Molecular and biochemical differences underlying the efficacy of lovastatin in preventing the onset of superficial scald in a susceptible and resistant *Pyrus communis* L. cultivar

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Running Title: The role of lovastatin in preventing superficial scald in different pear cultivars.

ABSTRACT

The molecular and biochemical events underlying the onset of superficial scald in two pear cultivars with different susceptibility (‘Blanquilla’ and ‘Conference’), was investigated in fruit untreated, treated with lovastatin, 1-MCP or ethylene. ‘Conference’ pears were characterized by higher content of flavonols and linolenic acid (18:3), two metabolites related to chilling injury resistance. In this cultivar, the expression level of three genes belonging to the ascorbate glutathione pathway (*APX*, *DHAR* and *MDHAR*) were constitutively over-expressed, highlighting the role that endogenous antioxidant potential played in scald control. In the scald-susceptible cultivar (‘Blanquilla’) the lovastatin treatment, in contrast to 1-MCP, effectively prevented superficial scald development and α-farnesene production without affecting fruit ripening. Moreover, lovastatin stimulated an increased the production of ethanol and oleic+cis vaccenic acid (18:1), both compounds being also involved in cold stress tolerance. In both cultivars, and in contrast to 1-MCP, lovastatin did not impair the expression level of the genes devoted to ethylene production (*ACO*, *ACS*) and perception (*ERS1*, *ERS2*). As a consequence, the expression levels of the genes involved in texture modifications (*PG1*) and volatile emission (*LOX*, *HPL*, *ADH* and *AAT*) were maintained in lovastatin-treated samples allowing the fruit to reach an adequate final quality.

The results from this study are discussed to highlight the complex regulatory network underlying superficial scald development in different pear cultivars.

KEYWORDS

superficial scald, pear, cold storage, chilling injury, ripening, antioxidant content

1.0 INTRODUCTION

By using cold storage in combination with controlled atmosphere or other postharvest strategies, pears (*Pyrus communis*) can be commercialized throughout the year, similarly to apple and other fleshy fruits (Little and Holmes, 2000). Unlike other *rosaceae* fruit, most pear cultivars are distinguished by the requirement of a chilling period or ethylene treatment for the completion of the ripening process (El-Sharkawy et al., 2004; Lelièvre et al., 1997; Villalobos-Acuña and Mitcham, 2008). However, prolonged low temperature storage can induce several physiological disorders, among which superficial scald is one of the most dramatic in terms of economical losses in pome fruit (Lurie and Watkins, 2012; Wang, 2016; Whitaker, 2008).

The symptoms of superficial scald are characterized by the development of brown patches on the fruit skin generally appearing after the fruit is removed from cold storage and placed at room temperature conditions (Lurie and Watkins, 2012) and caused by the oxidation of chlorogenic acid through the action of polyphenol oxidase (PPO) (Busatto et al., 2014; Giné-Bordonaba et al., 2020). In detail, the reaction between PPO and chlorogenic acid leads to the accumulation of quinones in the cytoplasm, reacting together to form the brown pigment melanin (Busatto et al., 2014). Despite the deep comprehension of the symptom appearance, mainly investigated in apples (Lurie and Watkins, 2012), the mechanism related to the etiological cause leading to the scald development in pears is still not completely elucidated in pears. Recent studies shed light on the physiological details related to the scald development and on the molecular mechanism underlying the basis of the scald resistance induced by 1-Methylcyclopropene (1-MCP) treatment in apple (Busatto et al., 2018). 1-MCP, a competitive inhibitor of ethylene, is among the most effective strategies to prevent the development of superficial scald (Lurie and Watkins, 2012; Watkins, 2006). The regulation of superficial scald through the action of ethylene is supposed to rely on the ability of this hormone to mediate the expression of α-farnesene synthase 1 gene (AFS1), the limiting step in the production of α-farnesene. Therefore, the effectiveness of 1-MCP in preventing the onset of superficial scald

was initially accounted to the inhibition of the ethylene perception induced by this ethylene analog (Lurie and Watkins, 2012). However, it has recently been shown that 1-MCP treatment is also able to promote a deep transcriptional reprogramming inducing a specific group of genes involved in the cold stress response finally leading to the establishment of a cold tolerance phenotype (Busatto et al., 2018). 1-MCP is also routinely used in the post-harvest management to increase the fruit storability, slowing down softening as well as other multiple ripening associated events (Ikiz et al., 2018; Watkins, 2006). The application of 1-MCP in pear can, however, dramatically impair the progression of the fruit ripening and affect several ethylene-dependent fruit quality related processes, such as the production of volatile organic compounds (VOCs) and fruit softening thereby compromising consumer acceptance. Indeed, while juiciness and crispiness are generally the most important apple quality traits in terms of consumer acceptance, consumers demand pears with a buttery and juicy texture. In this context, several strategies have been employed in the past to prevent the irreversible block of ethylene caused by 1-MCP yet achieving unsuccessful results (Chiriboga et al., 2011).

Consequently, the search of novel treatments using specific compounds able to reduce the impact of post-harvest physiological disorders, such as superficial scald, without impairing the pear ripening capability is a key factor for an innovative pear post-harvest management. Even if the etiology of superficial scald is still matter of speculation, a positive correlation between superficial scald onset and the presence of 6-Methyl-5-hepten-2-one (6-MHO) is well documented in literature. 6-MHO, together with the conjugated trienes hydroperoxides, are thought to be the major products of the α-farnesene autoxidation (Farneti et al., 2015; Rowan, 2011; Rowan et al., 2001) leading to the appearance of superficial scald symptoms. Therefore, the possibility of reducing the incidence of this disorder disrupting the accumulation of α-farnesene without interfering with the ethylene signaling, could represent a valuable strategy to promote or better maintain fruit quality.

