UNRAVELLING THE COLD-INDUCED REGULATION OF ETHYLENE AND α-FARNESENE AND ITS INVOLVEMENT WITH THE DEVELOPMENT OF SCALD-LIKE DISORDERS IN DIFFERENT PEAR CULTIVARS

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Abstract

To better understand the cold-induced regulation of scald-like disorders in pears and the specific roles played by ethylene and α-farnesene, three pear cultivars with different patterns of ethylene production and chilling requirement were used in this study. Fruit were treated with 1-MCP (ethylene inhibitor) and Lovastatin (α-farnesene inhibitor) and stored at -0.5 °C and 90 % RH during 6 months. Changes in targeted metabolites, enzymes and genes were monitored periodically up to 120 d of storage and superficial scald incidence was assessed after this time and after 180 d of cold storage. 1-MCP treatment induced in the three cultivars a down-regulation of *PcACS1*, *PcACO1*, *PcERF1* and *PcAFS1* gene expression, but also a significant up-regulation of *PcETR*1 and *PcEIN2* that led in all cases to the inhibition of the disorder incidence. In contrast, Lovastatin treatment caused diverse molecular or biochemical responses depending on the cultivar. In 'Blanquilla' pears, this treatment completely inhibited superficial scald reinforcing the idea that ethylene-α-farnesene interaction plays a decisive role in this specific cultivar. In contrast to 1-MCP, Lovastatin treatment did not control the disorder incidence in 'Flor d'Hivern' pears. Inversely, 1-MCP inhibited the development of the disorder, showing then that the inhibition of ethylene biosynthetic and signalling pathway may control superficial scald even in cultivars producing very low or undetectable ethylene levels. Finally, the inefficacy of both treatments to prevent the disorder development in 'Conference' pears, suggests the existence of a disorder different from that observed for the other cultivars whose biochemical basis remain unknown. Collectively our results show that the regulatory processes triggered by cold stress in pears are complex and cultivar dependent.

Keywords: superficial scald,1-MCP, Lovastatin, cold induction, storage

1. INTRODUCTION

Low-temperature storage is a common postharvest practice aiming to prolong the storage life and then the availability of pears in the market (Saquet, 2019). As for many other fruit, low-temperature storage can however lead to the appearance of chilling injury (CI) disorders (Benichou et al., 2018; Ma and Chen, 2003). Superficial scald is by far one of the main CI of pears accounting for important postharvest losses worldwide (Lurie and Watkins, 2012; Wang and Dilley, 1999). This physiological disorder manifests as brown-dark patches on the fruit skin, yet the susceptibility and severity of the symptoms can largely vary among cultivars (Fig.1; Larrigaudière et al., 2016; Lindo-García et al., 2020) and within each cultivar depending on the fruit maturity at harvest (Calvo et al., 2015; Lindo-García et al., 2020). Previous studies have characterised superficial scald or scald-like disorders in pears both at the morphological and biochemical level (Lindo-García et al., 2020; Zoffoli et al., 1998). Generally, symptoms are visible in most cultivars (i.e. 'Blanquilla', 'Abate Fetel', 'Packham's') upon rewarming and after relatively long periods of cold storage (Calvo et al., 2015; Larrigaudière et al., 2019, 2016), yet for some cultivars (i.e 'Flor d'Hivern') symptoms can appear even during cold storage (Lindo-García et al., 2020).

The most accepted theory to explain scald development relates the disorder to the formation and oxidation of α-farnesene into conjugated trienols (Farneti et al., 2015; Mir et al., 1999; Rowan et al., 2001). Under this scenario, ethylene plays a key role by controlling the production of α-farnesene via an up-regulation of α-farnesene synthase gene (*AFS*) (Gapper et al., 2006; Lurie et al., 2005; Pechous et al., 2005). This said, several studies suggest that α-farnesene may also accumulate independently of ethylene, directly in response to cold stress, but to a different extent depending on the cultivar (Calvo et al., 2015; Larrigaudière et al., 2019; Lindo-García et al., 2020). Such response in cold may be explained by the fact that terpenes such as α-farnesene are induced in plants under abiotic stress conditions (Holopainen and Gershenzon, 2010; Torregrosa et al., unpublished) in an attempt to stabilize membranes and prevent the cold-induced cell disruption.

The involvement of either ethylene or α-farnesene in scald development is further sustained by the fact that treatments with the ethylene inhibitor 1-methylcyclopropene (1-MCP) or with Lovastatin (a specific inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA reductase) clearly inhibit scald development in apples and pears (Busatto et al., 2014; Giné-Bordonaba et al., 2020; Ju and Curry, 2000a; Larrigaudière et al., 2019). Likewise, the oxidative nature of the disorder is also evident since treatments with synthetic antioxidants (i.e. diphenylamine) clearly control the appearance of the disorder without altering ethylene biosynthesis (Karagiannis et al., 2018).

Albeit the ethylene / α-farnesene theory is still valid, recent studies pointed out that others multiple complex metabolic changes are ultimately responsible for the development of the disorder. For instance, the oxidation of specific phenolic compounds (i.e. chlorogenic acid) via polyphenol oxidase (PPO) or the metabolism of cryoprotectants (i.e. sorbitol), volatiles or antioxidants seem to be also crucial pathways associated with the development of the disorder (Busatto et al., 2018, 2014; Giné-Bordonaba et al., 2020; Wang et al., 2018)

Little information is currently available about how cold storage may trigger these metabolic changes finally leading to the development of superficial scald. Accordingly, this study aimed to investigate the cold-induced regulation of both ethylene and α-farnesene biosynthesis, both at the biochemical and molecular level. Specific inhibitors (1-MCP and Lovastatin) were used to define the way by which these two compounds participate individually or collectively to the development of scald-like disorders in the three studied cultivars.

2. MATERIAL AND METHODS

2.1. Plant material and experimental design

'Blanquilla', 'Conference' and 'Flor d'Hivern' pears (*Pyrus communis* L.) were chosen based on their differential susceptibility to skin browning disorders (Lindo-García et al., 2020) but also given their different ethylene production pattern and chilling requirements. Fruits were harvested at a firmness values of 57.1 N, 62.5 N and 49.3 N, respectively, on a commercial orchard near Lleida (Catalonia, Spain). Harvest date corresponded to the commercial harvest date (CHD; about 125, 135 and 173 d after full bloom for 'Blanquilla', 'Conference' and 'Flor d'Hivern', respectively), based on standard local recommendations (Lindo-García et al., 2020; Torregrosa et al., 2019).

2.2. Treatments

Immediately after harvest, fruit from each variety were divided into three different batches of 240 fruit each. One batch (240 fruit) was placed in a sealed plastic container and treated with 300 nL L^{-1} 1-MCP during a minimum of 18 h at 0 °C and using the product Smartfresh^{TM} (Agrofresh Inc.). Lovastatin treatment was done on 240 fruit by dipping them into a 1.25 mmol L^{-1} solution (Giné-Bordonaba et al., 2020) during 2 minutes. The lovastatin formulation was prepared by dissolving 30.3 g of Lovastatin (98 %), 240 g of sunflower oil, 240 g of glycerol and 720 g of Tween-80 in 2.4 L of hot water and then adding water until 60 L. Finally, a batch of untreated fruit served as a control. After treatments, fruit were stored at -0.5 °C and 90 % RH until further physiological or biochemical analyses.

2.3. Determination of α-farnesene (AF) and conjugated trienol 281 (CT_{281})

AF and CT_{281} were analysed as described by Anet (1972) with some modifications (Larrigaudière et al., 2019). At harvest and after 7, 15, 30, 60 and 120 d of cold storage, 9 fruit of each treatment were removed and a strip of 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), 281 and 290 nm (conjugated trienol - CT_{281}) were 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} peel.

2.4. Ethylene production

Ethylene production (nmol kg^{-1} s^{-1}) was measured after 60 d of cold storage as described by Giné-Bordonaba et al. (2014). Briefly, 2 fruit per replicate and 3 replicates per treatment and removal time were placed in 1.5 L flasks continuously ventilated with humidified air at a flow rate of 1.5 L h^{-1}. Gas samples (1 mL) were taken of effluent air using a 1 mL syringe and injected into a gas chromatograph (CG; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an Alumina column F1 80/100 (2 m x 1/8 x 2.1, Tecknokroma, Barcelona, Spain). The oven temperature was 140 °C while the injector and detector were kept at 180 and 280 °C, respectively.

2.5. Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) levels and ACC synthase and ACC oxidase activity

Flesh tissue from 3 individual fruit per replicate and 3 replicates per treatment was frozen in liquid nitrogen at harvest and after 7, 15, 30, 60 and 120 d of cold storage, and kept at -80 °C until further biochemical assays.

1-aminocyclopropane-1-carboxylic acid (ACC) was extracted and analysed as described by Bulens et al. (2011) with some modifications as specified in Lindo-García et al. (2019).

