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THE RELATIONSHIP BETWEEN ETHYLENE- AND OXIDATIVE-RELATED MARKERS AT HARVEST WITH THE SUSCEPTIBILITY OF PEARS TO DEVELOP SUPERFICIAL SCALD

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ABSTRACT

To better understand the specific biochemical pathways involved in superficial scald susceptibility, changes in ethylene biosynthesis, antioxidant, oxidative related processes and sugar metabolism were investigated for two scald sensitive pear cultivars ('Blanquilla' and 'Flor d'Hivern') with distinct postharvest ripening patterns at different harvest dates. Both cultivars developed symptoms of scald after 4 months of storage at -0.5 ºC, but the biochemical basis underlying susceptibility were different. In the summer pear 'Blanquilla', capable of ripening even on the tree, scald susceptibility was higher in fruit of advanced maturity and was associated with the action of ethylene on triggering the expression of *PcAFS1* gene. In this cultivar, the levels of ACC, ACS enzyme activity and *PcAFS1* at harvest were strongly correlated to scald incidence. In contrast, in the winter pear, 'Flor d'Hivern', with little or no ethylene-production capacity even after cold storage, scald symptoms were already visible when fruit were removed from cold storage, regardless of the fruit maturity. In this pear cultivar, scald symptoms were not dependent on ethylene, but rather associated with higher lipoxygenase (LOX) activity at harvest, an enzyme often associated with responses to chilling injury, and lower content of sorbitol, a compound that may act as cryoprotectant preventing cell damage during cold storage.

Keywords: ACC metabolism, chilling injury, peroxidive damage, sorbitol, superficial scald

1. INTRODUCTION

Superficial scald is considered one of the major cold-storage disorders affecting pome fruit and leading to substantial economic losses worldwide (Lurie and Watkins, 2012). The disorder is generally induced during cold storage and the symptoms, which manifests as brown or dark patches on the fruit skin, generally appearing after cold storage (Hui et al., 2016). Senescent scald is another storage disorder described in pear fruit, which manifests with similar symptoms on the fruit skin yet quickly progressing to the flesh when the fruit is left at room temperature (Whitaker et al., 2009; Zoffoli et al., 1998).

Research on superficial scald of pome fruit is abundant (Lurie and Watkins, 2012; Larrigaudière et al., 2016; Whitaker, 2013; Zhou et al., 2017) and, for decades, it has been assumed that the physiological or biochemical processes that determine scald susceptibility in apples and pears were equivalent. For instance, it was generally recognized that ethylene plays a determining role in scald development through the up-regulation of α-farnesene synthase (AFS) gene expression (Gapper et al., 2006; Pechous et al., 2005). Although this regulatory mechanism has been confirmed in different apple cultivars (Lurie et al., 2005), controversial information has been described for pears. For instance, 'Beurré d'Anjou' pears, although producing very low ethylene levels compared to 'Packham's Triumph', tend to accumulate similar α-farnesene levels during cold storage (Larrigaudière et al., 2016). A similar lack of relationship between ethylene and α-farnesene was also found in 'Beurré d'Anjou' pears picked at different harvest dates (Calvo et al., 2015), but also in apple lines suppressed for ACC metabolism (Pesis et al., 2009) or apple selections resistant and susceptible to superficial scald (Rao et al., 1998). Indeed, an ethylene-independent regulation of superficial scald has been recently described for apples (Busatto et al., 2018; Karagiannis et al., 2018).

The role that α-farnesene and its oxidation products (CTols) play in the development of scald has also been questioned. Given that scald is considered the result of an oxidative damage (Lurie and Watkins, 2012), such disorder was initially attributed to the oxidative action of CTols (Huelin and Murray, 1966). Indeed, numerous studies have shown that higher accumulation of this oxidation products was accompanied by greater scald incidence in apples (Giné Bordonaba et al., 2013; Moggia et al., 2010) and pears (Hui et al., 2016; Whitaker et al., 2009). However, studies describe inconsistencies in the α-farnesene hypothesis (Knee and Hatfield, 1981; Rao et al., 1998; Whitaker et al., 2000). It has been suggested that α-farnesene oxidation is a mere consequence of free radical reactions occurring during chilling stress and that α-farnesene is not required for the induction of scald but rather in aggravating the symptoms in fruit already compromised by oxidative stress (Rao et al., 1998; Rupasinghe et al., 2000). Busatto et al. (2014) found that scald in apples was related to a specific accumulation of chlorogenic acid and to its further oxidation in brown pigments by polyphenol oxidase (PPO) enzyme and that CTols were acting as signalling molecules. Busatto et al. (2018) hypothesized that scald resistance in 1-MCP-treated fruit was linked to higher levels of unsaturated long chain fatty acids as well as sorbitol, a compound stabilising the cell membranes and likely acting as cryoprotectant, thereby providing the fruit with greater tolerance to chilling injury damage.

It has also been suggested that scald mainly arises from an imbalance between the fruit capacity to produce and/or regenerate antioxidants and hence scavenge reactive oxygen species produced during cold stress (Guerra et al., 2012; Ju and Bramlage, 1999; Silva et al., 2010). In this sense, interesting associations were found with increased levels of p-coumaryl fatty-acid esters (Du and Bramlage, 1993; Whitaker, 1998), α-tocopherol (Rudell et al., 2009) and ascorbate levels in fruit skin (Larrigaudière et al., 2016; Wang

et al., 2018), and reduced scald incidence in different pome fruit cultivars. Nevertheless, attempts to link scald development with changes in the fruit enzymatic antioxidant potential have been so far inconclusive.

Most efforts dedicated to find predictive markers for scald susceptibility in apple and pears have been carried out by analysing changes in quality-related or biochemical markers during the first months of cold storage (Giné Bordonaba et al., 2013). Interesting prediction techniques based on CTol kinetics (Giné Bordonaba et al., 2013), chlorophyll fluorescence and colorimetric parameters (Guerra et al., 2012) as well as the emission of specific volatile compounds (Farneti et al., 2015) have been proposed in different cultivars. Few studies have attempted to discriminate the fruit susceptibility to scald based on performing a biochemical characterisation at harvest (Barden and Bramlage, 1994; Emongor et al., 1994).

Accordingly, the aim of this study was to explore the relationships between the concentration of specific compounds in pear fruit from different cultivars, harvest dates and superficial scald susceptibility. Emphasis was given to investigate biochemical compounds or the expression of genes involved in ethylene, antioxidant, and α-farnesene metabolisms but also in other metabolic pathways recently hypothesized to play an important role on scald development such as sorbitol and PPO.

2. MATERIAL AND METHODS

2.1. Plant materials and experimental design

About 156 fruit of 'Blanquilla' and 156 fruit (52 fruit per replicate) of 'Flor d'Hivern' pears (*Pyrus communis* L.) were harvested from 3 replicates of 5 trees per replicate on a commercial orchard near Lleida (Catalonia, Spain). Fruit were harvested at four different dates: about 5 d before the initial commercial harvest date (CHD-5), at the initial commercial harvest date (CHDi), the final commercial harvest date (CHDf; 3 days after CHDi) and 4 d after the final commercial harvest date (CHD+4)). Immediately after harvest, fruit were transferred to the laboratory for biochemical analysis. Sixty fruit per harvest and cultivar (3 replicates of 20 fruit each) were stored in semi-commercial chambers routinely aired at -0.5 °C and 90 % of relative humidity for 4 months to evaluate storage disorders and 6 fruit (3 replicates of 2 fruit each) were also stored at the same conditions to evaluate the ethylene production after 30 d of cold storage. To analyse the quality parameters at harvest, 60 fruit (4 pseudo-replicates per each of three replicates of 5 fruit each) were used. The ethylene production capacity was evaluated on 6 fruit (3 replicates of 2 fruit each) whereas 9 fruit (3 replicates of 3 fruit each) were used to analyse the α-farnesene and conjugated trienols content. In parallel, 15 fruit (3 replicates of 5 fruit each) were used for biochemical measurements. To do so, 30 g of flesh from the equatorial zone of the fruit and 30 g of peel tissue were removed and immediately frozen in liquid nitrogen before being kept at -80 °C.