Some studies have investigated the effects of lovastatin treatment on α-farnesene and ethylene biosynthesis, VOC production, and fruit color changes during apple ripening showing that

lovastatin is capable to reduce the production of α-farnesene and sesquiterpenes without affecting the ethylene synthesis and the ripening progression (Ju and Curry, 2001; Kader, 1999; Pechous and Whitaker, 2004; Rudell et al., 2009; Savran and Koyuncu, 2016). Lovastatin is a statin inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG-CoA reductase*), an enzyme devoted to the conversion of HMG-CoA to mevalonate and a potent cholesterol-lowering pharmaceutical in animals. In higher plants, the biosynthesis of the C5 universal sesquiterpene precursor, isopentenyl diphosphate (IPP), is synthetized, in the cytosol, through the mevalonate pathway (Ju and Curry, 2001; Vranová et al., 2013). IPP is, in turn, converted to the α-farnesene precursor, farnesyl diphosphate (FPP) and then accumulated in the wax layer of the pear skin during cold storage, where undergoes progressive autoxidation processes (Giné Bordonaba et al., 2013; Larrigaudière et al., 2016).

In this work, we investigated the role of lovastatin in reducing the development of superficial scald and the treatment effect on major fruit quality traits of two pear cultivars, 'Blanquilla' and 'Conference', characterized by a distinct superficial scald susceptibility (Lindo-García et al., 2020b). For comparative purposes, fruit were treated with lovastatin, 1-MCP and ethylene prior to storage and gene expression and secondary metabolite analysis were done on fruit after removing the fruit from cold storage and further shelf-life.

2.0 MATERIALS AND METHODS

2.1 Plant materials, storage protocols and treatments

'Blanquilla' and 'Conference' pears were harvested in a commercial orchard located in Lleida (Spain). Trees, at the time of the analysis, were in the full bearing stage, trained and grown following standard horticultural practice for canopy management, pruning, fruit thinning and pest-disease control. Homogeneous fruit, in terms of both ripening stage and shape, were sampled at commercial maturity based on local grower standards mainly based on firmness and starch index

values (6.3 for ‘Conference’ and 4,1 for ‘Conference’) (Lindo-García et al., 2020b). A batch of thirty fruit was used for initial fruit quality assessment including fruit firmness, starch content, total soluble solids, and acidity. The remaining pears were divided in four batches of 200 fruit each and used for each of the diverse treatments. One batch, represented by untreated fruit, was employed as control (CT) while the other three subsets of fruit were treated with: ethylene (ET) (200 µL L^{-1} for 24h; 1-methylcyclopropene (1-MCP) (300 nL L^{-1}) applied as Smartfresh™ (Agrofresh Inc., PA, USA) and lovastatin (LOV) (1.25 mmol/L, dipping for 2 min). After treatments, fruit boxes were ventilated and placed in cold storage at +0.5°C with 95% relative humidity for four months in regular atmosphere. After 4 months of cold storage, fruit were place at room temperature conditions (20°C) for further 5 days (shelf-life). From each batch, thirty fruit were selected for RNA and metabolites extractions, while an additional batch of 54 fruit per treatment (3 biological replicates of 3 fruit each x 6 sampling points) were used to quantify α-farnesene and conjugated trienes (CTols) during storage. The remaining fruit from each treatment were used to monitor the fruit ethylene production capacity upon removal from 2 and 4 months of cold storage.

2.2 Standard quality, ethylene production and superficial scald incidence evaluations

A standard Penetrometer (Effegi penetrometer FT 327) was employed for profiling mechanical signatures of each set of ‘Blanquilla’ and ‘Conference’ pears.

The pear juice of a blend of 5 fruit per replicate and 4 replicates per sampling was used for measuring the total soluble solids (SSC; %) with a digital hand-held refractometer (Atago, Tokyo, Japan) whereas acid content (TTA) was obtained on the same juice samples by titration using Na OH 0.1N. The results were expressed as g malic acid L^{-1} sample.

Per each treatment at harvest and upon removal from cold storage, the ethylene production (nmol Kg^{-1} s^{-1}) was quantified in an acclimatized chamber at 20 °C. Two pears were placed in 1.5 L respiration flasks continuously ventilated with humidified air at a flow rate of 1.5 L h^{-1}. Ethylene production was determined on 4 replicates of two pears each. One mL of effluent air from the flasks

was sampled using a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) coupled with an FID detector and an alumina column 80/100 (2 m × 3 mm, Tecknokroma, Barcelona, Spain).

The superficial scald incidence was evaluated by visual inspection after 4 months of cold storage plus 5 days of shelf life following the methodology described elsewhere (Giné-Bordonaba et al., 2020).