1-Aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) was extracted as described by Lindo-García et al. (2019). The enzyme activity was analysed as described by Giné-Bordonaba et al. (2017) and results expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

The extraction and analysis of the activity of 1-aminocyclopropane-1-carboxylic acid synthase enzyme (ACS) was determined as also described by Lindo-García et al. (2019).

2.6. Determination of superficial scald incidence

Scald incidence for each treatment was estimated visually after 6 months of cold storage plus 7 d of shelf life (20 °C) as described by Larrigaudière et al. (2019). Superficial scald incidence was expressed as the percentage of damaged fruit, but also establishing the severity of the damage according to a 0 to 4 scale in which:

S0 = No damaged fruit; S1 = <10% of the skin surface damaged; S2 = <25% of the skin surface; S3 = <50% of the skin surface and S4 = >50% of the skin surface.

The final index was calculated with the following formula:

$$\text{Severity} = \frac{\sum \text{S0x0} + \sum \text{S1x1} + \sum \text{S2x2} + \sum \text{S3x3} + \sum \text{S4x4}}{\text{Total number of fruit}}$$

2.7. RNA extraction and Gene expression analysis

Peel tissue from 3 individual fruit per replicate and 3 replicates per treatment was frozen in liquid nitrogen at harvest and after 15, 30, and 60 d of cold storage, and kept at -80 °C until further molecular assays.

Total RNA was extracted 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) and both absence of contaminant DNA and RNA integrity were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). First-strand cDNA synthesis was performed with an oligo-dT primer on 1 μg of RNA using the

SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) on a Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Gene 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 and with the following conditions: 95 ºC (10 s) followed by 40 cycles of 95 ºC (15 s) and 60 ºC (1 min). Most of the oligonucleotides used for RT-qPCR analysis were adopted from Busatto et al. (2019), *PcHMGR* and *PcETR1* were adopted from Giné-Bordonaba et al. (2020) and Chiriboga et al. (2013), respectively, and *PcEIN2* was designed using the Primer-BLAST tool (Ye et al., 2012). *Md8283* was used as independent reference gene based on previous studies (Botton et al., 2011; Longhi et al., 2012; Busatto et al., 2019, 2018) but also given the constant expression along cultivars and treatments shown in preliminary trials. The primers used in this study are listed in Supplementary Table 1. Primer efficiency was confirmed to be >90 % using 3-fold cDNA dilutions in triplicate and primer specificity was checked by analyzing the melting curves at temperatures ranging from 60 to 95 ºC. A non-template control (NTC) was included using water instead of DNA. Relative gene expression was expressed as Mean Normalized Expression (MNE) and calculated using the method described by Muller et al. (2002).

2.8. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute. Comparisons between time samplings and/or treatments for each variety were done by Tukey's test at a significant level of $p \leq 0.05$ (*) and $p \leq 0.01$ (**). Least significant difference values (LSD; $p = 0.05$) for the interaction treatment*samplings of cold storage were calculated for mean separation using critical values of t for two-tailed tests.

3. RESULTS

Important differences in scald-like or superficial scald disorder incidence were found for the different cultivars investigated herein (Fig. 1). In 'Blanquilla' pears, superficial scald incidence was relatively low after 6 months of cold storage (28.3 %; data not shown) but rapidly increased thereafter reaching 95 % after 7 d of shelf-life (Fig. 1D). In contrast, scald-like incidence in 'Flor d'Hivern' was very high already upon removal from cold storage (76.7 %; data not shown) and a slight increase during shelf-life (85.0 % at 7 d, Fig.1E). Conference pears showed little disorder incidence after 6 months of cold storage (13.3 %, data not shown), regardless of initial harvest maturity (data not shown), as well as minor changes in the disorder incidence when the fruit were left to ripen at 20 ºC (21.7 % at 7 d, Fig. 1F).

In general, the results showing the severity of the disorder paralleled those of the disorder incidence (higher severity associated to higher number of damaged fruit), except for 1-MCP treated 'Flor d'hivern' pears, that presented very low incidence yet relatively high severity index after 6 months of cold storage.

3.1. Biochemical and molecular events involved in the development of scald-like disorder in 'Blanquilla' pear

3.1.1. <u>Treatment effect on scald-like disorder incidence</u>

Clear differences between treatments were observed in 'Blanquilla' pears after 4 (data not shown) and 6 months of cold storage (Fig. 1D). 1-MCP treatment completely inhibited the disorder incidence in this cultivar (only 1.67 % of damaged fruit after 6 months of cold storage plus 7 d of shelf life) while control fruit showed an incidence of 95 %. Lovastatin treatment also effectively reduced superficial scald incidence (5% of affected fruit) after cold storage and shelf-life.

3.1.2. Cold-induced regulation of ethylene biosynthesis in untreated and treated 'Blanquilla' pears

The patterns of ethylene production in untreated fruit and Lovastatin-treated fruit after 2 months of cold storage were similar, reaching the climacteric peak at 4 d of shelf-life (Suppl. Fig. 1). In contrast, 1-MCP clearly inhibited the fruit ethylene. The inhibition of ethylene production observed in 1-MCP-treated pears after removal from cold storage (Suppl. Fig. 1) was associated to a down-regulations of *PcACS1* and *PcACO1* genes occurring throughout cold storage (Fig. 2 and Suppl. Fig. 3A and 3D), and a decrease of the respective enzyme activities, especially ACO (27- and 9.5-fold lower ACO activity in 1-MCP-treated fruit compared to control fruit at day 60 and 120, respectively; Fig. 2 and Suppl. Fig. 2D). On the other hand, Lovastatin treatment did not affect the gene expression of either *PcACS1* or *PcACO1* (Fig. 2 and Suppl. Fig. 3A and 3D) but rather significantly enhanced ACO enzyme activity at day 120, showing values more than 2-fold higher in Lovastatin-treated than in untreated fruit (Fig. 2 and Suppl. Fig. 2D). No clear pattern was observed for ACC content in any treatment until day 30. From this day, 1-MCP-treated fruit showed a decrease in ACC content, reaching values 3.5- and 4.1-fold lower if compared to untreated and Lovastatin-treated fruit, respectively (Fig. 2).

Ethylene signalling and perception was also differentially affected by the treatments. 1-MCP-treated fruit exhibited a slight up-regulation of *PcETR1* after 15 d of cold storage (Fig. 2 and Suppl. Fig. 3J) and of *PcEIN2* later at 30 and 60 d (Fig. 2 and Suppl. Fig. 3G). A down-regulation of the ethylene response factor, *PcERF1*, was also observed in 1-MCP-treated fruit if compared to untreated fruit at day 60 (Fig. 3 and Suppl. Fig. 2M). Inversely to 1-MCP, Lovastatin treatment led to a slight down-regulation of *PcEIN2* and *PcETR1* during all the storage period (Fig. 2 and Suppl. Fig. 3G and 3J) and a slight and transitory up-regulation of *PcERF1* at 15 and 30 d compared to untreated fruit (Fig. 2 and Suppl. Fig. 3M).

3.1.3. Regulatory processes related to α-farnesene biosynthesis in 'Blanquilla' pears

A clear relationship between α-farnesene, CT_{281} levels and superficial scald incidence was observed in 'Blanquilla' pear. 1-MCP inhibited the accumulation of α-farnesene, yet showing a slight increase from 64 to 114 μmol kg^{-1} during cold storage and values 7.4-fold lower than untreated fruit after 120 d at -0.5 °C (Fig. 2). A similar tendency was observed for CT_{281} values, where 1-MCP-treated fruit showed basal levels compared to control fruit (Fig. 2). The lower values of these metabolites observed in 1-MCP-treated fruit was related to a down-regulation of both *PcHMGR* and *PcAFS1* gene expression (Fig. 2 and Suppl. Fig. 4A and 4D).

Although Lovastatin treatment also caused a clear inhibition of α-farnesene and CT_{281} accumulation throughout cold storage, this treatment did not affect *PcHMGR* and even caused an up-regulation of *PcAFS1* gene expression if compared to untreated fruit (Fig. 2 and Suppl. Fig. 4A and 4D).

Overall, our data show a classical association between ethylene and *PcAFS1* suggesting that ethylene is a key factor involved in the regulation of superficial scald in 'Blanquilla' pears.

3.2. Biochemical and molecular events involved in the development of scald-like disorders in 'Conference' pears

3.2.1. Treatment effect on scald-like disorder incidence

Conversely to the results observed in 'Blanquilla', 'Conference' pear did not showed clear differences in the disorder incidence between treatments. Control fruit exhibited 21.7 % of damaged fruit while 1-MCP- and Lovastatin-treated fruit even showed a higher, yet no significant, disorder incidence (33.3 % of damaged fruit for both treatments; Fig. 1F).