2.2. Quality evaluations at harvest

Fruit firmness was evaluated with a hand-held penetrometer (T.R. Turoni srl., Italy) equipped with and 8 mm probe as described by Chiriboga et al. (2011). Total soluble solids (TSS; %) were measured on pear juice using a digital hand-held refractometer (PAL-1, Atago, Tokyo, Japan) whereas titratable acidity (TA) concentrations were

142 measured on the same juice by titration using 0.1 N NaOH and the results expressed as g
143 malic acid L^{-1}. The starch index was evaluated using 5 fruit per replicate as described by
144 Lindo-García et al. (2019).

145 **2.3. Ethylene production**

146 Ethylene production (nmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al.
147 (2014) with some modifications. Briefly, fruit were placed in 1.5 L flasks continuously
148 ventilated with humidified air at a flow rate of 1.5 L h^{-1}. Gas samples (1 mL) were taken
149 of effluent air using a 1 mL syringe and injected into a gas chromatograph (CG; Agilent
150 Technologies 6890, Wilmington, Germany) fitted with a FID detector and an Alumina
151 column F1 80/100 (2 m x 1/8 x 2.1, Tecknokroma, Barcelona, Spain). The oven
152 temperature was 140 °C while the injector and detector were kept at 180 and 280 °C,
153 respectively.

154 **2.4. Enzymes related to ethylene metabolism**

155 1-Aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-
156 carboxylic acid oxidase (ACO) enzymes were extracted from frozen flesh tissue and
157 analysed as described by Lindo-García et al. (2019). Enzyme activity was expressed as
158 nmol C_2H_4 kg^{-1} s^{-1} on a fresh weight basis. 1-Aminocyclopropane-1-carboxylic acid
159 (ACC) and malonyl-ACC (MACC) were extracted as described by Bulens et al. (2011)
160 with some modifications. Briefly, 2 g of frozen flesh tissue were homogenized with 4 mL
161 of a 5 % (w/v) sulfosalicylic acid solution. The samples were gently shaken for 30 min at
162 4 °C and then were centrifuged at 8,000 *g* for 10 min at 4 °C. Subsequently, the
163 supernatant was stored at -80 °C until analysis. An aliquot of 0.5 mL of the supernatant
164 was hydrolysed by adding 0.2 mL of 6M HCl, vortex 5 s and incubated at 99 °C for 3
165 hours. Thereafter, tubes were removed from the dry bath and allow them to cool down
166 for 15 min at room temperature. Then the reaction was neutralized by adding 0.2 mL of

6 M NaOH and vortex 5 s. The samples were centrifuged for 15 min at 20,000 *g* at 20 °C and the supernatant stored at -20 °C until analysis. ACC and MACC extracts reading was analysed as described by Bulens et al. (2011) and the results expressed as μmol C_2H_4 kg^{-1} on a fresh weight basis.

2.5. Determination of α-farnesene and conjugated trienols (CTols)

AF and CTols were analysed as described by Larrigaudière et al. (2019) with some modifications. A strip of the peel was removed from the equatorial zone of each fruit and 6 discs (10 mm diameter) prepared using a cork borer. The discs were immersed in 5 mL of HPLC grade hexane for 10 min with constant stirring and then the solution was filtered and mixed with hexane until a final volume of 5 mL. Measurements were performed calibrating first the equipment with HPLC grade hexane. Absorbance at 232 nm (α-farnesene) and 281-290 nm (conjugated trienols) was recorded using a UV-spectrophotometer (1001 Plus, Milton Roy, USA). Concentrations of α-farnesene and conjugated trienols were calculated using the molar extinction coefficients E_{232nm}= 27,700 for α-farnesene and $E_{281\text{-}290nm}$ = 25,000 for conjugated trienols and the results expressed as μmol kg^{-1} on a fresh weight basis.

2.6. Fruit antioxidant capacity and antioxidant enzymes

Antioxidant capacity was analysed from peel tissue using the Ferric Reducing Antioxidant Power (FRAP) assay as previously described by Giné-Bordonaba and Terry (2016). Results were expressed as g Fe^{3+} g kg^{-1} of fresh weight. Ascorbic acid was extracted and analysed as described by Rassam and Laing (2005) with some modifications. Briefly, 3 g of peel tissue was homogenized with 5 mL of metaphosphoric acid suspension (3% MPA, 8% acetic acid) and then centrifuged at 20,600 *g* for 22 min at 4 °C. The supernatant was filtered using a 0.22 μm Millipore filter. Total ascorbic acid were prepared mixing 950 μL of extract with 50 μL of 40 mM Tris [2-carboxiethyl]

192 phosphine hydrochloride (TCEP·HCl) during 3 h at room temperature. Levels of total
193 ascorbic acid were determined by injection of 10 µL of sample on an Agilent 1260 Infinity
194 II liquid chromatograph UHPL measuring at 254 nm. Separation was carried out on a
195 Poroshell 120 EC-C18 (3 x 100 mm, particle size 2.7 µm, Agilent) at a flow-rate of 0.125
196 ml min^{-1} using 10 % of methanol (v/v) as mobile phase.
197 Total peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) were
198 extracted mixing 5 g of peel tissue with 10 mL of phosphate buffer (0.1M pH 6) with 0.5
199 mM cysteine and 5 % (w/v) PVPP. The extract was filtered through two layers of
200 Miracloth and centrifuged at 20,000 *g* for 15 min at 4 °C. A 2.5 mL sample of the
201 supernatant was loaded into a Sephadex G-25 column (PD 10; Pharmacia, Madrid, Spain)
202 that had previously been equilibrated with 10 mL phosphate buffer pH 6 and the enzyme
203 was eluted with 3.5 mL of the same buffer. The activity was analysed as described by
204 Giné-Bordonaba et al. (2017). Lipoxygenase (LOX, EC 1.13.11.12) extraction and
205 analysis were carried out accordingly to Larrigaudière et al. (2001) with some
206 modifications. Peel frozen tissue (5 g) was mix with 15 mL of extraction solution
207 containing 0.1 M phosphate buffer pH 7.5, 2 mM DTT, 1 mM EDTA, 0.1 % (v/v) Triton
208 X-100 and 1 % (w/v) PVPP. The homogenized was filtered through two layers of
209 Miracloth and centrifuged at 25,000 *g* for 15 min at 4 °C. The enzyme activity was
210 measured spectrophotometrically at 234 nm using a solution containing 0.1 M phosphate
211 buffer pH 8, 8.6 mM linoleic acid, 0.25 % (v/v) tween-20 and 10 mM NaOH.
212 For the extraction of catalase (CAT, EC 1.11.1.6), 5 g of peel frozen tissue were
213 homogenized with 15 mL 0.1 M phosphate buffer pH 7.8, 2 mM DTT, 0.5 mM EDTA
214 and 1.25 mM polyethylene glycol. Homogenates were filtered through two layers
215 Miracloth and centrifuged at 20,000 *g* for 15 min at 4 °C. Samples were loaded into a
216 Sephadex G-25 column and the activity measured as described by Giné-Bordonaba et al.

(2017). Enzyme activity was expressed in Unit of Activity (UA) per milligram of protein, with one UA representing the quantity of enzyme responsible for a change in 1 absorbance unit per minute.

2.7. Fructose and sorbitol content

Fructose and sorbitol were extracted from frozen flesh tissue as described by Giné-Bordonaba et al. (2017). The supernatants were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase), sucrose (β-fructosidase) and sorbitol (sorbitol dehydrogenase) using commercial kits (BioSystems S.A., Barcelona, Spain and Megazyme, Co. Wicklow, Ireland, respectively) and following the manufacturer's instructions.

2.8. Determination of superficial scald incidence

Scald incidence was estimated visually after 4 months of cold storage and after 7 d of shelf life (20 °C) for each harvest as described by Larrigaudière et al. (2019), and calculated as a percentage of the total number of fruit.

