	1.3 ± 0.06 b	1.8 ± 0.15 b	2.3 ± 0.07 a
28		1.1 ± 0.05	1.8 ± 0.12 b	2.4 ± 0.10 a

602
603

604 **LIST OF FIGURES**

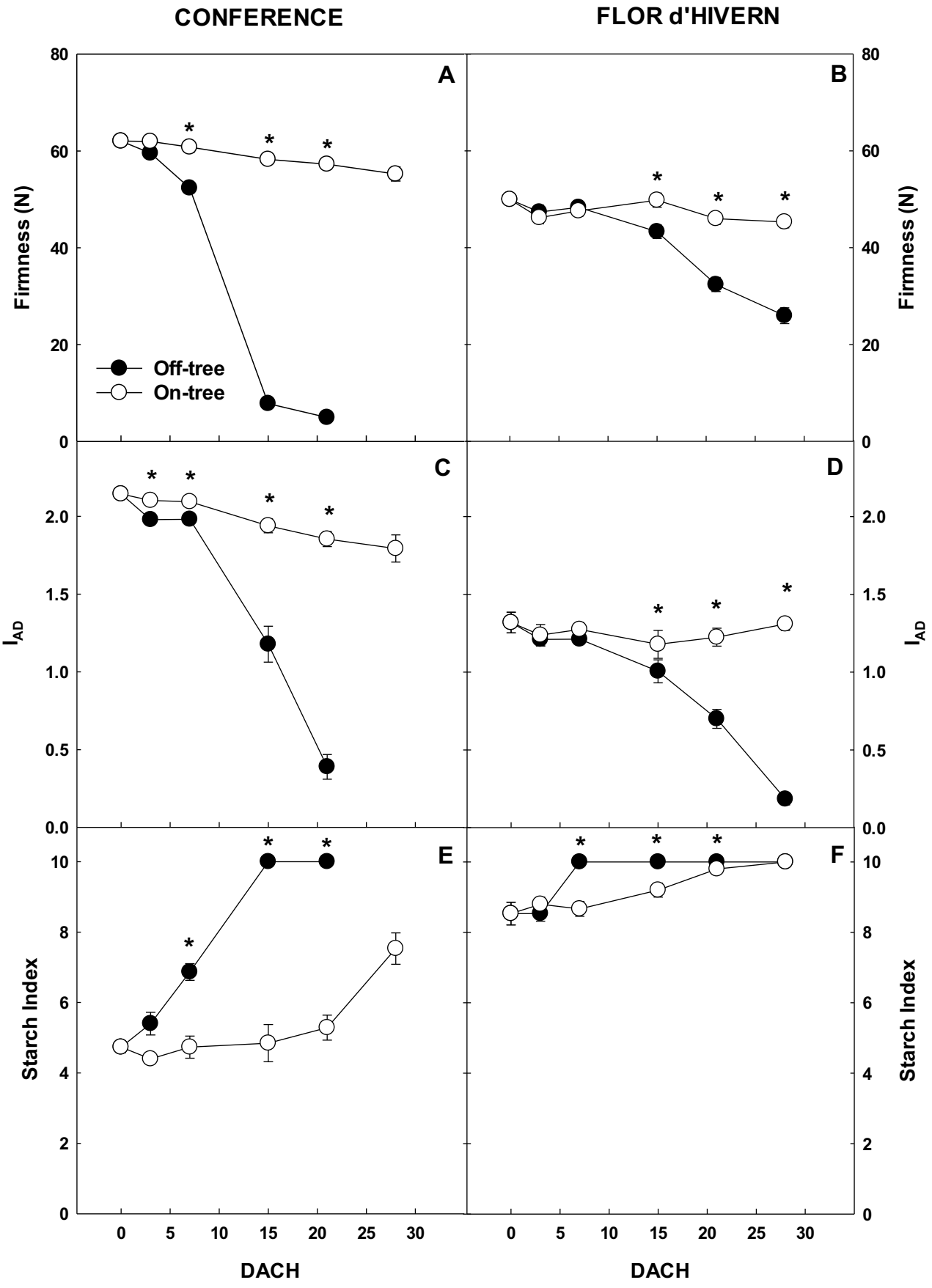
605 **Figure 1**. Changes in fruit firmness (A and B), index of absorbance difference (I_{AD}; C
606 and D) and starch index (E and F) during off-tree (●) and on-tree (○) ripening for
607 'Conference' (left) and 'Flor d'Hivern' (right) cultivars. DACH stands for Days After
608 Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars
609 indicate significant differences at $p \leq 0.05$. LSD values ($p = 0.05$) for the interaction
610 ripening condition*DACH for figures A, B, C, D, E and F were: 3.02, 5.13, 0.21, 0.11,
611 0.95 and 0.40, respectively.