2.3 Pear VOC analysis

Pear skin VOCs, from 3 technical replicates from each of the three biological replicates, were measured with a PTR-ToF-MS 8000 apparatus (Ionicon Analytik GmbH, Innsbruck, Austria). 0.5 g of powdered frozen tissue were rapidly inserted into a 20 mL glass vial equipped with PTFE/silicone septa (Agilent, Santa Clara, CA, USA) and mixed with 0.5 mL of deionized water, 200 mg of sodium chloride, 2.5 mg of ascorbic acid, and 2.5 mg of citric acid, and then preserved at 4°C until assessment. The sample headspace was withdrawn through PTR-MS inlet with 40 sccm flow for 60 cycles resulting in an analysis time of 60 s/sample. Pure nitrogen was flushed continuously through the vial to prevent pressure drop. Each measurement was conducted automatically after 20 min of sample incubation at 40°C. All steps of measurements were automated by an adapted GC autosampler (MPS Multipurpose Sampler, GERSTEL) coupled to PTR-ToF-MS. The analysis of PTR-ToF-MS spectral data proceeded as follows. Count losses due to the ion detector dead time were corrected off-line through a Poisson statistics-based method (Cappellin et al., 2011a), while internal calibration was performed according to the procedure described in previous studies (Cappellin et al., 2011b).

2.4 Extraction and characterization of the skin lipid composition

Lipids were characterized following the protocol reported in previous studies (Della Corte et al., 2015). Lipids extracted from three biological replicates were separated and quantified through

183 an ultra-high-performance liquid chromatography (UHPLC) Dionex 3000 (Thermo Fischer
184 Scientific Germany), with a RP Ascentis Express column (15 cm 9 2.1 mm; 2.7 lm C18) applying
185 30-min of multistep linear gradient. The UHPL chromatographic system was coupled to an API
186 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an ESI
187 source. Lipids were identified based on reference standards and retention time, and further
188 quantified as μg/g of fresh weight.
189
190 2.5 Profiling of phenolic compounds
191 The analysis of phenols followed the protocol described in Vrhovsek et al. (Vrhovsek et al., 2012)
192 with a simplified sample extraction (Giné-Bordonaba et al., 2019), and using three biological
193 replicates. For this assessment a Waters Acquity UPLC system (Milford, MA, USA) coupled to a
194 Waters Xevo TQMS mass spectrometer (Milford, MA, USA) was employed. The capillary voltage
195 was 3.5 kV in the positive mode and −2.5 kV in the negative mode. Each compound was analyzed
196 under the optimized MRM conditions (precursor and product ions, quantifiers and qualifiers,
197 collision energies, and cone voltages) as described (Vrhovsek et al., 2012). Waters MassLynx 4.1
198 and TargetLynx software were used to process the phenolic data and each phenolic compound was
199 characterized on the base of reference compounds and expressed as mg/Kg of fresh weight.
200
201 2.5 Gene expression profiling by RT-qPCR
202 The peel from 5 fruit per replicate and per each treatment and sampling point was isolated,
203 immediately frozen with liquid nitrogen, grinded into a fine powder, and finally stored at -80°C
204 until processing. RNA extraction was carried out using Spectrum Plant total RNA kit (Sigma-
205 Aldrich Co., St Luis, MO, USA). The RNA, extracted by two biological replicates (of five fruit
206 each), was quantified and assessed with a NanoDrop ND-8000 spectrophotometer (Thermo
207 Scientific, Waltham, MA, USA). For each sample, 1 μg of total RNA was treated with 1 Unit of
208 Ambion rDNAse I (DNA free kit, Life Technologies, Carlsbad, CA, USA) and used as a starting

template to synthetize cDNA using the "Super-Script VILO cDNA Synthesis Kit" (Life Technologies, Carlsbad, CA, USA). The transcript relative quantification was obtained using ViiA7™ instrument (Life Technologies, Carlsbad, CA, USA) and FAST SYBR GREEN MASTER MIX (Life Technologies, Carlsbad, CA, USA). The thermal conditions applied during the PCR were: initial incubation at 95°C for 20 sec, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. In the end a final amplification cycle at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec was applied to determine the melting curve. The final Ct is represented by the average of two independent normalized expression values for each sample, carried out using the software provided with the ViiA7™. The gene expression was reported by the mean normalized expression through the use of equation 2 of the "Qgene" software. Actin gene (Md8283) was employed as housekeeping (Botton et al., 2011). For each gene a couple of discriminant and specific primer was designed, using the online software Primer3 (http://primer3.ut.ee) and Primique (http://cgi-www.daimi.au.dk/cgi-chili/primique/ front.py). The primer list as well as the description of the set of genes analyzed (retrieved by Busatto et al., 2019; Giné-Bordonaba et al., 2020 and Lindo-García et al., 2020a) is reported in the Suppl. Table 1.

2.6 Data analysis

Data were analyzed using R.3.4.1 (R Core Team (2017). R Foundation for Statistical Computing, Vienna, Austria). In particular, the PCA were done using ChemometricsWithR packages. The heatmaps depicting the gene expression data combined with the polyphenol quantifications were calculated and visualized trough Gene Cluster 3.0 and Java Tree software, respectively. Metabolite profiles were processed using the Water MassLynx 4.1 and Target Lynx software. Student-Newman-Keuls test ($\alpha = 0.05$) were performed using the software R in order to indicate significative differences between treatments and genotypes for each specific sampling.

3.0 RESULTS

235

236 3.1 Effect of the treatments on scald incidence and fruit quality in ‘Blanquilla’ and ‘Conference’
237 pears.