2.9. RNA extraction and gene expression analysis

Total RNA was extracted from peel tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, MO, USA). RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific). 43Gene expression analysis was performed as described by Baró-Montel et al. (2019) using KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA) as polymerase master mix. Oligonucleotides used for RT-qPCR analysis were designed as described by Busatto et al. (2019). *Md8283* was used as independent reference gene in all the experiment (Busatto et al., 2019, 2018). Results were expressed as Mean Normalized Expression (MNE) and calculated using the method described by Muller et al. (2002).

2.10. Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute. Comparisons between harvest time for each variety were done by Tukey's test at a significant level of $p \leq 0.05$. Least significant difference values (LSD; $p < 0.05$) were calculated for mean separation using critical values of t for two-tailed tests.

3. RESULTS

3.1. Influence of the fruit maturity at harvest on disorder incidence

The quality parameters determined at harvest are presented in Table 1. Flesh firmness decreased progressively from 69.6 N at CHD-5 to 53.5 N at CHD+4 in 'Blanquilla' pears (-1.79 N/d during on-tree ripening). In contrast, no significant differences were observed in 'Flor d'Hivern' pears between harvest dates, which maintained the same firmness values during on-tree ripening (50 N). Only slight differences in TSS values were found for the two cultivars. However, TSS values were always higher in 'Flor d'Hivern' pears than in 'Blanquilla' pears, especially at CHD+4 (Table 1). The TA slightly decreased in 'Blanquilla' pears whereas 'Flor d'Hivern' values remained stable. The SI increased as 'Blanquilla' pears ripened on-tree, and no clear pattern was observed for 'Flor d'Hivern' pears, yet SI values for this cultivar were generally higher than those observed in 'Blanquilla' pears (Table 1).

After 4 months of cold storage, only minimal differences in scald incidence were found among harvest dates for 'Blanquilla' pear that exhibited 0 to 6 % of the disorder incidence (Fig.1A). However, after 7 d of shelf life, superficial scald incidence sharply increased, observing 33 % , 71 %, 78 % and 98 % of incidence for CHD-5, CHDin, CHDfin and CHD+4 respectively (Fig. 1A). Scald incidence in 'Flor d'Hivern' pears was very high upon removal from cold storage (ranging from 70 to 75 %), and reached nearly 100 % after 7 d of shelf life regardless of the fruit maturity at harvest (Fig. 1B).

3.2. Changes in ethylene metabolism

The ethylene production after harvest was notably different between cultivars. 'Blanquilla' pears produced ethylene immediately after harvest and the days needed to initiate ethylene production and reach the climacteric peak were reduced as the harvest date progressed. Fruit harvested at CHD-5 reached the ethylene peak (*ca.* 0.10 nmol kg^{-1}

s^{-1}) after 20 d at 20ºC, whereas those harvested at CHD+4 need only 12 d at 20 °C (Fig. 2A). After 30 d of cold storage, 'Blanquilla' pears reached the ethylene peak at day 5, showing a maximum ethylene production of 0.10 nmol kg^{-1} s^{-1} (Fig. 2A, insert). In this pear variety, a short cold period did not lead to higher ethylene production values but rather to a faster initiation of the climacteric rise. Conversely, 'Flor d'Hivern' pears did not produce ethylene at harvest (maximum ethylene production of *ca.* 0.014 nmol kg^{-1} s^{-1}; Fig. 2B) or even after a 30 days cold stress (max. 0.0062 nmol kg^{-1} s-1; Fig.2B insert) regardless of the harvest date.

Differences in ethylene production rates between cultivars were related to differences in ACC metabolism. In 'Blanquilla' pears, ACC content increased by 15-fold from the earliest to the latest harvest date, reaching values of 6 µmol kg^{-1} at CHD+4. In contrast, in 'Flor d'Hivern' pears, ACC remained at very low levels of about 0.08 µmol kg^{-1} for all harvest dates (Fig. 3A), suggesting that the inability of this pear cultivar to produce ethylene was likely related to a reduced substrate availability. Regarding MACC content, no differences were observed between cultivars and harvest dates (with average values of 2 µmol Kg^{-1}; Fig. 3B).

ACS activity in 'Blanquilla' pears increased from 0.01 nmol kg^{-1} s^{-1} at CHD-5 to 0.04 nmol kg^{-1} s^{-1} at CHD+4 hence supporting the results obtained for ACC. In contrast, ACS activity in 'Flor d'Hivern' pears remained constant (ca. 0.01 nmol kg^{-1} s^{-1}) at all the harvest dates (Fig. 3C). It is of interest to note that ACO activity showed an opposite pattern when comparing the cultivars. In 'Blanquilla' pears, ACO activity remained stable whereas in 'Flor d'Hivern' the enzyme activity increased by 4-fold, from the earliest (0.009 nmol kg^{-1} s^{-1}) to the latest harvest (0.04 nmol kg^{-1} s^{-1}; Fig. 3D). Although higher *PcACS1* gene expression was observed in 'Flor d'Hivern' pears compared with 'Blanquilla' pears (Fig. 3E), no significant differences among harvest dates were

observed for the cultivars. Furthermore, a clear up-regulation of *PcACO1* was observed in 'Flor d'Hivern' pears but expression of the gene was not affected by harvest date for either cultivar (Fig. 3F).

3.3. α-Farnesene metabolism

α-Farnesene concentrations remained constant in 'Blanquilla' pears as the fruit ripened on-tree (100 μmol kg^{-1}; Fig. 4A). In contrast, an increase, but not statistically significant, from 80 to 160 μmol kg^{-1} was observed in 'Flor d'Hivern' pears from CHD-5 to CHD+4. CTol 281 content in 'Flor d'Hivern' pears also slightly increased in relation to the harvest date whereas in 'Blanquilla' pears this compound decreased by 2-fold from the earliest to the latest harvest (Fig. 4B).

PcAFS1 gene expression was differentially regulated as harvest dates progressed depending on the cultivar. A clear up-regulation was found in 'Blanquilla' pear, especially from CHD-5 to CHDin, while no clear trend was observed for 'Flor d'Hivern' pears (Fig. 4C).

3.4. Antioxidants and oxidative-related changes

Although 'Flor d'Hivern' pears exhibited higher antioxidant potential than 'Blanquilla' pears (Fig. 5A), harvest date had no effect for either cultivar. A similar trend was found for total ascorbate contents that were higher in 'Flor d'Hivern' pears but not affected by harvest date (Fig. 5B).

LOX activity was nearly 2-fold higher in 'Flor d'Hivern' than in 'Blanquilla' pears being about 15 UA mg^{-1} protein compared to 8 UA mg^{-1} protein in ''Blanquilla' pears, again not affected by harvest date (Fig. 5C). Finally, an inverse behaviour was found for CAT activity that was slightly higher in 'Blanquilla' than in ''Flor d'Hivern' pears regardless of harvest date (Fig. 5D).

An interesting behaviour was recorded for PPO and POX activity in 'Blanquilla' pears. In particular, PPO activity in 'Blanquilla' pears was significantly higher at earlier harvest (CHD-5; 550 UA mg^{-1} protein) if compared to the following harvests (200 UA mg^{-1} protein). In contrast, in 'Flor d'Hivern' pears and despite higher values at CHDi, PPO activity was not affected by harvest date. (Fig. 5E).

POX activity values in 'Blanquilla' pears were almost 500 UA mg^{-1} protein at the earliest harvest date (CHD-5) and 2 to 3-fold lower ($p<0.05$) in the successive harvest (Fig. 5F). No effect of harvest date was detected for 'Flor d'Hivern' pears, activities ranging from 210-350 UA mg^{-1} protein.