612 **Figure 2**. Ethylene metabolism scheme showing the ethylene production, ACC synthase
613 activity, ACC content and ACC oxidase activity during off-tree (●) and on-tree (○)
614 ripening for 'Conference' and 'Flor d'Hivern' cultivars. DACH stands for Days After
615 Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars
616 indicate significant differences at $p \leq 0.05$. LSD values ($p = 0.05$) for the interaction
617 ripening condition*DACH for ACS, ACC, ACO and ethylene production were: 0.007,
618 0.02, 0.16 and 0.54, respectively for 'Conference' pear and 0.008, 0.02, 0.26 and 0.06,
619 respectively, for 'Flor d'Hivern' pears.

620 **Figure 3.** Changes in the concentration of malondialdehyde (A and B), antioxidant
621 capacity (C and D) and changes in hydrogen peroxide (E and F) during off-tree (●) and
622 on-tree (○) ripening for 'Conference' (left) and 'Flor d'Hivern' (right) cultivars. DACH
623 stands for Days After Commercial Harvest. Error bars represent the standard errors of the
624 means (n=3). Stars indicate significant differences at $p \leq 0.05$. LSD values ($p = 0.05$) for
625 the interaction ripening condition*DACH for figures A, B, C, D, E and F were: 1.10,
626 1.42, 0.13, 0.20, 19.80 and 17.17, respectively.

627 **Figure 4**. Changes in malic acid content (A and B), D-Glucose (C and D), D-fructose
628 levels (E and F) and sucrose levels (G and H) during off-tree (●) and on-tree (○) ripening

for ‘Conference’ (left) and ‘Flor d’Hivern’ (right) cultivars. DACH stands for Days After Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars indicate significant differences at $p \leq 0.05$. LSD values ($p = 0.05$) for the interaction ripening condition*DACH for figures A, B, C, D, E, F, G and H were: 0.54, 0.32, 7.74, 6.23, 13.15, 20.50, 1.67 and 1.40, respectively.



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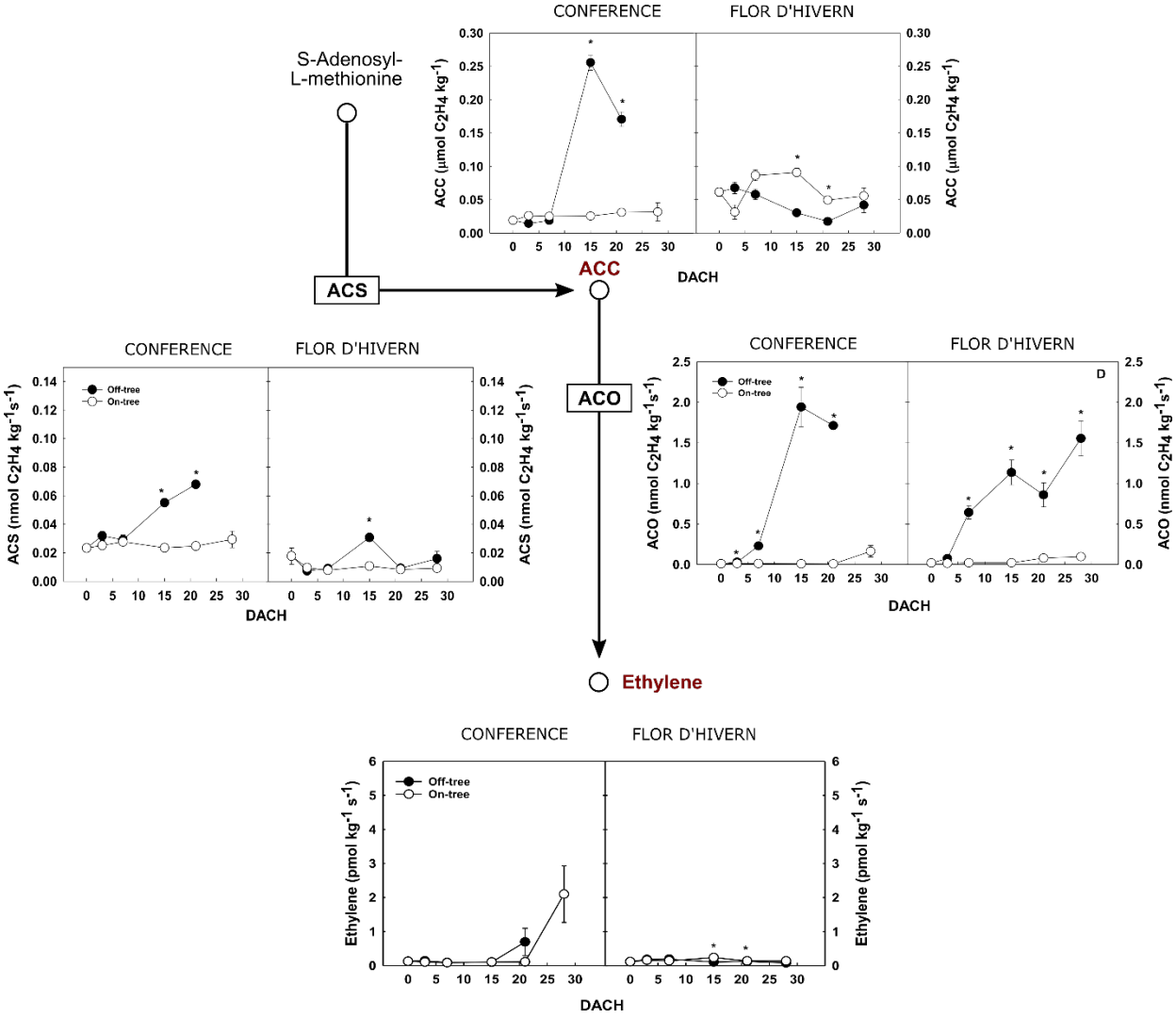
638 **Figure 1:**