Symptoms of the disorder were also slightly different to those observed in ‘Blanquilla’ fruit (Fig.1). The symptoms in ‘Blanquilla’ were more diffuse and brown in colour while the symptoms in ‘Conference’ were darker, less diffuse and seemed not to affect the lenticels. Taken together, these results suggest that superficial scald in ‘Blanquilla’ and scald-like disorder in ‘Conference’ are likely two different disorders yet showing similar symptoms.

3.2.2. Cold-induced regulation of ethylene biosynthesis in untreated and treated ‘Conference’ pears

Control and Lovastatin-treated fruit showed similar ethylene production patterns after 2 months of cold storage, reaching the climacteric peak after 7 d at 20 °C (*ca.* 0.43 nmol kg^{-1} s^{-1}), while 1-MCP treatment completely inhibited the ethylene production upon removing the fruit from cold storage (Suppl. Fig. 1). Similarly to that observed in ‘Blanquilla’ pears, the ethylene inhibition by 1-MCP was related to lower ACS and especially ACO enzyme activities during storage (Fig.3 and Suppl. Fig. 2B and 2E). This inhibition was also related to a significant down-regulation of both *PcACS1* and *PcACO1* gene expression during cold storage (Fig. 3 and Suppl. Fig. 3B and 3E) and lower ACC content (5.5-fold lower at day 120 if compared to control fruit; Fig. 3). On the other hand, Lovastatin treatment did not affect the transcript levels of *PcACS1* (Fig. 3 and Suppl. Fig. 3B and 3E) nor the ACS or ACO enzyme activities compared to untreated fruit (Fig. 3 and Suppl. Fig. 2B and 2E).

At the signalling and perception level, the results observed in ‘Conference’ pear were similar to those previously described for ‘Blanquilla’. 1-MCP treatment caused an up-regulation of *PcEIN2* gene expression at the beginning of the cold storage (Fig. 3 and Suppl. Fig. 3H) and also a slight up-regulation of *PcETR1* (Fig.3 and Suppl. Fig. 3K). On the contrary, a clear down-regulation of *PcERF1* was observed in 1-MCP-treated

'Conference' pears compared to control fruit (Fig.3 and Suppl. Fig. 3N). A complete opposite behaviour was found in Lovastatin-treated fruit. In detail, Lovastatin-treated fruit exhibited a down-regulation of both *PcEIN2* and *PcETR1* together with an up-regulation of *PcERF1* gene expression levels in comparison to untreated fruit (Fig. 3 and Suppl. Fig. 3H, 3K and 3N).

3.2.3. Regulatory processes related to α-farnesene biosynthesis in 'Conference' pear

Conversely to 'Blanquilla', no clear relationship between α-farnesene, CT_{281} levels and scald-like disorder incidence was observed in 'Conference' pear. Higher values of α-farnesene and CT_{281} were observed in control fruit at day 120, suggesting that both 1-MCP and Lovastatin inhibited the accumulation of these metabolites in 'Conference' pears, yet to a lesser extent than in 'Blanquilla' (Fig. 3).

1-MCP clearly down-regulated the expression of *PcAFS1* and impaired the up-regulation of *PcHMGR* at day 60 (Fig. 3 and Suppl. Fig. 4B and 4E). In turn, Lovastatin treatment induced a slight down-regulation of *PcHMGR* and an up-regulation of *PcAFS1* especially at the end of cold storage in comparison to untreated fruit Fig. 3 and Suppl. Fig. 4B and 4E).

3.3. Biochemical and molecular events involved in the development of scald-like disorders in 'Flor d'Hivern'

3.3.1. Treatment effect on scald-like disorder incidence

Clear differences in superficial scald incidence were observed in 'Flor d'Hivern' pears between treatments. After 6 months of cold storage plus 7 d of shelf life, control fruit showed an incidence of 85 % similar to that observed in Lovastatin-treated fruit (90 %). 1-MCP, in contrast, clearly controlled scald incidence, showing only 2% of the disorder incidence after 6 months of cold storage plus 7 d of shelf-life (Fig. 1E).

3.3.2. Cold-induced regulation of ethylene biosynthesis in untreated and treated 'Flor d'Hivern' pears

Despite exhibiting a very high incidence of scald-like disorder, this cultivar did not produce detectable amounts of ethylene after 2 months of cold storage (Suppl. Fig. 1). The lack of ethylene production in untreated and Lovastatin-treated fruit were not explained by a repression of either ACS or ACO enzyme activities nor by the expression of their respective genes during storage, since similar levels to that observed in 'Blanquilla' and 'Conference' pears were found in this cultivar. ACC levels increased both in Lovastatin-treated and untreated fruit but the levels reached in control fruit at 120 d were 2.92- and 1.83-fold lower than those observed in 'Blanquilla' and 'Conference', respectively (Fig. 4).

Despite not affecting the fruit ethylene production, 1-MCP treatment induced a clear inhibition of ACO enzyme activity and also of *PcACS1* and *PcACO1* gene expression levels. This said, an increase in ACS activity from day 60 together with limited ACO activity in 1-MCP-treated fruit resulted in enhanced ACC levels from day 60 to 120.

Although not producing detectable amounts of ethylene, the genes involved in the ethylene signalling and perception pathway showed a similar pattern to those observed in 'Blanquilla' and 'Conference' pears. Concretely, 1-MCP-treated fruit showed a time-consistent up-regulation of *PcEIN2* and *PcETR1* (Fig. 4 and Suppl. Fig. 3I and 3L) and a slight down-regulation of *PcERF1* (Suppl. Fig. 3O). On the contrary, Lovastatin treatment caused a down-regulation of *PcEIN2* and *PcETR1* ((Fig. 4 and Suppl. Fig. 3I and 3L) but did not affect the expression level of *PcERF1* (Fig. 5 and Suppl. Fig. 3O).

3.3.3. Regulatory processes related to α-farnesene biosynthesis in 'Flor d'Hivern' pear

Control and Lovastatin-treated fruit showed a similar pattern of α-farnesene accumulation during cold storage (Fig. 4). A similar tendency was also observed for CT_{281} even though control fruit reached values 1.83-fold higher than Lovastatin treated-fruit after 120 d of cold storage. As observed in the other cultivars, 1-MCP treatment strongly inhibited the accumulation of both α-farnesene and CT_{281}. These results were in agreement with the disorder incidence since a similar scald incidence was observed between control and Lovastatin treatment (85-90 %), while 1-MCP strongly inhibited the disorder incidence (2 %).

From a molecular perspective, both 1-MCP and Lovastatin regulated *PcHMGR* in a similar manner, down-regulating its expression at 30 and 60 d of cold storage if compared to untreated fruit. 1-MCP clearly down-regulated *PcAFS1* gene expression throughout cold storage (Fig. 4 and Suppl. Fig. 4F) in comparison to both untreated or Lovastatin-treated fruit.

4. DISCUSSION

Even though superficial scald is one of the most studied physiological disorders in apples and pears (Calvo et al., 2002; Emongor et al., 1994; Lurie and Watkins, 2012; Xie et al., 2014), its molecular or biochemical basis has been mainly studied after cold storage when the symptoms are visible (Busatto et al., 2018; Gamrasni et al., 2010; Giné-Bordonaba et al., 2020; Villalobos-Acuña et al., 2011; Zhou et al., 2020). Albeit the disorder appears after relatively long-term cold storage, depending on the cultivar and the fruit maturity at harvest (Calvo et al., 2015; Lindo-García et al., 2020), its induction is thought to occur mainly during the first weeks at low temperature (Lurie and Watkins, 2012). Accordingly, our study was directed to better understand these primary events and especially the specific role that ethylene and α-farnesene may play in the induction of superficial scald in different pear cultivars.

4.1. Cold-induced regulation of ethylene and its involvement in superficial scald development

The involvement of ethylene in superficial scald development has been deeply studied over the past decades since this hormone regulates the expression of the *α*-farnesene synthase 1 (*AFS1*) gene, involved in the last step of the *α*-farnesene biosynthetic pathway (Lurie et al., 2005; Pechous et al., 2005). Indeed, treatments with the ethylene inhibitor 1-MCP reduce the accumulation of *α*-farnesene (Isidoro and Almeida, 2006; Larrigaudière et al., 2019; Zhi and Dong, 2018) and is among the most effective treatments to prevent the appearance of the disorder both in apples and pears (Busatto et al., 2018; Calvo et al., 2018; Du et al., 2017). To further understand the role of ethylene in superficial scald development, three different cultivars with known differences in their ethylene production rates were selected in this study (Lindo-García et al., 2020).

'Blanquilla' pear is a typical summer cultivar able to produce ethylene already at harvest (Lindo-García et al., 2019) and highly susceptible to superficial scald (Giné-Bordonaba et al., 2020; Larrigaudière et al., 2019). 'Flor d'Hivern' pears, belong to the winter cultivar type and also develop high incidence of scald-like disorders despite producing very low ethylene levels even after prolonged cold storage (Lindo-García et al., 2020). 'Conference' pears finally, represent an intermediate cultivar requiring short-term cold storage to produce ethylene and much more resistant to the development of scald-like disorders.