3.5. Characterisation of fructose and sorbitol content at harvest

Sorbitol metabolism was different between cultivars (Figure 6). In 'Blanquilla' pears, a lower sorbitol content (*ca.* 15 g kg^{-1}) was observed at CHD-5 compared with the other harvest (*ca.* 23 g kg^{-1}; Fig. 6A). In contrast, no significant differences were observed for 'Flor d'Hivern' pears where values remained relatively constant at 13 g kg^{-1} for all harvest dates, meaning about 1.2-1.8-fold lower than in 'Blanquilla' pears. The lower sorbitol contents in 'Flor d'Hivern' pears were associated with significant up-regulation of *PcSDH* especially at CHD_{+4} (Fig. 6C).

Fructose contents were similar for the cultivars and generally remained unchanged (*ca*. 30 g kg^{-1}) during on-tree fruit ripening (Fig. 6B).

4. DISCUSSION

4.1. Differences in symptom development and its relationship with the initial harvest date indicate that distinct types of superficial scald

The way by which the scald disorders appeared for the two cultivars was clearly different (Figure 1). 'Blanquilla' pear is highly susceptible to scald and it is commonly used as the model for this storage disorder in a summer-type pear. On the other hand, 'Flor d'Hivern' is a local cultivar, which does not produce ethylene even after long periods of cold storage (winter-type pear behaviour) but also has very high susceptibility to scald (Figure 1). In 'Blanquilla' pears, disorder incidence depended on the initial fruit maturity at harvest and was expressed only after removal from cold storage. In contrast, disorder incidence in 'Flor d'Hivern' cultivar developed during cold storage and regardless of initial harvest date.

Such a behaviour in 'Blanquilla' pears is in accordance with previous studies (Calvo et al., 2015; Villalobos-Acuña et al., 2011) and with the idea that scald development in pome fruit is strongly determined by the fruit physiological maturity at harvest (Calvo et al., 2015). It is directly linked to the fruit capacity to ripen on-tree (Lindo-García et al., 2019) and to the fact that ethylene may trigger specific metabolic changes ultimately associated to scald development. In contrast, the different pattern of scald development observed in 'Flor d'Hivern' pears, shows that the etiology of scald is cultivar dependent and that the same symptoms (brown patches on the fruit surface) can result from multiple and different metabolic shifts yet always induced by cold storage. It is unlikely that the symptoms observed in 'Flor d'Hivern' refer to other disorder such as senescent scald since the fruit susceptibility was not dependent on the maturity stage at harvest and the symptoms exclusively affected the fruit surface without altering the flesh or the fruit taste. To further elucidate the physiological and molecular basis of scald development in these two

371 cultivars, as well as to identify putative markers at harvest, targeted compounds and
372 genes, previously identified as playing a key role in the development of scald (Busatto et
373 al., 2018, 2014; Lindo-García et al., 2019) were analysed in fruit from both cultivars and
374 different maturities.

375 **4.2. Ethylene related markers may predict scald in 'Blanquilla' pears but not in**
376 **'Flor d'Hivern'**

377 The ability of 'Blanquilla' pears to ripen on-tree, was clearly mediated by the increasing
378 levels of ACC resulting from higher ACS enzyme activity (Fig. 3A and Fig. 3C,
379 respectively), that is the key factor limiting ethylene metabolism (Yang and Hoffman,
380 1984). *PcACO1* gene expression in this cultivar remained down-regulated during on-tree
381 ripening (Fig.3F) and hence did not explain the differences observed for the kinetics of
382 ethylene production among harvests. In 'Blanquilla' fruit, ACS and ACC were positively
383 correlated with disorders incidence (r^2=0.634 and 0.709, respectively; $p \leq 0.05$) thereby
384 confirming that the capacity of the fruit to ripen or at least to produce ethylene upon
385 harvest may be a good indicator of the fruit susceptibility to scald. However, further
386 studies are still needed to determine the critical ACC and ACS thresholds at harvest below
387 which 'Blanquilla' pears may not develop scald during storage.

388 The specific behaviour of 'Flor d'Hivern' pears regarding ethylene metabolism revealed
389 a higher regulatory complexity. In this cultivar, ACC levels remained very low during
390 on-tree ripening regardless of the harvest date, a result that likely explain the incapacity
391 of these fruit to produce ethylene upon harvest. However, and despite of these low
392 ethylene rates, ACS activity was not negligible and ACO activity was even higher than
393 that observed in 'Blanquilla' pear. Since the low levels of ACC were not associated to an
394 ACS inhibition, increased ACO activity and/or higher synthesis of MACC (Fig.3B), we
395 may hypothesize that ACC depletion in this cultivar may be regulated up-stream (Van de

Poel and Van Der Straeten, 2014). The lack of ethylene production in 'Flor d'Hivern' may also explain the lack of fruit softening at different harvest dates (Table 1). Accordingly, further studies determining the roles play by methionine and S-adenosyl methionine in the regulation of ethylene production in 'winter pears' are needed to better understand this specific behaviour.

Concomitantly, a clear up-regulation of *PcACS1* and *PcACO1* gene expression likely due to ACC deprivation was also observed in 'Flor d'Hivern' pears as harvest dates progressed. As 'Flor d'Hivern' pear did not produce ethylene nor accumulate ACC, we may speculate that some alterations at the post-translation level are responsible for the lack of association between gene expression and enzyme activity during 'Flor d'Hivern' on-tree ripening.

Our results also show that compounds related to ACC metabolism in 'Flor d'Hivern' pears, in contrast to 'Blanquilla' pears, were not related to the susceptibility of this pear cultivar to scald. The results indicate that scald development in 'Flor d'Hivern' pears was ethylene independent, since this cultivar behaves like a non-climacteric fruit, hence in agreement with previous studies (Busatto et al., 2018; Karagiannis et al., 2018; Rao et al., 1998) and further suggesting that others regulatory mechanism triggered by cold stress are likely involved in the development of this disorder. Besides, this is the first study showing that a non-climacteric like pear cultivar was very susceptible to superficial scald. That said, it is important to note that albeit not strictly being ethylene dependent, 1-MCP treatment controls scald in 'Flor d'Hivern' (Dupille, E.; personal communication) as also does in 'Blanquilla' pears (Larrigaudière et al., 2019).

4.3. *PcAFS1* as a key marker of scald sensitivity of 'Blanquilla' pears at harvest

419 The accumulation of α-farnesene and its oxidation into CTols has for long time been
420 recognized to be involved in scald development either as a causal agent (Lurie and
421 Watkins, 2012) or as an intermediate or signalling process (Busatto et al., 2014).
422 In our study, no clear relationships were found between α-farnesene, CTol281 levels and
423 the differences in disorder incidence for the different harvest dates for any of the cultivars
424 investigated. In 'Blanquilla' pears, however, a clear relationship was found between
425 *PcAFS1* gene expression, the fruit capacity to produce ethylene, and the disorder
426 incidence. These results are in accordance with previous studies which reported that
427 ethylene induces α-farnesene biosynthesis (Ju and Curry, 2000; Lurie and Watkins, 2012)
428 up regulating AFS1 gene expression (Gapper et al., 2006; Pechous et al., 2005). However,
429 considering that the up regulation of AFS1 in this work was not associated to increased
430 α-farnesene levels at harvest, we hypothesize that this regulatory mechanism leading to
431 higher α-farnesene content is evident only during cold storage. That said, changes in
432 AFS1 gene expression during on-tree ripening were clearly related to scald incidence (r^2
433 = 0.565; $p \leq 0.05$,), suggesting then that this parameter may be a potential marker to
434 predict, at harvest, scald susceptibility in 'Blanquilla' pears.
435 In 'Flor d'Hivern' pears and despite of similar and higher levels of α-farnesene and CTols
436 than in 'Blanquilla' pears, *PcAFS1* gene expression remained low regardless of the
437 harvest date. This result is likely related to the lower ethylene values observed in this
438 cultivar and hence to its inability to trigger *PcAFS1*. Our results suggest then, that in
439 contrast to 'Blanquilla' pears that follow a typical scald model linked to ethylene and α-
440 farnesene accumulation and oxidation, other regulatory mechanisms are likely involved
441 in the regulation of scald disorder in 'Flor d'Hivern' pears.
442 **4.4. The relationship between oxidative-related markers at harvest and superficial**
443 **scald susceptibility**