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640 **Figure 2:**

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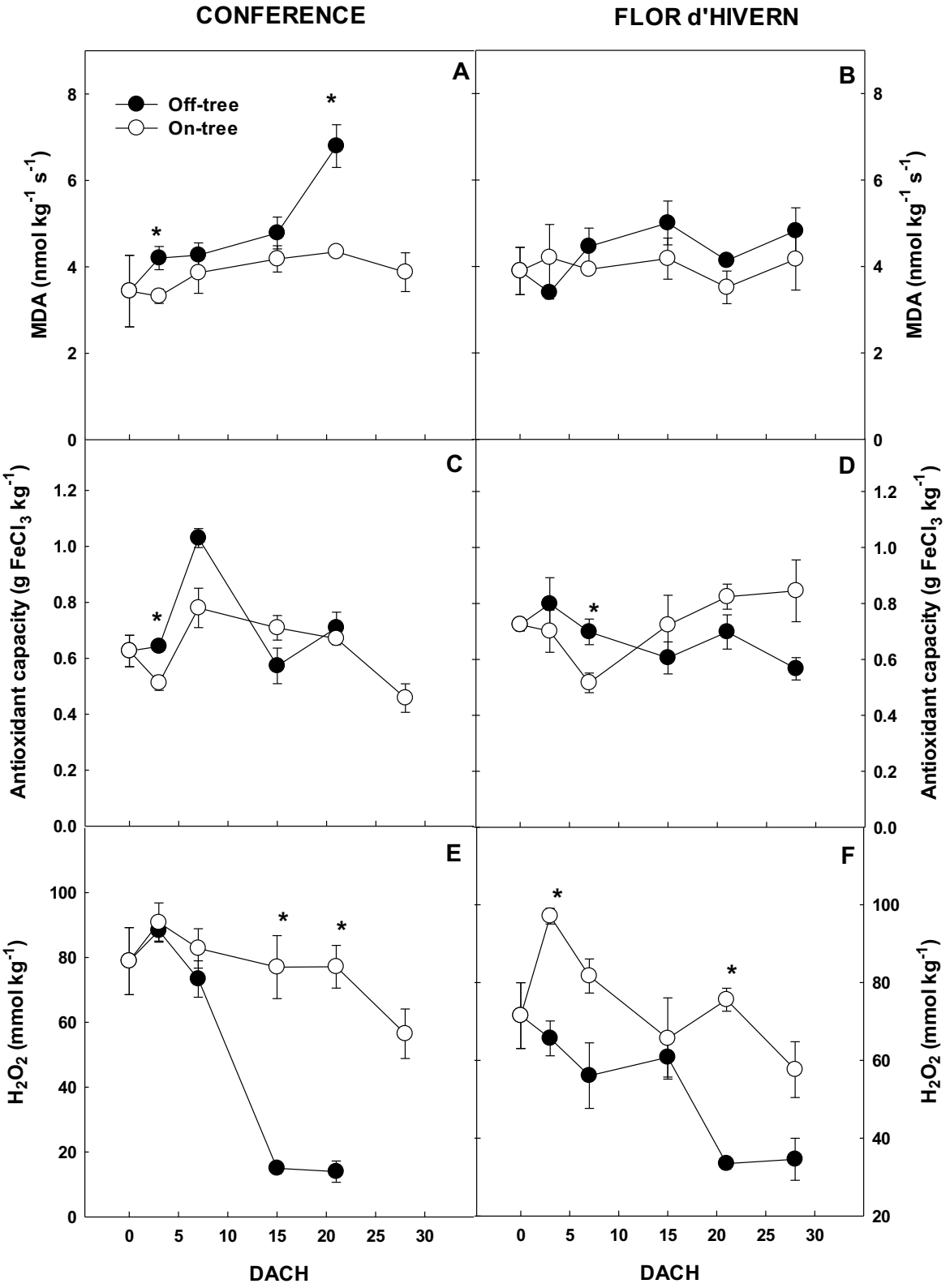