238 After four months of cold storage and shelf-life the susceptibility of the fruit to superficial scald
239 was significantly different for ‘Blanquilla’ and ‘Conference’ pears (Fig. 1a). Prolonged cold storage
240 severely affected the scald development in untreated ‘Blanquilla’ fruit (78%) and almost entirely
241 the ethylene treated fruit (96%) upon shelf-life. The application of both 1-MCP and lovastatin
242 efficiently alleviated the scald development, with a complete reduction of the symptoms (0%) in the
243 1-MCP treated fruit. Fruit treated with lovastatin, showed low incidence of superficial scald (11%)
244 after 5 days of shelf-life (Fig. 1a). On the contrary, ‘Conference’ pears were significantly less prone
245 to develop superficial or scald-like disorders, and none of the treatments applied led to lower scald-
246 like incidence in comparison to untreated fruit. While ‘Blanquilla’ achieved a complete prevention
247 of the scald symptoms in 1-MCP treated fruit, and a reduction of 7.0 and 8. 6-fold in lovastatin
248 samples if compared to CT and ET-treated fruit respectively, ‘Conference’ pear showed a variation
249 of scald for 1-MCP of 0.63 and 1.41-fold (compared to CT and ET) and for lovastatin of 0.56 and
250 1.25-fold (compared to CT and ET), respectively (Fig.1a).

251 In order to verify the impact of the different treatments on fruit quality and ripening progression,
252 fruit firmness (Fig. 1b), titratable acidity (TTA - Suppl. Table2) and soluble solid content (SSC –
253 Suppl. Table2) were measured. TTA and SSC did not show any significative variation among
254 treatments for any of the cultivars investigated. On the contrary, a completely different behavior
255 was observed for the fruit firmness. In ‘Blanquilla’ an important firmness loss occurred in all
256 samples, except for 1-MCP treated fruit during (1.76-fold) and after cold-storage (3.52-fold). In
257 contrast, ‘Conference’ pears did not show any firmness loss during cold storage, but it sharply
258 declined as the fruit were moved at 20ºC shelf-life (80% of firmness loss; Fig. 1b). Slightly yet
259 significantly higher firmness values were observed for 1-MCP treated ‘Conference’ pears after 4
260 months of cold storage and 5 days of shelf-life in comparison to the other treatments.

Ethylene production of ‘Blanquilla’ and ‘Conference’ pear significantly differed during storage. While after 4 months of cold storage the fruit ethylene production was quite consistent between the two cultivars, a more pronounced production of ethylene was observed in ‘Conference’ following 5 days of shelf-life. At this stage, untreated ‘Conference’ pears showed an ethylene production of 0.72 nmol $Kg^{-1}s^{-1}$, while untreated ‘Blanquilla’ fruit showed a 3.3-fold lower amount (0.22 nmol $Kg^{-1}s^{-1}$) (Fig. 1c). The production of ethylene was, as expected, severely reduced in 1-MCP-treated fruit, with a stronger effect in ‘Blanquilla’ than in ‘Conference’. Application of lovastatin and ethylene, instead, did not show any particular effect on the fruit ethylene production (Fig. 1c).

3.2 Effect of the treatments on the gene expression profile of ‘Blanquilla’ and ‘Conference’pears.

The transcriptional changes underlying the onset of superficial scald deveopment between the two cultivars was assessed through the investigation of 19 genes belonging to six different metabolic pathways, such as ethylene biosynthesis and perception, auxin signaling, polyphenol biosynthesis and oxidation, volatile biosynthesis, ROS scavenging and cell wall disassembling (Suppl. Table1).

The PCA score plot, accounting for 64.2% of the total gene expression variance (Fig 2a) clearly revealed the impact of the different treatments and genetic background (cultivar) on the transcriptional dynamics occurring during the two postharvest stages (cold-storage and shelf-life). The different treatments were distinguished by the first principal component, with harvest and 1-MCP treated sample plotted on the positive PC1 area and the rest on the negative part, exception made for the samples of ‘Blanquilla’ treated with lovastatin and assessed during shelf-life. PC2, instead, clearly characterized the two sampling stages, with samples collected after 4 months of cold storage plotted on the PC2 positive part of the 2D-PCA plot, and the samples collected after additional 5 days of shelf-life located on the PC2 negative part of the PCA distribution, for both cultivars (Fig. 2a). The analysis of the expression pattern for each of the 19 genes highlighted a cultivar specific gene regulation in response to the different treatments or post-cold storage ripening. From the variable projection depicted in Fig. 2b, it is interesting to underline the

correlation between the expression pattern of the genes related to ethylene and the two main genes involved in superficial scald metabolism, such as the polyphenol oxidase (PPO) and the α-farnesene synthase (AFS) genes. Genes involved in pathways directly affected by lovastatin (HMG2) as well as those related to ascorbic acid metabolism (MDHAR and DHAR) were instead orthogonally projected with regards to the first group of ethylene related genes (Fig. 2b).

During the cold storage and shelf life in 'Blanquilla', 1-MCP treatment strongly reduced the activity of all genes related to the ethylene domain such as ACS, *ACO*, *ERS1*, *ERS2*, *ERF1* and *ERF2* as well as the genes involved in the phenylpropanoid pathway (*PAL* and *PPO*), production of volatiles (*LOX*, *HPL*, *ADH* and *AAT*) and α-farnesene (*HMG2* and *AFS1*) or involved in the softening process (*PG1*) (Fig. 3a, Supp. Fig. 1). However, 1-MCP application also increased the expression level of genes involved in the ascorbate-dependent antioxidant pathway (*APX*, *DHAR*, *MDHAR*). Although the gene regulation observed in the samples treated with ethylene or lovastatin was similar to that observed in untreated fruit, lovastatin had a significant effect on repressing the genes involved in the superficial scald development such as *PAL*, *PPO*, *HMG2* and *AFS1*. Interestingly, lovastatin slightly downregulated also *ACS, ACO* and *ERS1* yet only during shelf-life.