444 Given that scald is known to be the result of an oxidative process (Lurie and Watkins,
445 2012), antioxidants may play a decisive role in scald prevention. Ascorbate for instance
446 was found to play a key role to explain the differences in scald susceptibility between
447 pear cultivars (Larrigaudière et al., 2016). In accordance, a clear relationship was found
448 in this work in 'Blanquilla' pears between the initial levels in ascorbate within the fruit
449 skin and the disorder incidence after cold storage. Combined with the lower POX activity
450 observed after CHDi, these results might explain, at least in part, the increasing scald
451 susceptibility observed in 'Blanquilla' pears in relation to the fruit maturity at harvest.
452 The others parameters such as global antioxidant potential, LOX and CAT activities seem
453 to play a secondary role, in the same way that PPO activity at harvest is unlikely to act as
454 a limiting factor.
455 The results obtained in 'Flor d'Hivern' pears were again more complex. Although
456 presenting higher initial antioxidant potential and ascorbate levels, higher disorder
457 incidence was observed at all maturity stages. Similar results were reported in a previous
458 study with 'Beurre d'Anjou' pears, where the authors suggested that the initial antioxidant
459 potential of the fruit was not directly related to the capacity of the fruit to develop
460 superficial scald (Calvo et al., 2015). However, the higher POX and especially LOX
461 activity observed in this cultivar, compared with 'Blanquilla', may suggest an enhanced
462 lipid peroxidation as previously described in Japanese pear (Li and Wang, 2009), and
463 hence partially explaining the development of this disorder.
464 In contrast to 'Blanquilla' pears that exhibited a typical scald disorder associated to
465 ethylene and α-farnesene metabolism, scald-like disorders in 'Flor d'Hivern' seem to be
466 rather related to a classical chilling injury damage in which membrane integrity plays a
467 predominant role. This is further supported by the fact that scald in 'Flor d'Hivern' occurs
468 after short storage durations and becomes evident even during cold storage (Figure 1).

469 **4.5. The involvement of sorbitol and related enzymes with superficial scald**
470 **susceptibility**
471 Considering these last results and the new information generated in the recent articles of
472 Busatto et al. (2018) in apples, we also analysed the role that cryoprotectants such as
473 sorbitol may play in this context.
474 Only slight differences in sorbitol and fructose contents were found among harvest dates
475 for the two cultivars (Fig.6A and 6B). Nevertheless, sorbitol contents were lower and
476 fructose contents slightly higher in 'Flor d'Hivern' compared with 'Blanquilla'.
477 Accordingly, these results suggest that 'Flor d'Hivern' already had lower capacity to
478 prevent chilling damage at harvest, since higher concentrations of this compound have
479 been shown to increase the resistance to chilling injury in model species (Busatto et al.,
480 2018). Furthermore, a sharp up-regulation of *PcSDH* was observed in 'Flor d'Hivern' but
481 not in 'Blanquilla'. The higher conversion of sorbitol into fructose, a phenomenon likely
482 occurring during cold storage, may further determine the storage potential of this cultivar
483 limiting its fruit resistance to chilling injury. Combined with the increased peroxidative
484 problems showed earlier, the lack in cryoprotectants in 'Flor d'Hivern' likely determined
485 the higher susceptibility of this cultivar to develop the disorder.
486

CONCLUSIONS

The results from this study demonstrate that superficial scald among different pears cultivars with distinct ripening patterns may have different etiologies. Comparing the concentration of ethylene-related or oxidative compounds at harvest of two highly susceptible cultivars, we clearly distinguished two key casual features involved in scald development. The first one, associated to the action of ethylene on ripening and up-regulation of *PcAFS1* gene expression, was only noticeable when the fruit were able to initiate fruit ripening on-tree. This is the case of 'Blanquilla' pears for which ACC concentrations, ACS enzyme activity and *PcAFS1* expression level increased as fruit ripened on-tree in parallel to scald and thereby showing potential to be used at harvest as markers of the fruit susceptibility to scald. In the case of 'Flor d'Hivern', a typical winter pear cultivar, scald development was in a first instance ethylene independent but clearly associated to higher LOX and POX activities, that likely promote membrane peroxidation. This increase in membrane peroxidation coupled to a reduced content of specific compounds generally acting as cell cryoprotectants, such as sorbitol, may explain the development of the disorder in this pear cultivar.

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699 **Table 1:** Flesh firmness, total soluble solids (TSS), titratable acidity (TA) and starch
700 index (SI) for 'Blanquilla' and 'Flor d'Hivern' at different harvest date (CHD for
701 Commercial Harvest Date). Means ± standard error followed by the same letter are not
702 significant different at $p \leq 0.05$ (n=3) for each cultivar. LSD values ($p < 0.05$) for the
703 interaction cultivar*harvest date for firmness, TSS, TA and SI were 2.52, 0.48, 0.38 and
704 1.12, respectively.
705

Cultivar	Harvest	Firmness (N)	TSS (%)	TA (g malic L^{-1})	SI
Blanquilla	CHD-5	69.6 ± 0.92 a	12.9 ± 0.08 c	3.6 ± 0.08 a	3.3 ± 0.21 c
	Initial CHD	62.3 ± 0.72 b	13.3 ± 0.06 bc	3.3 ± 0.07 ab	5.0 ± 0.02 b
	Final CHD	57.8 ± 0.61 c	13.8 ± 0.04 a	3.2 ± 0.07 ab	6.2 ± 0.20 a
	CHD+4	53.4 ± 0.64 d	13.5 ± 0.07 ab	3.0 ± 0.09 b	6.6 ± 0.13 a
Flor d'Hivern	CHD-5	49.8 ± 0.87 a	13.7 ± 0.15 b	2.9 ± 0.09 a	6.8 ± 0.50 ab
	Initial CHD	47.8 ± 0.80 a	13.9 ± 0.15 b	3.2 ± 0.10 a	6.6 ± 0.48 b
	Final CHD	49.2 ± 0.59 a	13.7 ± 0.19 b	3.0 ± 0.11 a	7.8 ± 0.29 ab
	CHD+4	51.5 ± 0.78 a	14.8 ± 0.09 a	3.3 ± 0.11 a	8.4 ± 0.29 a

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LIST OF FIGURES

Figure 1: Scald incidence (%) in 'Blanquilla' (A) and 'Flor d'Hivern' (B) cultivars after 4 months of cold storage and 7 d of shelf life at 20 °C. Error bars represent the standard error of the mean (n=3). Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. (C) Visual appearance of scald-like disorders in 'Blanquilla' (left) and 'Flor d'Hivern' (right) pears after 4 months of cold storage (-0.5 ºC) plus 7 days of shelf-life (20 ºC). CHD stands for Commercial Harvest Date.