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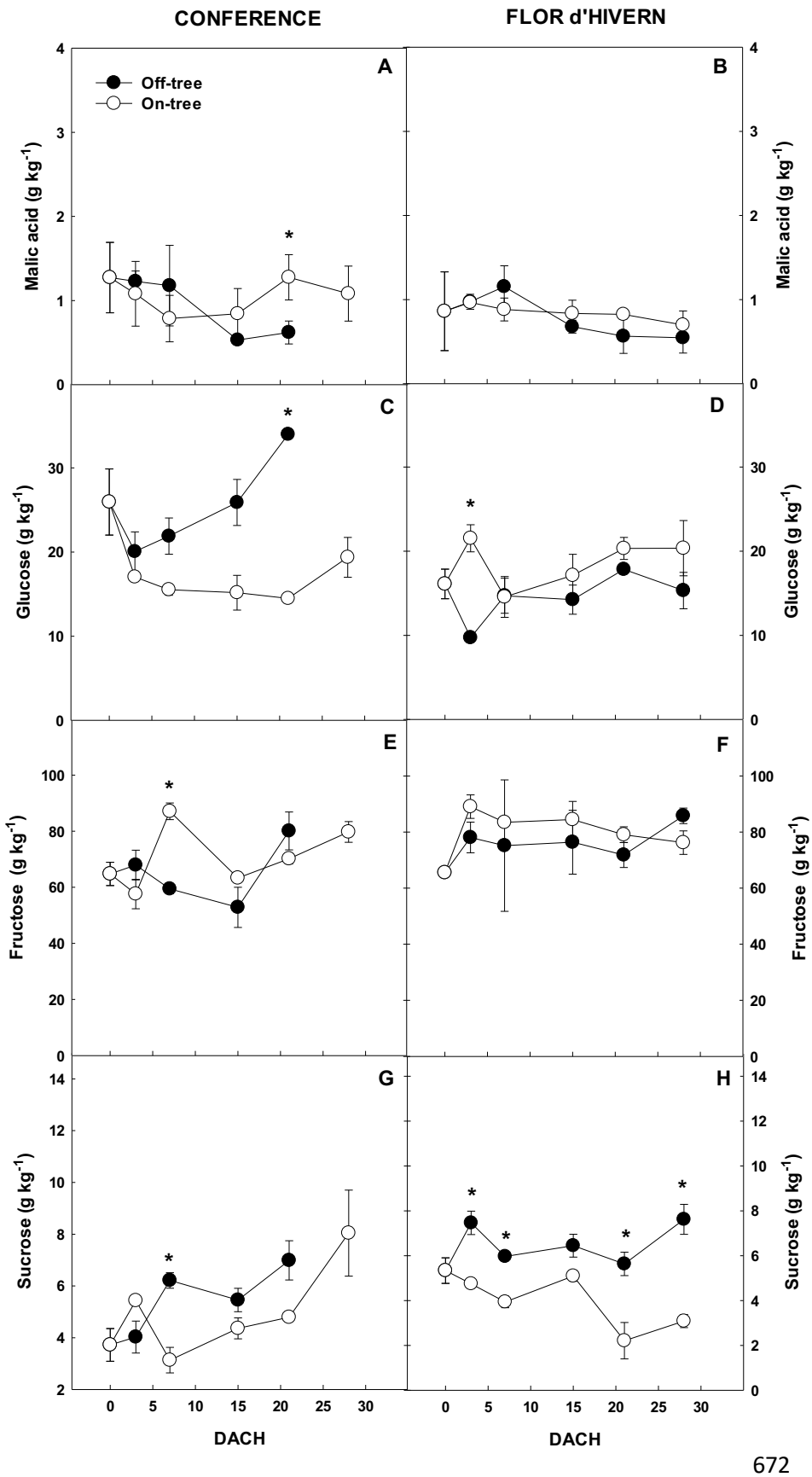
643 **Figure 3:**



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673 **Figure 4:**