Despite their differences in ethylene production, 1-MCP treatment in general led to similar down-regulation of ethylene biosynthetic genes and enzymes in all the cultivars throughout cold storage (Figs. 2, 3 and 4 and Suppl. Fig. 2 and 3), hence consistent with the literature (Busatto et al., 2014; Chiriboga et al., 2013b; Gamrasni et al., 2010; Xie et al., 2016; Zhao et al., 2020). Likewise, a similar 1-MCP effect on the ethylene perception and signalling pathways was observed in all cultivars with treated fruit showing a slight up-regulation of *PcEIN2* and *PcETR1* genes (Figs. 2, 3 and 4 and Suppl. Fig. 3; Chiriboga et al., 2013b; Zhou et al., 2017). By contrast, 1-MCP-treated fruit from the three cultivars studied showed a clear down-regulation of the *PcERF1* gene expression (Figs. 2, 3 and 4 and Suppl. Fig. 3). Such changes in ethylene signalling pathway are likely related to the fact that 1-MCP completely inhibits ethylene production. In this way, the up-regulation of *PcETR1* is likely the result of ethylene deprivation and the up-regulation of *PcEIN2*, that positively interacts with *PcETR1* (Bisson et al., 2009; Bisson and Groth, 2010), a consequence of the regulation of *PcETR1*. The down regulation of *PcERF1* is also likely the consequence of the general inhibition of the ethylene signalling pathway. However, and as *PcERF1* down-regulation was not observed in 1-MCP treated 'Blanquilla' pears after 4 months of cold storage (Giné-Bordonaba et al., 2020), the effect of 1-MCP on this specific gene is likely transitory and only observed during the first months of cold storage. Overall, our results indicated that the way by which superficial scald is induced is specific for each pear cultivar. In 'Flor d'Hivern' and 'Blanquilla' pears in which 1-MCP completely control the disorder development, superficial scald appeared to be linked to ethylene dependent processes taking place during cold storage. These processes likely play the main determining role in scald development. However, and as previously reported in apple (Karagiannis et al., 2018) and pear fruit (Giné-Bordonaba et al., 2020; Larrigaudière et al., 2019), we cannot discard the involvement of other ethylene independent processes likely associated to fruit acclimation. An ethylene-independent regulation of the disorder was instead observed in 'Conference' pears. Indeed, in this cultivar, 1-MCP effectively inhibited the ethylene production but also slightly enhanced the scald-like disorder incidence (Fig. 1). Similarly, Rizzolo et al. (2015) reported no incidence of superficial scald in 'Conference' pear after 4 months of cold storage and identified two different types of peel disorders (blackening and black speck), which were not inhibited by 1-MCP. Overall, our results are in accordance with the above-mentioned study and suggest that the disorder observed in 'Conference' pear is a scald-like type disorder, yet with completely different etiology. Further studies at the biochemical and molecular level are needed to better understand and characterise the disorder in this pear cultivar.

4.2. Cold-induced regulation of α-farnesene and its involvement in superficial scald development

In addition to ethylene, superficial scald is commonly related to α-farnesene metabolism and to a widely described relationship between ethylene and α-farnesene (Anet, 1972; Giné-Bordonaba et al., 2013; Whitaker et al., 2000). Albeit not working at the molecular level, Lovastatin is an inhibitor of α-farnesene biosynthesis that does not affect the ethylene production (Ju and Curry, 2000b) but effectively controls superficial scald both in apples and pears (Giné-Bordonaba et al., 2020; Ju and Curry, 2000a). This compound hence, is a very interesting tool to understand the specific role that α-farnesene may have on superficial scald development. In agreement to that mentioned above, our data shows that lovastatin effectively inhibited superficial scald development in 'Blanquilla' pears. It is, however, worth mentioning that the lovastatin treatment described herein was formulated as a vegetable oil emulsion (containing sunflower oil at 0.4%; v/v), and that this specific oil alone or in combination with glycerol and/or tween may directly influence superficial scald development. Indeed, previous studies working with different vegetable oils, yet at much higher concentrations (over 5-fold higher), have shown that to some extent oil-based treatments can tackle superficial scald development in apples and pears (Ju and Curry, 2000c and 2000d; Ju et al., 2000). While information is rather scarce for sunflower oil, evidence suggest that corn-oil based emulsion (at concentrations of 2.5% or higher) are effective in preventing superficial scald in both apples and pears (Ju and Curry, 2000c and 2000d; Ju et al., 2000). In the same studies, not only superficial scald but fruit ripening was altered in response to the treatments (Ju and Curry, 2000a) thereby pointing out that, to some extent, the effectiveness of such treatments was likely related to the oil's barrier effect towards oxygen. In our study, the lovastatin formulation did not inhibit the fruit ethylene production that depends on oxygen availability (Supplementary Figure 1). This result together with the results we obtained in a previous trial (Supplementary Figure 5) and with the recognized effect that lovastatin has on α-farnesene biosynthesis, suggest that the superficial scald inhibitory effect detailed herein was likely associated to lovastatin rather than other compounds included in the formulation. Nonetheless, future studies are needed to further corroborate if sunflower oil-based formulations, at the concentrations tested herein, are capable of altering α-farnesene biosynthesis in these specific pear cultivars.

From a biochemical perspective, several studies have reported that ethylene promotes the α-farnesene biosynthesis by its action on the *AFS1* gene expression (Gapper et al., 2006; Lurie et al., 2005; Pechous et al., 2005; Tsantili et al., 2007). Our results support these findings but also suggest that *AFS1* is directly activated by cold as soon as the ethylene metabolism at the molecular level is active. These results are in accordance to those observed in previous studies (Calvo et al., 2015; Larrigaudière et al., 2019, 2016) and highlight the idea that α-farnesene is synthesized in pears both in response to increased ethylene production but also in a constitutive way determined by the genetic potential of each cultivar and likely induced by cold stress.

In contrast to 1-MCP, the response to the Lovastatin treatment was cultivar dependent. In 'Blanquilla' pear, untreated and Lovastatin-treated fruit exhibited similar levels of ethylene production (Suppl. Fig. 1) but Lovastatin effectively inhibited the accumulation of α-farnesene and disorder incidence (Figs. 2 and 1D). Lovastatin also induced a clear increase of ACO activity (Fig. 2 and Suppl. Fig. 2D) that was not paralleled by higher *PcACO1* gene expression compared to control (Suppl. Fig. 3D). This said, such enhancement of ACO activity is likely transitory since no differences were reported after 4 months of cold-storage (Giné-Bordonaba et al., 2020). Based on our findings, superficial scald development in 'Blanquilla' pears was clearly related to the fruit capacity to produce ethylene and to its regulatory role on *PcAFS1* gene expression during cold storage. However, an improved cold-acclimation capacity associated to 1-MCP treatment (Busatto et al., 2018) or driven by genetic or environmental factors (Marc et al., 2020), is also likely of paramount importance for the prevention of the disorder. We cannot discard especially the possible involvement of diverse metabolic shifts participating in redox homeostasis and membrane stabilization that may determined the cultivar-specific resistance to superficial scald (Zubini et al., 2007). 1-MCP treatment for instance not only inhibits ethylene production but also consistently leads to enhanced antioxidant enzyme activities (Chiriboga et al., 2013a; Giné-Bordonaba et al., 2020; Vilaplana et al., 2006; Zhi and Dong, 2018; Zhou et al., 2017) and increases the levels of certain cryoprotectants facilitating the stabilization of membranes (Busatto et al., 2018; Giné-Bordonaba et al., 2020). Furthermore, and since ethylene has been shown to be an important repressive regulator of apoplastic H_2O_2 levels in apples (Zermiani et al., 2015), 1-MCP may also promote the expression levels of some genes involved in the ascorbate-glutathione cycle (Zermiani et al., 2015), leading then to higher potential to scavenge ROS and thereby prevent oxidative damage (Giné-Bordonaba et al., 2020; Wang et al., 2018). It is also known that 1-MCP inhibits or delays the gene expressions of glutathione-S-transferases (GSTs) (Karagiannis et al., 2020) and glutathione peroxidases (GPXs) (Wang et al., 2018; Zhou et al., 2017), two enzymes involved in the oxidation of conjugated trienes hydroperoxides to their alcohols (Dixon et al., 2010; Whitaker, 2013). Collectively these results show that the development of superficial scald in 'Blanquilla' pears results from the interaction of several factors and that ethylene, even playing an important role in the synthesis of α-farnesene, did not determine alone the disorder incidence. Future studies investigating the role that ROS scavenging may have in scald control in relation to the initial harvest maturity or to the use of different postharvest storage scenarios is envisaged.