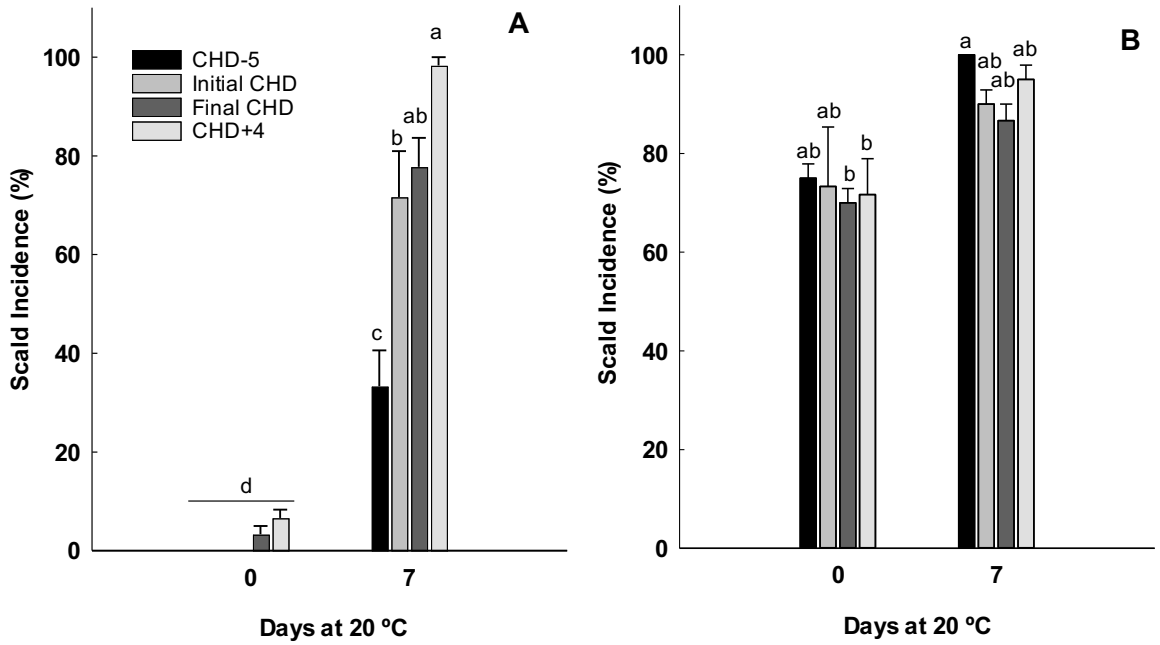
Figure 2: Ethylene production in 'Blanquilla' (A) and 'Flor d'Hivern' (B) cultivars at the different harvest dates. Error bars represent the standard error of the mean (n=3). Inserts describe the ethylene production at 20 ºC after 30 d of cold storage of fruit harvested at final CHD. CHD stands for Commercial Harvest Date.

Figure 3: Content of ACC (A), MACC (B), and ACC synthase activity (C), ACC oxidase activity (D), *PcACS1* gene mean normalised expression (MNE; E) and *PcACO1* gene mean normalised expression (F) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars) pears. Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, C, D, E and F were 0.80, 0.81, 0.03, 0.03, 0.006 and 0.22, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

Figure 4: α-Farnesene content (A), its oxidation products CTols (B) and *PcAFS1* gene mean normalised expression (C) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars). Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B and C were 74.94 and 1.99, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

Figure 5: Total antioxidant capacity (A), total ascorbate (B), LOX activity (C), CAT activity (D), PPO activity (E) and POX activity (F) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars). Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, C, D, E and F were 0.93, 2.01, 3.29, 2.57, 198.72 and123.39, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

Figure 6: Sorbitol content (A), fructose content (B) and *PcSHD* gene expression (C) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars) among the different harvest dates. Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, and C were 6.58, 9.04 and 0.04, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.



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751 **Figure 1:**

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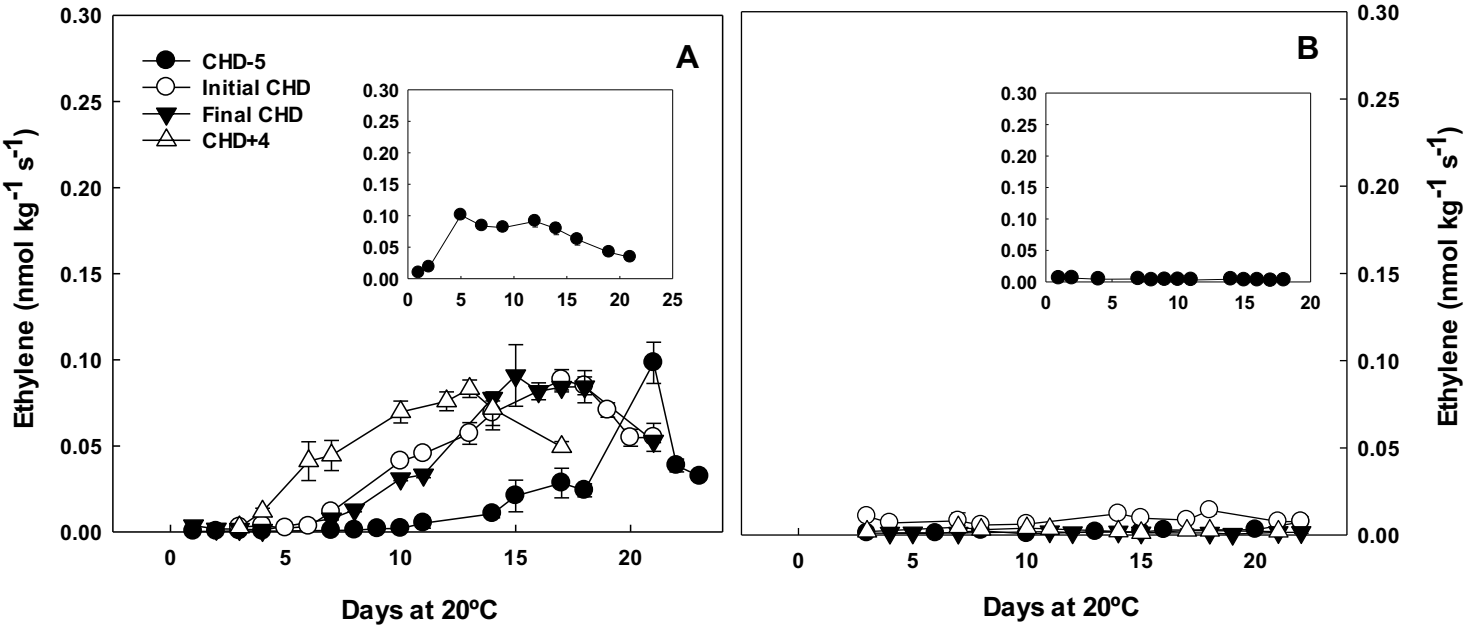
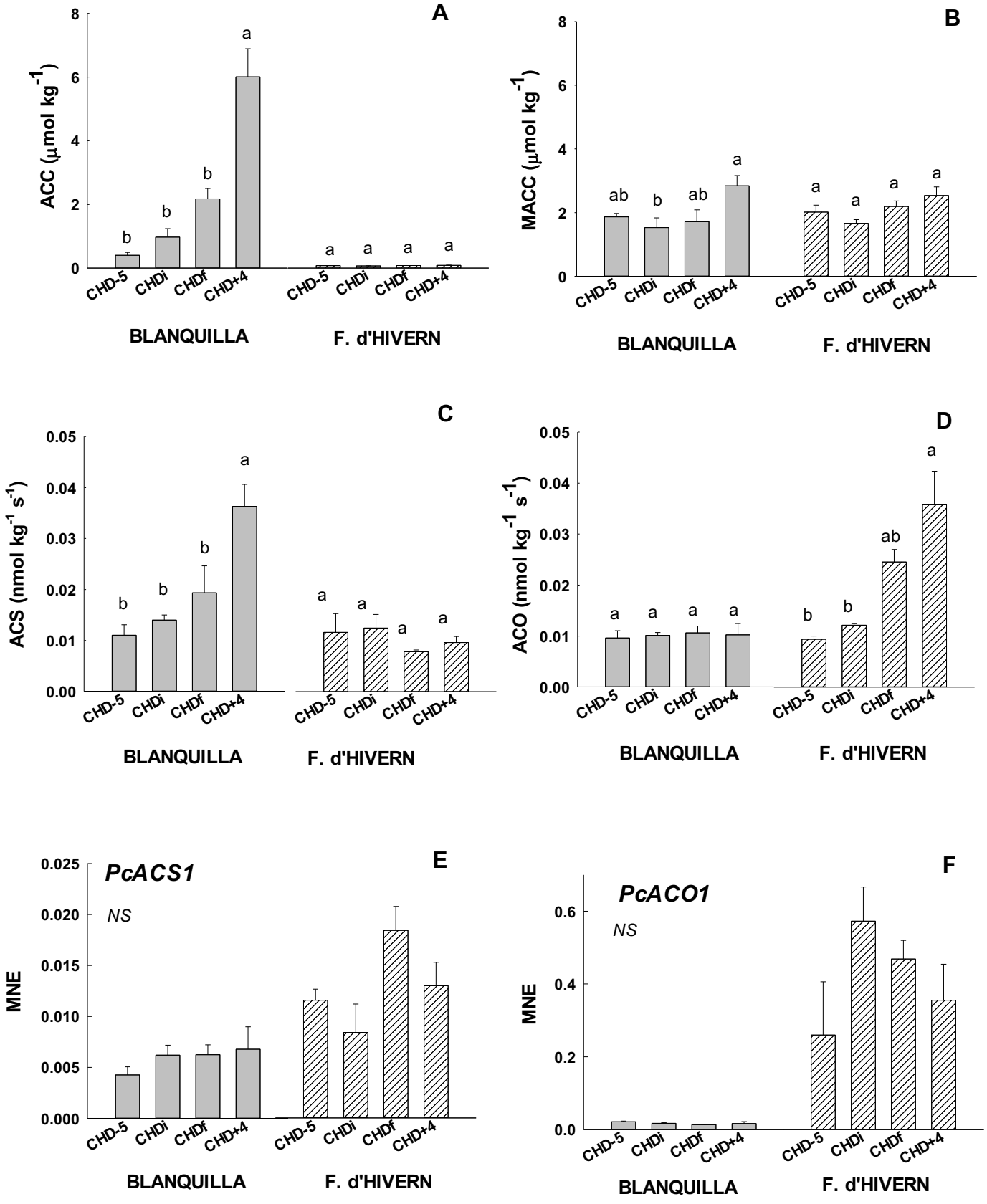