In 'Conference', a sub-set of genes, such as *APX*, *DHAR*, *PAL*, *HPL* and *LOX*, were rather strongly modulated by the shelf-life rather than by the treatments (Fig 3b). Moreover, in this cultivar, *HMG2* and *AFS1* were not significantly affected by the application of lovastatin.

3.3 Effect of the treatments on the volatile signature of 'Blanquilla' and 'Conference' pears.

The detection of 139 VOC mass peaks enabled a clear distinction of the samples over the 2D-PCA space (Fig. 4a). Samples of 'Conference' were mostly located in the positive PC1 – negative PC2 quadrant, exception made for LOV_4M_SL, while the samples of 'Blanquilla' were rather spread in the other three quadrants of the PCA plot. Between the two cultivars, 'Blanquilla' was characterized by a high concentration of specific compounds tentatively identified as butanal, cis-3-hexenyl

312 acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate, ethyl acetate, butanoic acid hexyl ester
313 and alcohols (hexanol, 1-butanol, ethanol) (Suppl. Table 2).
314 The PC2 values efficiently depicted the influence of the different treatments and storage stages for
315 both cultivars. Among the most relevant loadings, it is worthwhile to mention α-farnesene together
316 with some aldehydes, such as nonenal, 2-heptenal, octanal, 2,4-hexadienal, heptanal, heptadienal,
317 butenal, hexenal and 2-methyl butanal (Fig. 4b). Within the distribution of the samples based on the
318 volatilome variability, it was noted that the harvest samples for the two cultivars were closely
319 plotted together, and the distinction between cultivars based on their volatile profile only occurred
320 after postharvest storage. Samples from 'Blanquilla' at shelf-life were characterized by the highest
321 VOC production. The volatilome was also ethylene related. Treatment with 1-MCP lower down the
322 production of VOCs, while samples treated with lovastatin showed an intermediate production of
323 aromatic compounds. For aldehydes, a general decreased after harvest was observed for both cvs
324 (Fig.5a), with a slightly higher accumulation in 'Blanquilla' than in 'Conference' and showing
325 imperceptible changes in response to the different treatments. Also, for alcohols and esters, the
326 accumulation was higher in 'Blanquilla' than in 'Conference', which showed a significant higher
327 accumulation in control and ethylene treated samples after shelf-life (Fig. 5b and 5c). Particularly
328 interesting was the accumulation of ethanol in lovastatin-treated 'Blanquilla' pears, showing 1,8-
329 fold higher values than untreated fruit. α-farnesene content was greater in control and ethylene
330 treated samples and strongly inhibited by both1-MCP and lovastatin in both cultivars (Fig. 5d).
331 Likewise, the accumulation of 6-MHO was higher in control and ethylene treated 'Blanquilla'
332 samples and severely reduced by 1-MCP or to a lesser extent also by lovastatin (Fig. 5e). Especially
333 for the control and ethylene treated samples, the accumulation of 6-MHO in 'Blanquilla' was 2,56-
334 fold (in average) higher than 'Conference'.
335
336 3.4 Changes in the phenolic compounds and lipids induced by treatments with lovastatin, 1-MCP
337 and ethylene.

To characterize the array of secondary metabolite between ‘Blanquilla’ and ‘Conference’ samples, 20 phenolic compounds and 18 lipids were assessed. As depicted in Fig. 6a, the distribution of the samples based on the polyphenol variability (Fig. 6b) showed a clear separation of the cultivars over the 2D-PCA space. The ‘Blanquilla’ samples were characterized by negative values of PC1, whereas ‘Conference’ fruit were characterized by positive PC1 values. The effect of shelf life and treatments was instead represented by the PC2 (16.5% of the total variability).

‘Conference’ showed a higher accumulation of polyphenols in all the conditions analyzed, reaching the maximum peak in the ethylene treated sample after 4 months of cold storage (Fig. 7a). Similarly, chlorogenic acid content (Fig. 7b), a phenolic compound playing a key role in the metabolism of superficial scald, was 5.2-fold higher in ‘Conference’ than in ‘Blanquilla’ and generally was not affected by the treatments. Flavonols (Fig. 7c) including quercetin-3-glucoside, isoramnetina-3-glucoside, isoramnetina-3-rutinoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, were the predominant class of phenolic compounds detected in our study, accounting for 29% and 11% of the total phenolic composition in ‘Conference’ and ‘Blanquilla’ pears, respectively.

The multivariate analysis of PCA also illustrated the variability of the lipids analyzed across the several samples defined in this study (Fig. 8a). The two pear cultivars were distinguished along the PC2 axes, with ‘Blanquilla’ and ‘Conference’ samples located in the portion of the PCA described by positive and negative values of the PC2, respectively. The first principal component clearly differentiated the samples based on the different treatments, albeit with a cultivar-specific response. In fact, in ‘Blanquilla’, all the shelf life samples clustered together, in an area characterized by negative values of PC1 clearly separated from the samples from harvest and those treated with 1-MCP. In ‘Conference’ pears, the PC1 did not effectively discriminate the samples according to different sampling stages but rather by the influence of the treatment (Fig. 8a).

Interestingly, ‘Conference’ showed a noticeably increased content of linolenic acid (C18:3), a polyunsaturated fatty acid (Fig. 9a) that was highly accumulated in all the conditions analyzed in

this survey. Similarly, the monounsaturated lipids, oleic acid + cis-vaccenic acid (C18:1) were highly accumulated in the lovastatin treated samples (Fig. 9b), showing a pattern that was also observed in ‘Blanquilla’, although to a lesser extent.