In 'Conference' pears, Lovastatin also reduced the levels of α-farnesene and its oxidation products but did not affect the disorder incidence (Fig. 3 and 1F). These results suggest that α-farnesene is unlikely involved in the development of the disorder observed in this cultivar and further sustained the hypothesis mentioned earlier that the disorder observed in 'Conference' has a completely different etiology than superficial scald. Similar results were also observed by Rizzolo et al. (2015) that, on the basis of the symptom appearance and response to 1-MCP treatment, also suggested that this disorder was not superficial scald.

Finally, the Lovastatin treatment could not control the appearance of superficial scald nor the accumulation of α-farnesene in 'Flor d'Hivern' pears (Figure 1E and 4). Since the Lovastatin effect on *PcAFS1* was fairly similar in all cultivars (Suppl. Fig. 4), it is possible that α-farnesene accumulation in 'Flor d'Hivern', may be partly due to the synthesis of isopentenyl diphosphate (IPP), a precursor of α-farnesene in the mevalonate pathway, in the plastid via the MEP prior to being transported into the cytoplasm (Eisenreich et al., 2001). Under this scenario, Lovastatin would have little or no effect in the accumulation of α-farnesene in this specific pear cultivar.

5. CONCLUSIONS

The results from this study provide detailed information on the distinct processes involved in the cold-induced regulation of scald-like disorders in different pear cultivars. ‘Blanquilla’ pear showed typical superficial scald symptoms clearly related to the fruit capacity to produce ethylene and to the cold-mediated regulation of *PcAFS1* gene expression. This last link may be considered as a key inducing factor of superficial scald development in this cultivar yet other more complex mechanisms are also likely involved. In contrast to ‘Blanquilla’, scald control in ‘Flor d’Hivern’ pears seems to be mainly associated to an improved cold-acclimation process, since this specific cultivar produce undetectable ethylene levels at harvest or upon removal from cold storage. In ‘Conference’ pear, neither 1-MCP nor Lovastatin inhibited the development of a scald-like disorder and even enhanced it, suggesting the existence of a completely different disorder of unknown etiology that needs to be further investigated.

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LIST OF FIGURES

Figure 1: Scald-like disorder appearance and disorder incidence (%) in 'Blanquilla' (A and D), 'Flor d'Hivern' (B and E) and 'Conference' (C and F) pears. Numbers inside boxes in the lower panel indicate severity for each treatment. Error bars represent the standard error of the mean (n = 3). Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$.

Figure 2: Scheme of the regulatory mechanisms involved in scald development in 'Blanquilla' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments or sampling points. Single error bar in line plots depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

Figure 3: Scheme of the regulatory mechanisms involved in scald development in 'Conference' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments or sampling points. Single error bar in line depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

Figure 4: Scheme of the regulatory mechanisms involved in scald development in 'Flor d'Hivern' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments

or sampling points. Single error bar in line depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

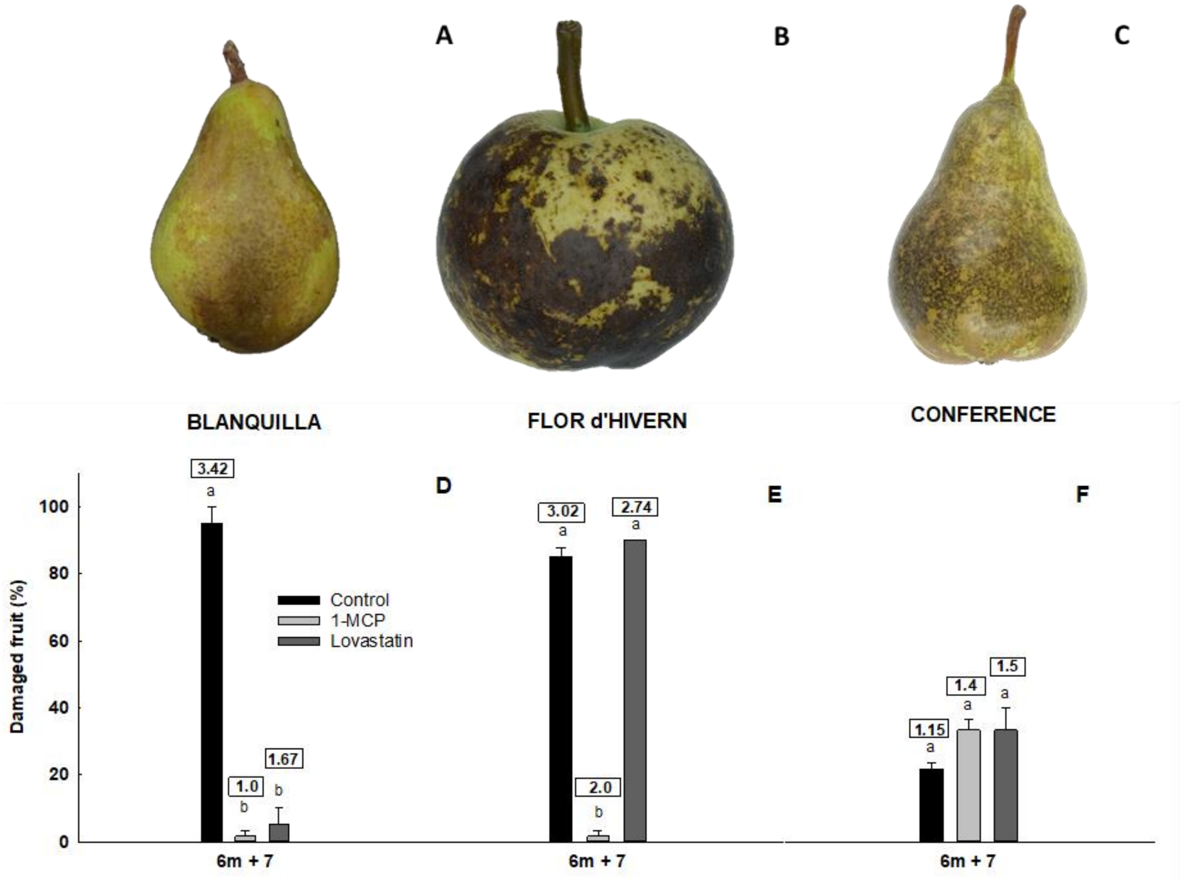
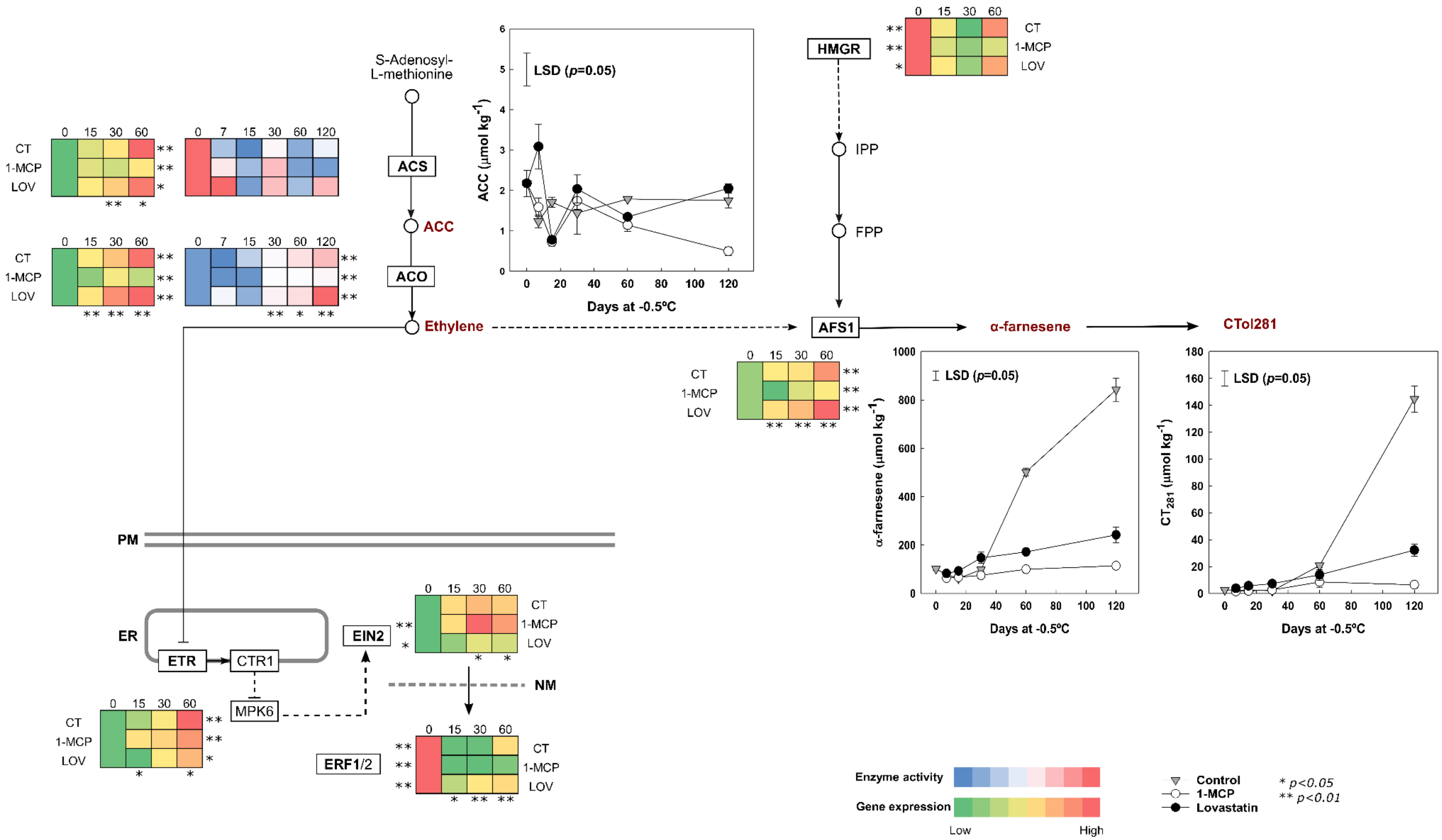