753

754 **Figure 2:**

755

756


A
ACC (µmol kg-1)
8
6
4
2
0
b
b
b
a
a
a
a
a
CHD-5
CHDi
CHDf
CHD+4
BLANQUILLA
F. d'HIVERN
B
MACC (µmol kg-1)
ab
b
ab
a
a
a
a
a
C
ACS (nmol kg-1 s-1)
0.05
0.04
0.03
0.02
0.01
0.00
D
ACO (nmol kg-1 s-1)
E
PcACS1
NS
MNE
0.025
0.020
0.015
0.010
0.005
0.000
F
PcACO1
NS
0.6
0.4
0.2
0.0

757 **Figure 3:**

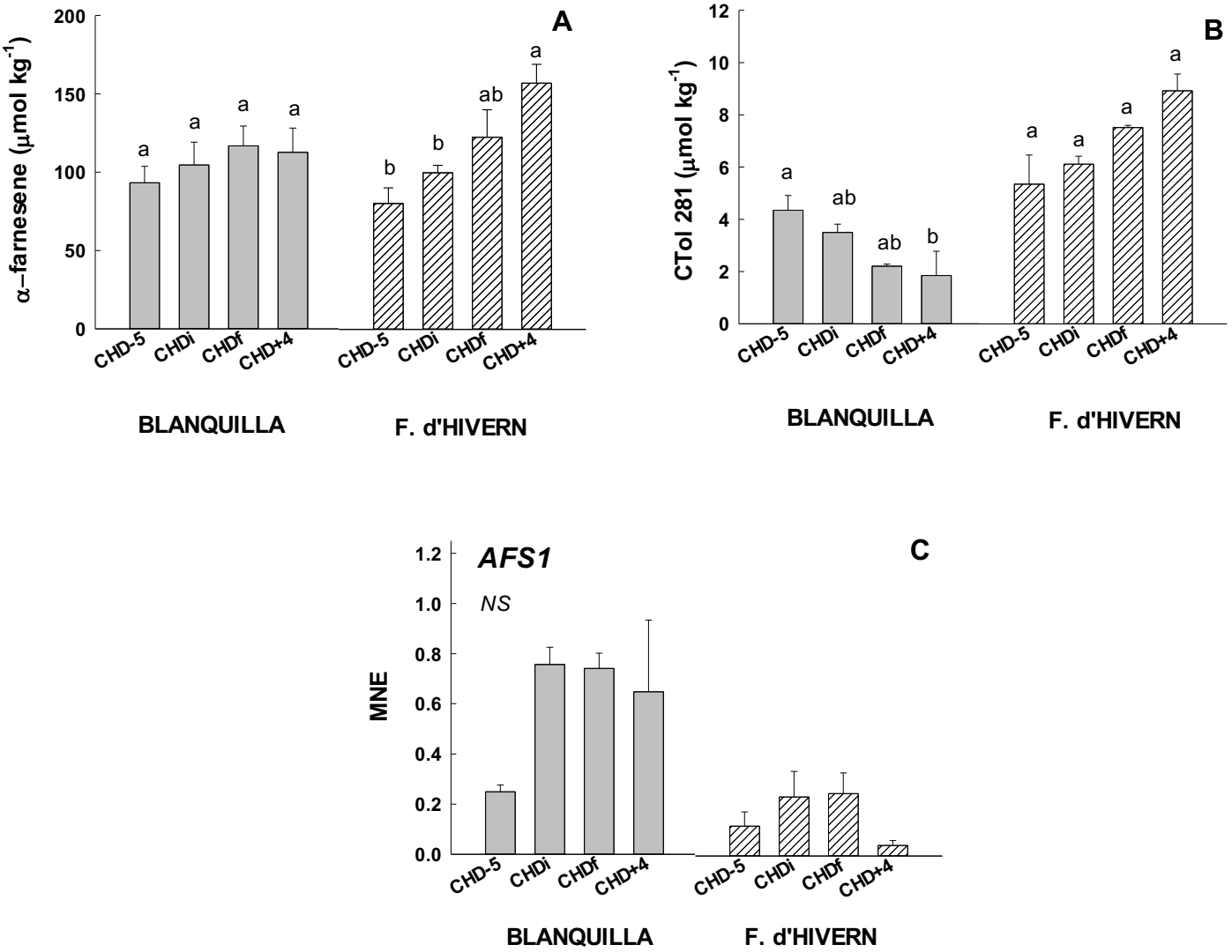
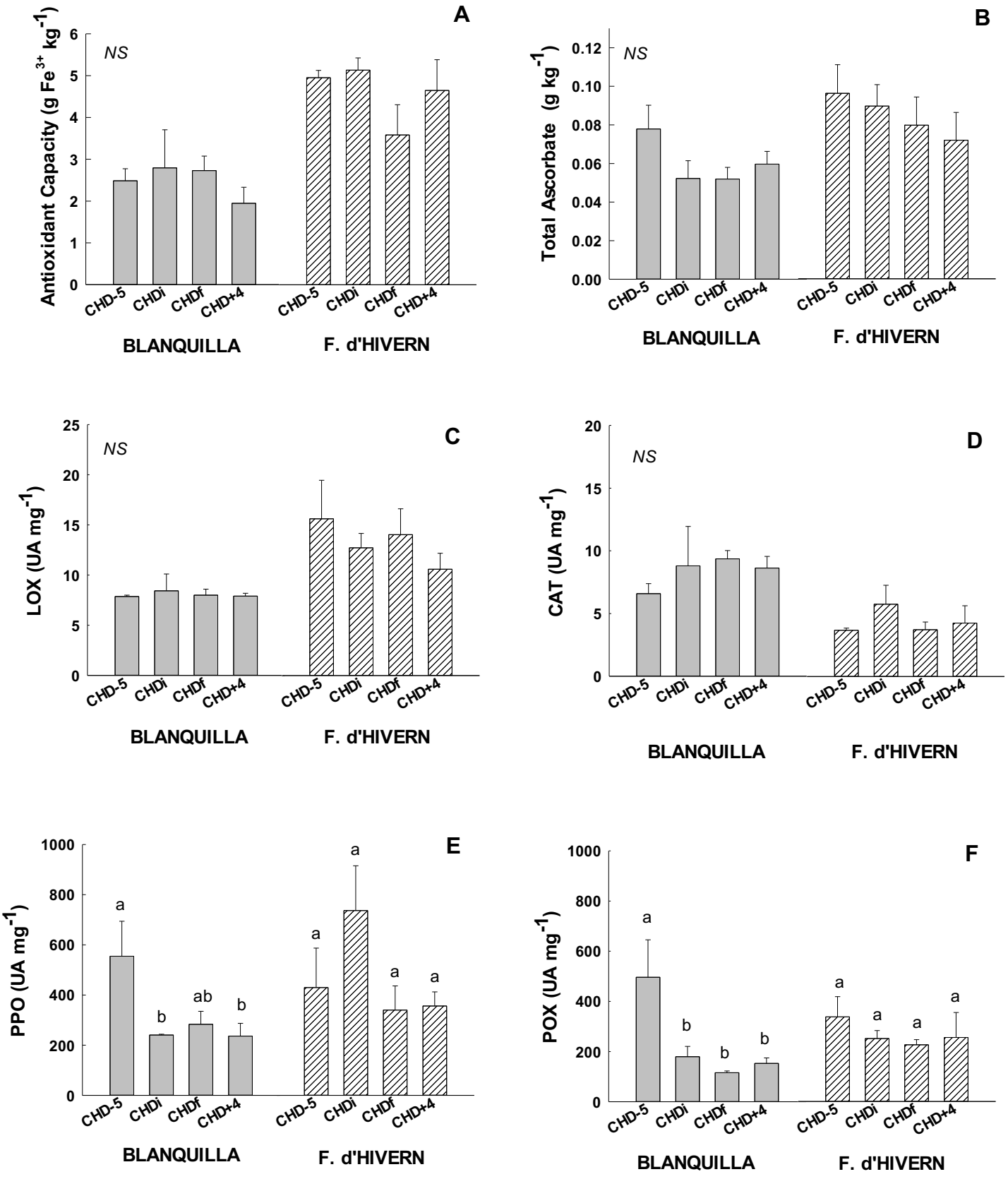
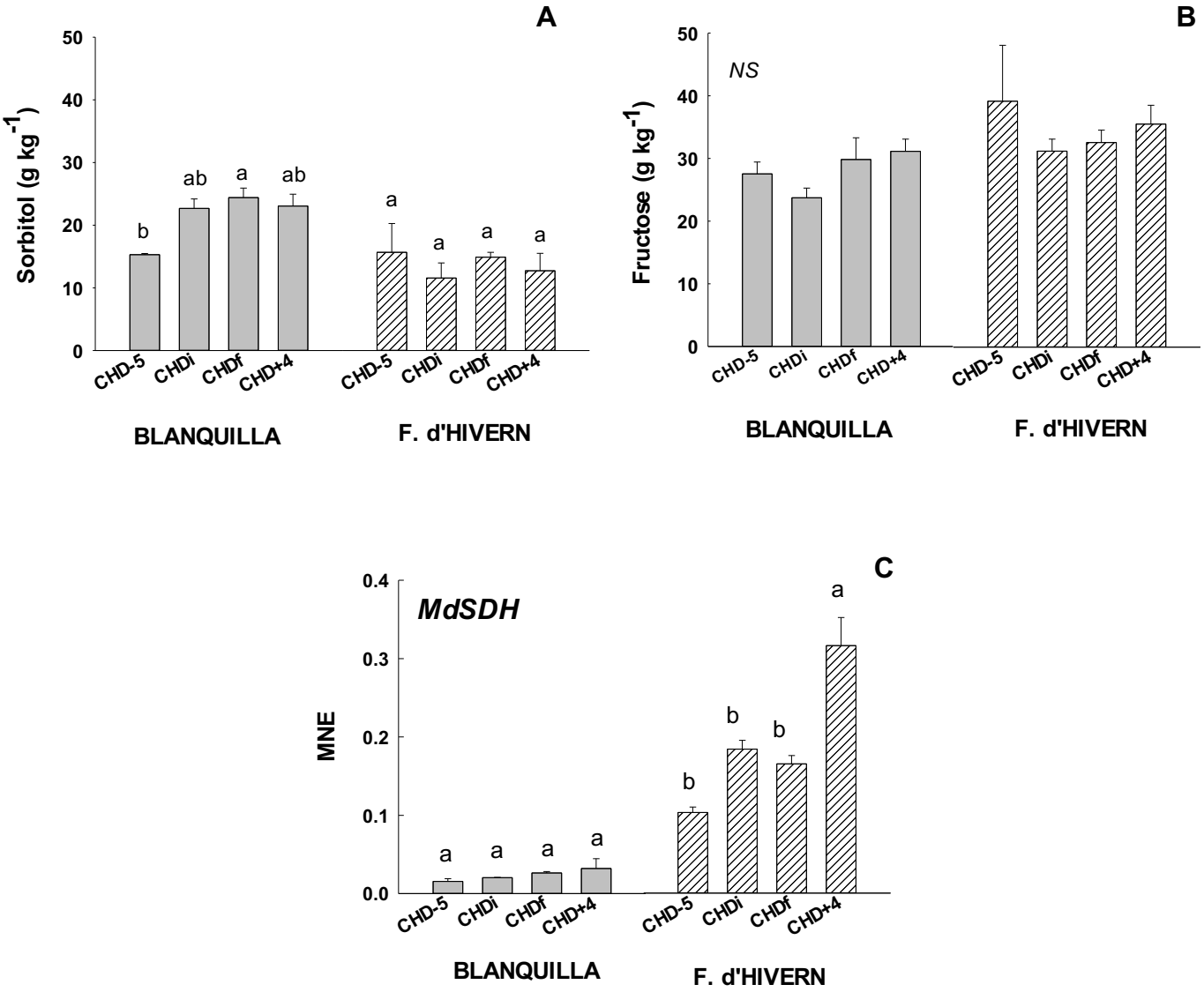

A
α–farnesene (µmol kg-1)
200
150
100
50
0
a
a
a
a
b
b
ab
a
CHD-5
CHDi
CHDf
CHD+4
BLANQUILLA
F. d'HIVERN
B
CTol 281 (µmol kg-1)
12
10
8
6
4
2
0
a
ab
ab
b
a
a
a
a
C
AFS1
NS
MNE
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Figure 4:



761

762 **Figure 5:**



763

764 **Figure 6:**

Supplementary Table 1: Primers used for quantitative PCR