4.0 DISCUSSION

4.1 The occurrence of superficial scald in pear is governed by the contribution of several metabolite pathways acting in a cultivar specific manner.

The development of superficial scald was strongly influenced by the type of treatment (1-MCP or lovastatin) as well as by the cultivar. In fact, while 78% of untreated ‘Blanquilla’ pears showed superficial scald symptoms, very low incidence (5%) was observed in ‘Conference’ fruit (Fig. 1a), confirming the differential susceptibility to superficial scald among cultivars reported in the literature (Larrigaudière et al., 2016; Lindo-García et al., 2020a) and suggesting a specific genetic control similar to what was already observed for apple (Busatto et al., 2018). Superficial scald is well known for being the result of a chilling injury (Lurie and Watkins, 2012) induced by low temperature storage. Indeed, to overcome chilling-triggered stresses, higher plants can respond through the activation of a series of complex mechanisms finally aimed to enhance cold tolerance (Sanghera et al., 2011; Schulz et al., 2016; Theocharis et al., 2012; Thomashow, 1999). Among such mechanism, the accumulation of specific compounds such as flavonoids seems to be determinant for freezing tolerance and cold acclimation in model species such as *A. thaliana* (Shulz et al., 2016). Accordingly, our data shows that ‘Conference’ pears had higher amounts of flavonols (Fig. 7c), a specific type of flavonoids, than ‘Blanquilla’, ranging from three to seven-fold higher values, yet depending on the specific compound, (Suppl. Table 2) accompanying the greater resistance of this cultivar to develop superficial scald. However, although the role of flavonols on cold acclimation has been intensively studied in *A. thaliana* (Schulz et al., 2016, 2015) and *T. hemsleyanum* (Peng et al., 2019), the molecular details on the link existing between them is still

390 unclear. Not only flavonoids but the total amount of phenolic compounds was generally greater in
391 ‘Conference’ than in ‘Blanquilla’ (Fig. 7a), and especially for chlorogenic acid (Fig. 7b). Previous
392 studies have shown that the accumulation of chlorogenic acid is correlated to the superficial scald
393 onset (Busatto et al., 2014), a result that cannot be confirmed in our study since ‘Conference’ pears
394 own higher content of this compound but displayed very limited scald symptoms. Discrepancies
395 between this and previous studies (Busatto et al., 2014) might be explained by the different
396 expression of the *PPO* gene deputed to encode for a protein responsible for the oxidation of this
397 hydroxycinnamic acid finally leading to the peel browning characteristics of superficial scald.
398 While in ‘Blanquilla’ PPO was highly expressed during the stage where superficial scald was
399 boosted (shelf-life after postharvest cold storage), in ‘Conference’ the expression of this gene was
400 severely down-regulated (Fig. 3a and Supp. Fig. 1). This result suggested a different genetic
401 regulation of the *PPO* gene among pear cultivars that warrants further investigation.
402 Besides phenolic compounds, the role of cis-vaccenic acid in enhancing cold resistance has been
403 demonstrated in several plant species, as for example in *Solanum lycopersicum* transgenic lines,
404 where the overexpression of cis-vaccenic acid induced an improved tolerance to freezing
405 temperatures (Badea and Basu, 2009; De Palma et al., 2008). The cold tolerance mechanism is also
406 regulated by the integrity of the internal lipidic membrane that during cold tolerance can
407 progressively loose permeability, with a consequent ion leaking coupled to the production of
408 reactive oxygen species. ROS can contribute to the peroxidation of lipids (Marangoni et al., 1996),
409 causing a loss of unsaturated fatty acids with an increased membrane rigidity due to the formation
410 of covalent bonds among lipid radicals (Alonso et al., 1997; Hara et al., 2003). The increase of the
411 unsaturated/saturated fatty acid ratio acid represents one of the key factors determining the
412 temperature at which the internal membrane changes from gel to liquid crystalline phase (Badea
413 and Basu, 2009; Browse, 2010; Marangoni et al., 1996). Interestingly, ‘Conference’ accumulated in
414 comparison to ‘Blanquilla’ higher amounts of linolenic acid (C18:3), a trienoic fatty acids having
415 three cis double bonds, which abundancy is frequently correlated with the maintaining a constant

fluidity of membranes and contributing to develop cold tolerance in higher plants (Hamada et al., 1998; Iba, 2002; Torres-Franklin et al., 2009), and likely reducing the scald susceptibility in this pear cultivar (Fig. 9a). In the same manner, the monounsaturated lipids, oleic acid+cis-vaccenic acid (C18:1) were also highly accumulated in the lovastatin treated samples (Fig. 9b), both in 'Conference' and to a lesser extent in 'Blanquilla'. The accumulation of this lipid was already observed in scald preventing mechanism stimulated by the application of 1-MCP in apple (Busatto et al., 2018), strengthening the hypothesis that despite the multiple differences between apples and pears regarding superficial scald (Busatto et al., 2018; Giné-Bordonaba et al., 2020; Larrigaudière et al., 2016) some physiological aspects may be sustained among both species.