A
B
C
BLANQUILLA
FLOR d'HIVERN
CONFERENCE
D
E
F
Damaged fruit (%)
100
80
60
40
20
0
Control
1-MCP
Lovastatin
3.42
a
1.0
b
1.67
b
3.02
a
2.74
a
2.0
b
1.15
a
1.4
a
1.5
a
6m + 7
6m + 7
6m + 7

Figure 1:



838

839 **Figure 2:**

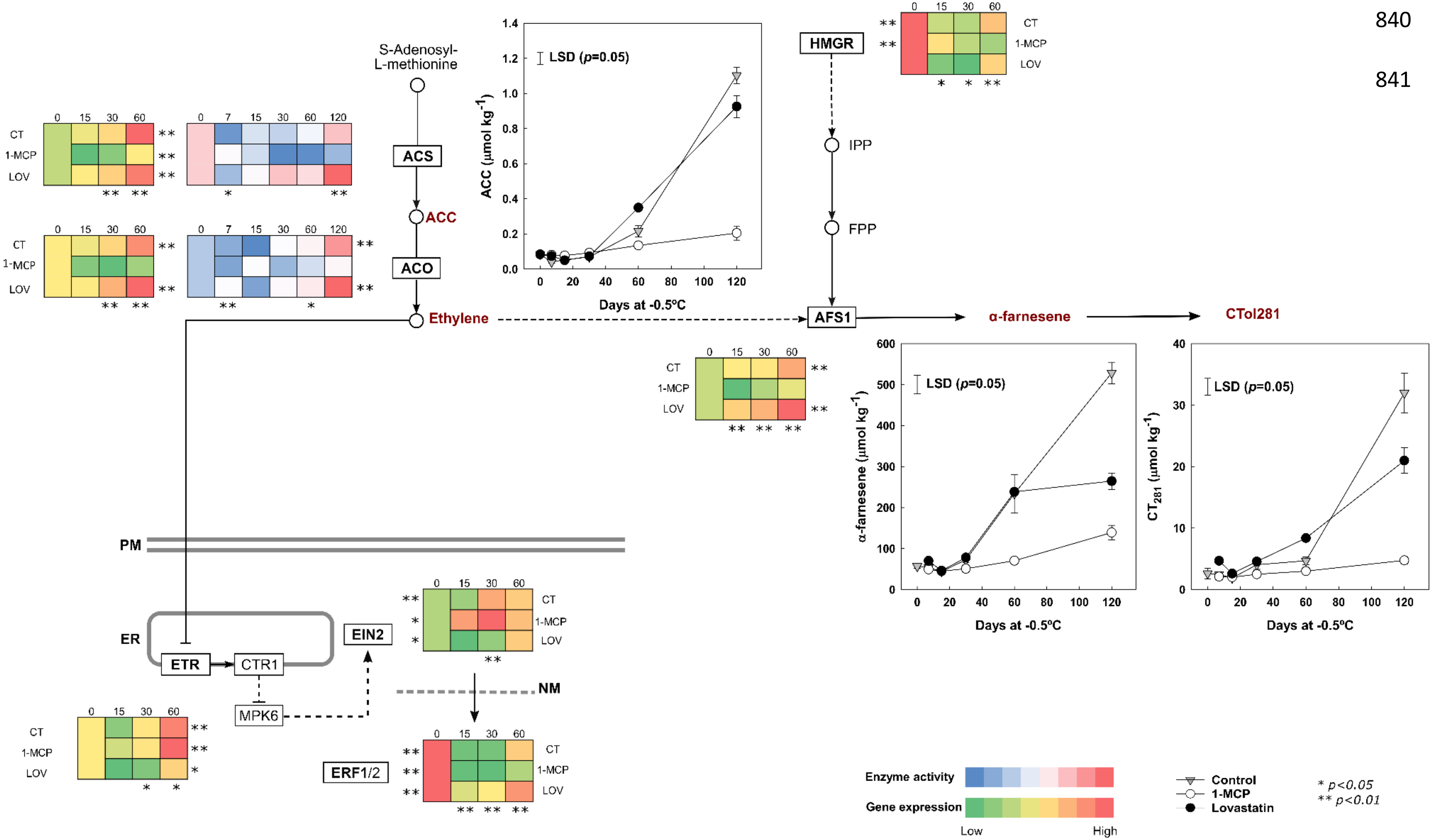
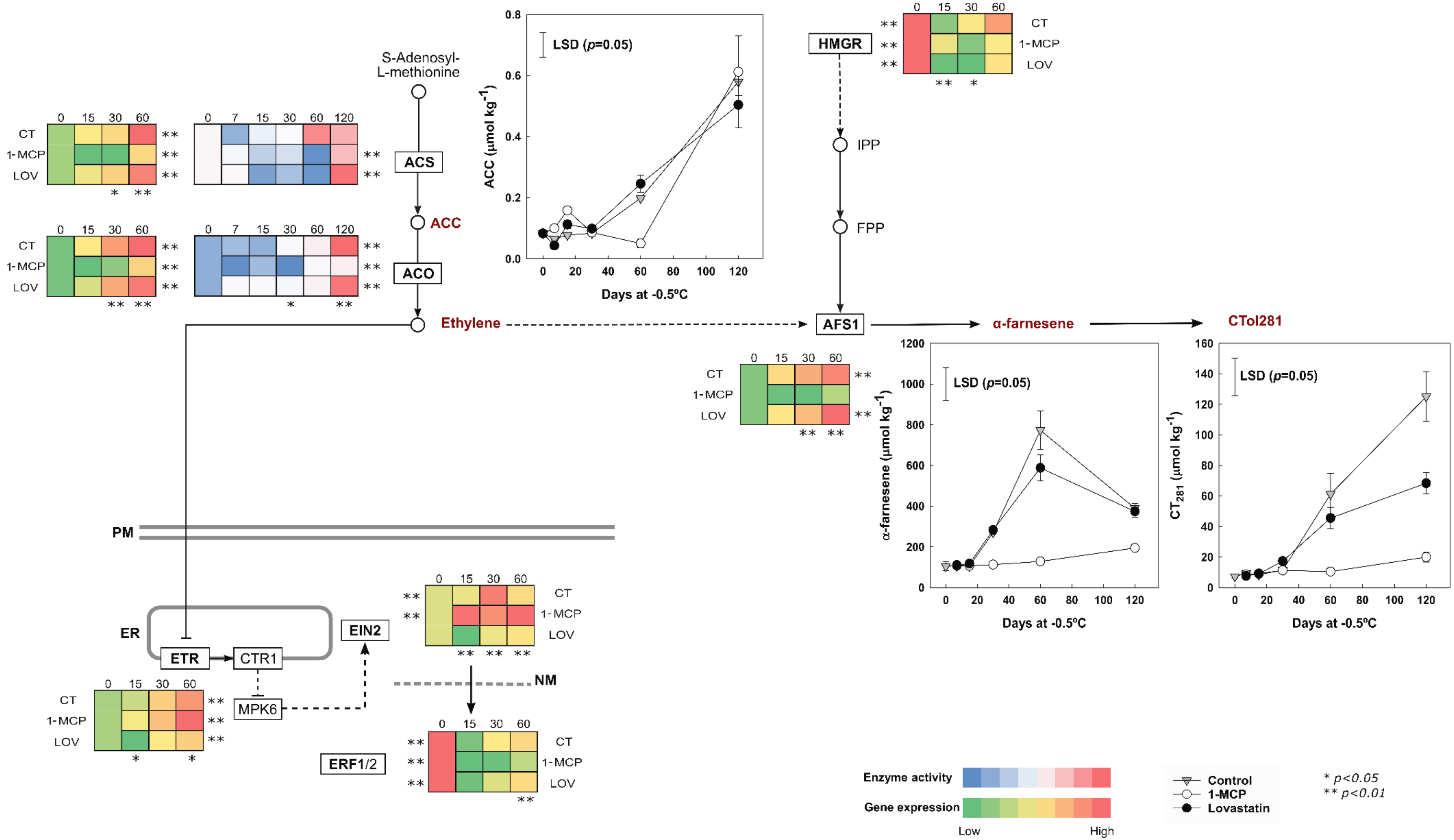