Gene	Annotation	Oligonucleotide sequences	Target gene	Metabolic pathway/Biological function
PcACS	Aminocyclopropane-1-carboxylic acid synthase	F5'-ATGCTGGCTTGTTCTGTTGG-3' R5'-AGGTTCCGTGCAATGACAAG-3'	PCP011500	Ethylene metabolism
PcACO	Aminocyclopropane-1-carboxylic acid oxidase	F5'-AAGGTCAGCAACTACCCTCC-3' R5'-TGTCATCCTGGAAGAGCAGG-3'	PCP011683	
PcAFS1	α-farnesene synthase	F5'- GAAAACTAGGCCTCGCGAAC-3' R5'- TTCGATAGCTGCAATGCCGT-3'	PCP028486	α-farnesene metabolism
PcLOX	Lipoxygenase	F5'- CTTCAACGGAGAATCAGGCG -3' R5'- TCGGTTATGTCATCGAGGGG-3'	PCP002320	Antioxidant capacity
MdPPO	Polyphenol oxidase	F5'-CCTACTCACAAAGCCCAAGC-3' R5'-CCTCCAAGACCAAGAAGCAC-3'	PCP039035	
MdSDH	Sorbitol dehydrogenase	F5'-ATGGTCACAGCCATTGGTCA-3' R5'-ACCTTGTCCTTGCCCAGAAG-3'	PCP002232	Sorbitol Metabolism
Md8283	Housekeeping	F5'-CTCGTCGTCTTGTTCCCTGA-3' R5'-GCCTAAGGACAGGTGGTCTATG-3'	PCP030439	-