Besides, the increased formation of ROS induced by cold stress can modulate the expression of various genes, including those encoding antioxidant enzymes (Suzuki et al., 2012). Among them, the transcriptional trend of three genes belonging to the ascorbate-glutathione pathway (*APX*, *DHAR* and *MDHAR*) was investigated. The ascorbate-glutathione pathway represents an essential component of the scavenging system for superoxide radicals and H_2O_2 in plants. It has been demonstrated that the overexpression of *APX* in tobacco induced the expression of both *DHAR* and *MDHAR*, increasing the cold tolerance (Wang et al., 2017). Recent studies on pears have indicated that changes in the expression level of *glutathione S-transferases* (*GSTs*) gene and mainly a downregulation of three genes encoding for *dehydroascorbate reductase* (*DHAR1, 2 and 4*) gene might participate in the development of superficial scald through regulating redox balance (Wang et al., 2018). In 'Conference' the expression level of *DHAR* and *MDHAR* did not change during the cold storage period or the shelf life (Fig. 3b, Suppl. Fig. 1), while in 'Blanquilla' a reduced transcription of both *DHAR* and *MDHAR*, with respect to the harvest was observed (Fig. 3a, Suppl Fig1). In this context, 'Conference' was characterized by a genetically higher antioxidant potential if compared to 'Blanquilla', likely conferring a better scald resistance.

441 4.2 Lovastatin and 1-MCP treatments have a different effect on the superficial scald onset and α-
442 farnesene production in ‘Blanquilla’ and ‘Conference’.
443
444 Lovastatin effectively prevented the scald development in ‘Blanquilla’ (Fig. 1a), promoting the
445 accumulation of ethanol during the shelf life period (Fig. 5f). Normally ethanol production is
446 associated with fermentation processes ongoing when fruit is stored under low-oxygen conditions
447 (Geigenberger, 2003) but it is also considered and efficient control agent of superficial scald in
448 apple (Ghahramani and Scott, 1998; Wang and Dilley, 2019, 2000) and pear (Larrigaudière et al.,
449 2019) as well as responsible for the induction of freezing tolerance in *Cucumis sativus* seedlings
450 (Frenkel and Erez, 1996).
451 In ‘Conference’, 1-MCP or lovastatin, were not capable to totally inhibit scald symptoms, even
452 though this cultivar generally displayed a much lower scald susceptibility (Fig. 1b). The cultivar
453 differential response to lovastatin detailed above was also observed at transcriptional level in the
454 regulation of *HMG2*, one of the rate limiting steps of the cytosolic mevalonate pathway for
455 isopentenyl diphosphate synthesis (Hedl and Rodwell, 2004), a compound involved in the synthesis
456 of α-farnesene (Liao et al., 2016). In ‘Blanquilla’ HMG2 was repressed both in 1-MCP and LOV
457 samples, whereas, in ‘Conference’ the expression level of *HMGR2* was lowered only in the 1-MCP
458 treated samples and not by lovastatin (Fig. 3b, Suppl Fig1). The activity of *AFS1*, the last
459 committed step devoted to the production of α-farnesene (Lurie et al., 2005), exhibited a
460 transcriptional pattern similar to *HMGR2*, in both cultivars. Moreover, the final quantification of α-
461 farnesene and 6-MHO production (Fig. 5e) showed a substantial decrease in both cultivars. The
462 discrepancy observed between the transcript profile and α-farnesene accumulation can be explained
463 by the mode of action of lovastatin. This compound physically bounds to the enzymes belonging to
464 the HMG-CoA reductase class (Hedl and Rodwell, 2004) regulating its activity at the protein level
465 and reducing the amount of available substrate used by *AFS1* for the synthesis of α-farnesene.
466 Therefore, the different transcriptional regulation of *HMG2* and *AFS1* in the two cultivars could be

467 explained by the complex tuning of the mevalonate pathway existing both in plants and animals
468 (Goldstein and Brown, 1990; Nakanishi et al., 1988). These findings suggested that the inhibition of
469 the functional HMGR2 protein could not be followed by a subsequent negative feedback in the
470 regulation of the gene expression, but instead by the continuation of the transcription in the attempt
471 to restore a more physiological condition in a cultivar specific manner.
472
473 4.3 Fruit ripening process and quality are not impaired by lovastatin treatment.
474 The residual effect of 1-MCP on the ripening recovery after cold storage (Chiriboga et al., 2011,
475 2013) is one of the major problems related to the use of this ethylene inhibitor when attempting to
476 increase the storability of pears (Busatto et al., 2017; Watkins, 2006).
477 The production of ethylene and the expression profiles of genes belonging to the ethylene domain
478 (*ACS*, *ACO*, *ERS1*, *ERS2*, *ERF1* and *ERF2*) were severely downregulated by the treatment with 1-
479 MCP in both cultivars (Fig 3a, 3b, Suppl. Fig. 1), leading to some extent to an impaired ripening, as
480 depicted by the transcription suppression of the group of genes related to fruit firmness and aroma
481 production (*PG1*, *ADH*, *AAT* and *HPL*). The impact of lovastatin on the aroma of the two cultivars
482 was much less dramatic than 1-MCP (Fig. 5a, 5b, 5c), enabling the production of aldehydes, esters
483 and alcohols, essential components of the aroma in pears (Busatto et al., 2019; El Hadi et al., 2013).
484 Likewise, the impact of lovastatin on ethylene and texture related genes was negligible and did not
485 interfere with the ripening progression (Fig. 3a, Suppl. Fig. 1b). Especially in 'Blanquilla', the
486 firmness values in the LOV samples were similar to the control, as also demonstrated by the
487 expression profile of *PG1*, one of the key gene involved in the softening process in European pears
488 (Hiwasa et al., 2004).
489 Among all the genes analyzed in this work, the auxin-regulated gene *AUX/IAA* was induced by all
490 the treatments (1-MCP, ET, LOV) during the shelf life in both cultivars. In apple this gene normally
491 decreases during late ripening (Busatto et al., 2017, 2016; Schaffer et al., 2013), but an increased
492 expression has been reported after treatment with 1-MCP. Surprisingly, not only 1-MCP but also

lovastatin and ethylene were able to induce the expression of this gene, thereby underlying the existence of differences between pear and apple ripening, despite their phylogenetic proximity.