S-Adenosyl-L-methionine
ACS
ACC
ACO
Ethylene
HMGR
IPP
FPP
AFS1
α-farnesene
CTol281
PM
ER
ETR
CTR1
MPK6
EIN2
NM
ERF1/2
CT
1-MCP
LOV
LSD (p=0.05)
ACC (µmol kg-1)
α-farnesene (µmol kg-1)
CT281 (µmol kg-1)
Days at -0.5ºC
Enzyme activity
Gene expression
Low
High
Control
1-MCP
Lovastatin
* p<0.05
** p<0.01

Figure 3:

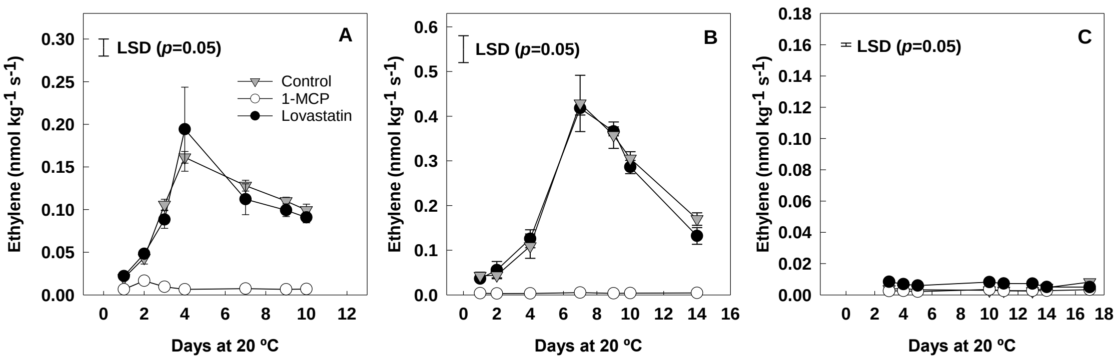


S-Adenosyl-L-methionine
ACS
ACC
ACO
Ethylene
HMGR
IPP
FPP
AFS1
α-farnesene
CTol281
PM
ER
ETR
CTR1
EIN2
MPK6
ERF1/2
NM
CT
1-MCP
LOV
LSD (p=0.05)
ACC (µmol kg-1)
Days at -0.5ºC
α-farnesene (µmol kg-1)
CT281 (µmol kg-1)
Enzyme activity
Gene expression
Low
High
Control
1-MCP
Lovastatin
* p<0.05
** p<0.01

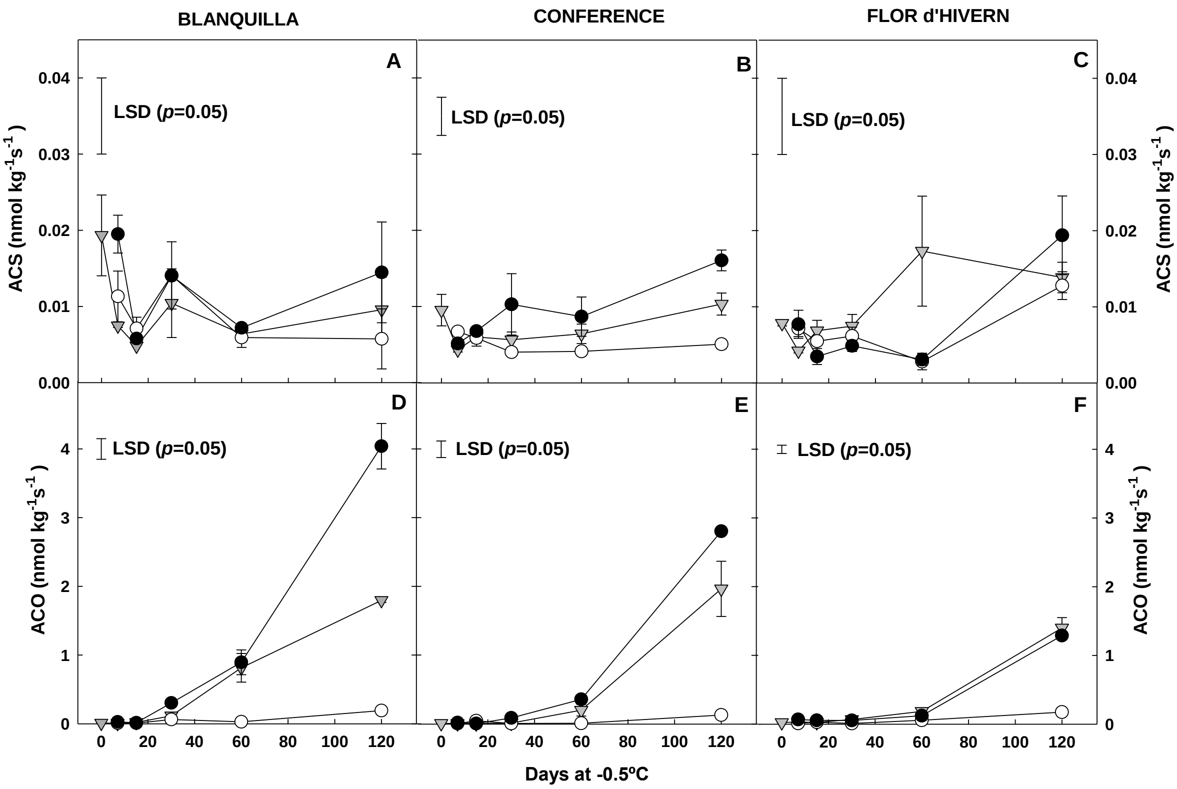
843 **Figure 4:**

Gene	Annotation	Oligonucleotide sequences	Target gene	Metabolic pathway/Biological function	References
PcACS1	Aminocyclopropane-1-carboxylic acid synthase	F5’-ATGCTGGCTTGTTCTGTTGG-3’ R5’-AGGTTCCGTGCAATGACAAG-3’	PCP011500	Ethylene biosynthesis	Busatto et al. (2018)
PcACO1	Aminocyclopropane-1-carboxylic acid oxidase	F5’-AAGGTCAGCAACTACCCTCC-3’ R5’-TGTCATCCTGGAAGAGCAGG-3’	PCP011683		Busatto et al. (2018)
PcAFS1	α-farnesene synthase	F5’-GAAAACTAGGCCTCGCGAAC-3’ R5’-TTCGATAGCTGCAATGCCGT-3’	PCP028486	α-farnesene biosynthesis	Busatto et al. (2018)
PcHMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase	F5’-ACGACGGCAAGGACCTTCATG-3’ R5’-GCAGGCTGCTTGTGATGCAAG-3’	PCP017787		Giné-Bordonaba et al. (2020)
PcERF1	ethylene response factor 1	F5’-AACATTCGAAACGGCGGAAG-3’ R5’-CGAGGACTGAGACGCATTTG-3’	PCP015040	Ethylene response factor	Busatto et al. (2018)
PcETR1	ethylene receptor 1	F5’-AGAACGAGGCGTTGTTGCAC-3’ R5’-CCATCATCCCCCATTGCTC-3’	PCP024250	Ethylene signaling	Chiriboga et al. (2013b)
PcEIN2	ethylene insensitive protein 2	F5’-ATCTCTTGTCGAAAGGGCCG-3’ R5’-ACGCTTGTAGCGTTTGAGGA-3’	PCP018637.1	Ethylene signaling	This study
Md8283	housekeeping	F5’-CTCGTCGTCTTGTTCCCTGA-3’ R5’-GCCTAAGGACAGGTGGTCTATG-3’	PCP030439	housekeeping	Busatto et al. (2019, 2018)

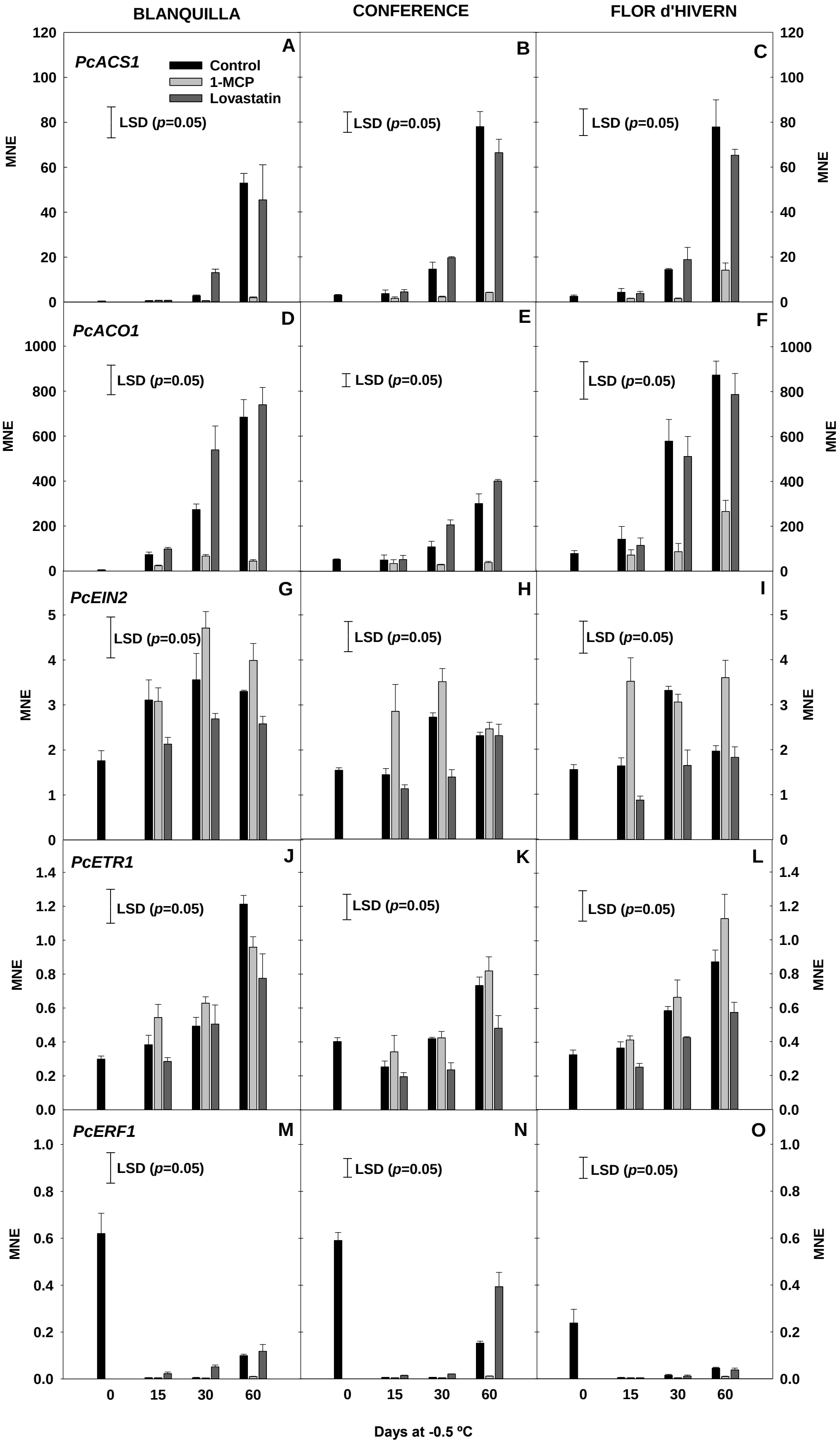
Supplementary Table 1: primers used for quantitative PCR.



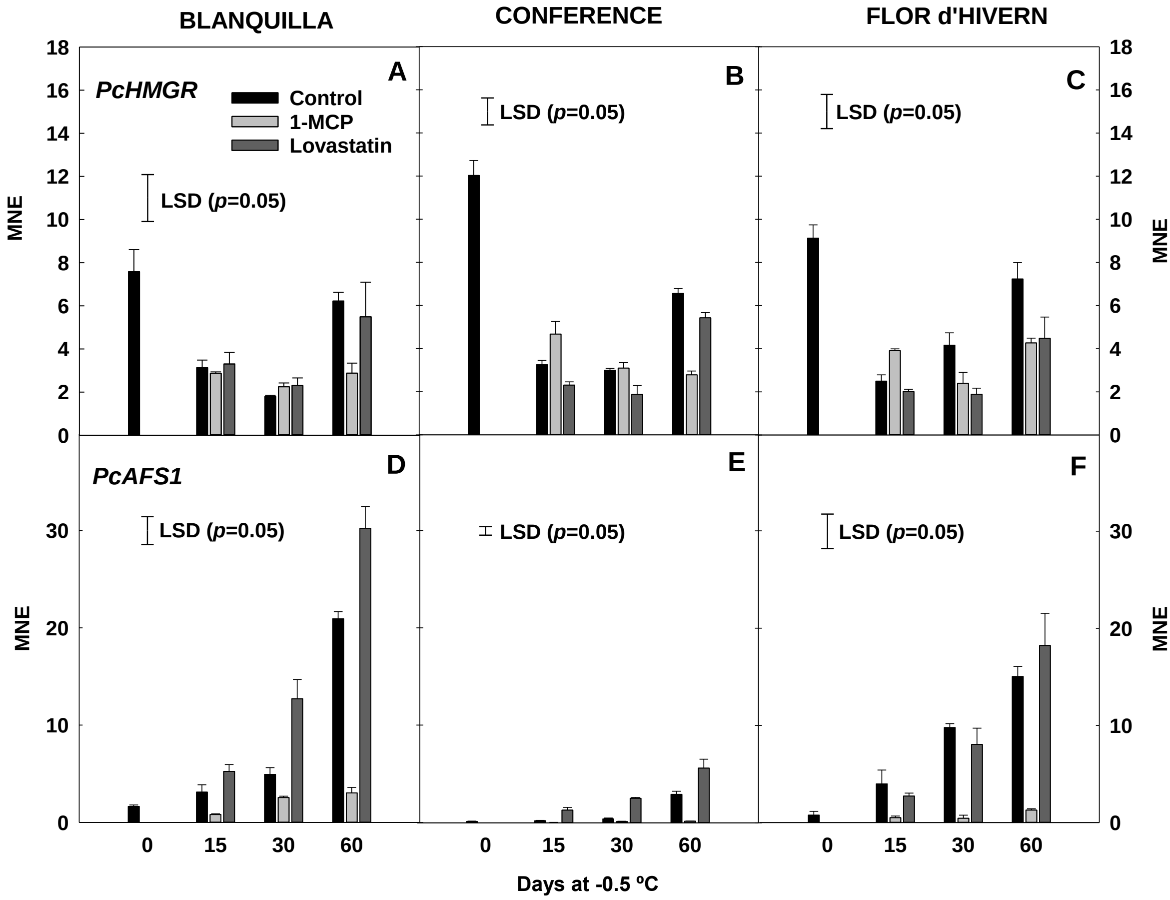
Supplementary Figure 1: Ethylene production upon removal at 20ºC after 60 d of cold storage in ‘Blanquilla’ (A), ‘Conference’ (B) and ‘Flor d’Hivern’ (C). Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (*p*=0.05) for the interaction treatment*sampling of cold storage.



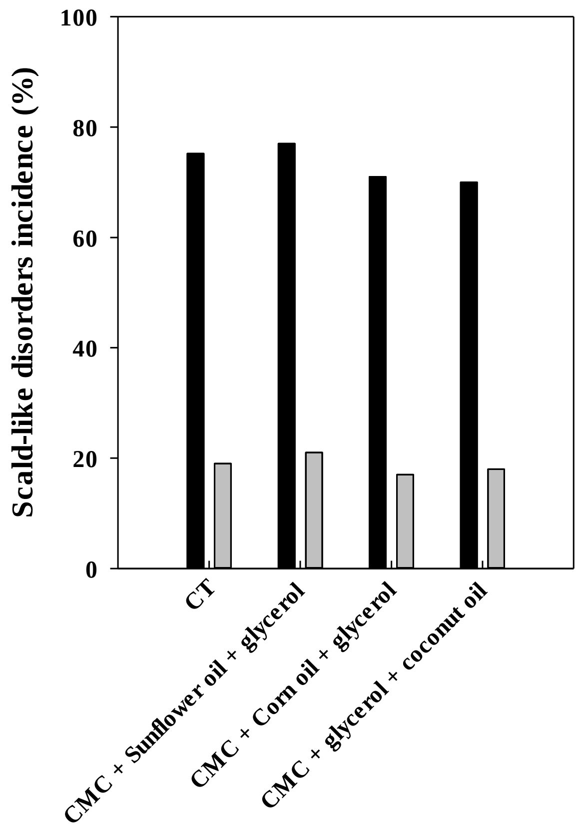
Supplementary Figure 2: ACC synthase (A, B and C) and ACC oxidase (D, E and F) activities in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 3: *PcACS1* (A, B and C), *PcACO1* (D, E and F), *PcEIN2* (G, H and I), *PcETR1* (J, K and L) and *PcERF1* (M, N and O) gene expressions in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 4: *PcHMGR* (A, B and C) and *PcAFS1* (D, E and F) gene expressions in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 5: Superficial scald incidence in untreated (CT) or pear fruit ('Blanquilla' (●) and 'Conference' (●)) treated with vegetable oil based (0.7-1.0%; v/v depending on the oil) formulations.