5.0 CONCLUSION

The use of 1-MCP to prevent superficial scald development in pear, despite its effectiveness, may represent for some cultivars, undesirable side-effects such as the inability of the fruit to properly ripen after cold storage thereby reducing the general fruit quality. Lovastatin is well known to interfere with the mevalonate pathway, and therefore with the production of α-farnesene, a sesquiterpene thought to be involved in the superficial scald etiology. Our results suggest that lovastatin can be therefore considered as a valid alternative for the control of superficial scald in pear, while ensuring the completeness of ripening and the achievement of high-quality features such as firmness and volatile production. Moreover, the metabolite and transcriptional comparison between 'Blanquilla' and 'Conference' pears highlighted the complexity of the molecular basis contributing to the specific scald susceptibility among cultivars. Future studies are encouraged to define the genetic factors associated to superficial scald susceptibility, for instance by comprehensively investigating the allelism of the key genes assessed herein. The putative characterization of the alleles associated to the genetic resistant to scald, such as the one showed by 'Conference', could be exploited in future breeding program oriented to ameliorate postharvest losses in pears.

AKNOWLEDGEMENTS

This work was partially supported by the CERCA programme from the Generalitat de Catalunya and by the Ministerio de Economía y Competitividad (MINECO; grant AGL2017-87923-R). JGB was the recipient of a José Castillejo Mobility Fellowship (CAS18/00186).

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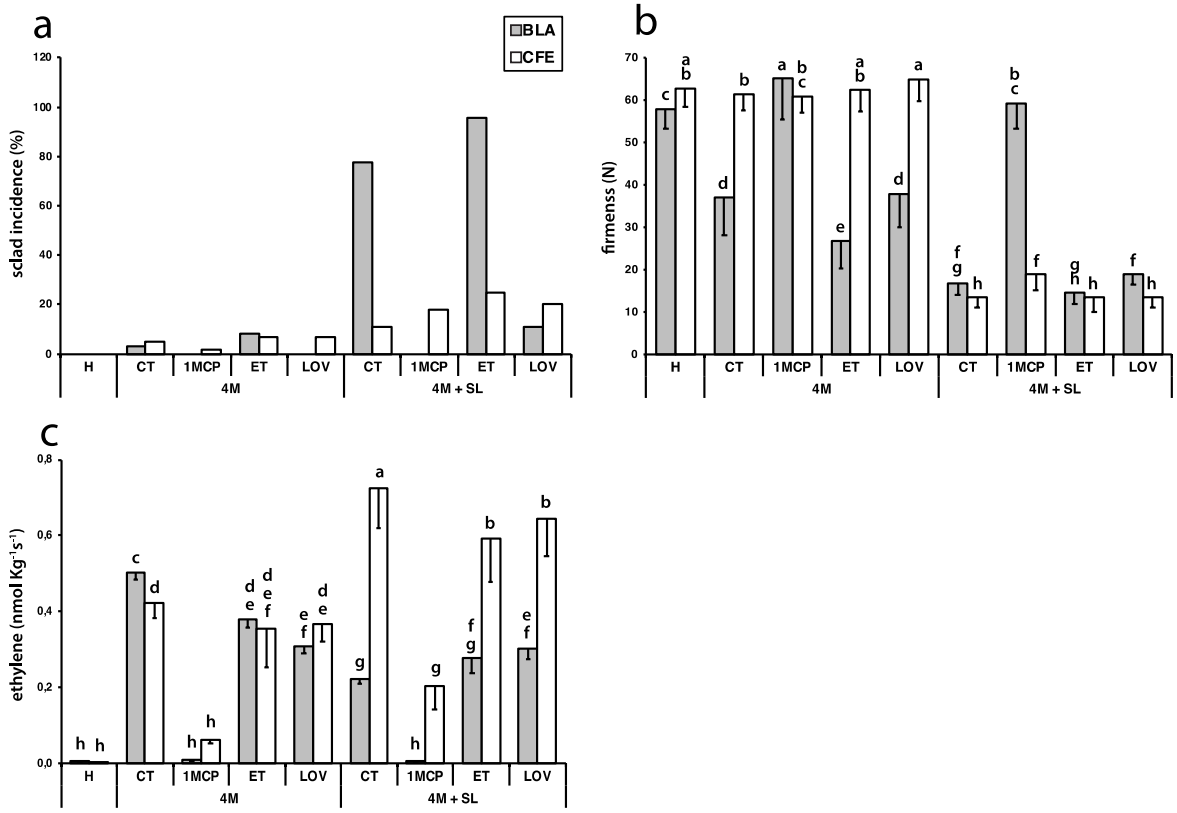
753 FIGURES



754

755 **Fig.1** Scald incidence (panel **a**) (% of affected fruit) in Blanquilla (BLA) and Conference (CFE)
756 pears, at harvest (H), and treated with 300 nL L^{-1} of the ethylene inhibitor 1-methylcyclopropene (1-
757 MCP), 1.25 mmol L^{-1} of the HMGR inhibitor lovastatin (LOV) or with 200 nL L^{-1} of exogenous
758 ethylene (ET), after 4 months of cold storage and after 4 months of cold storage (4M) plus 5 days of
759 ripening at 20ºC (4M+SL). Change in firmness and ethylene production are instead depicted in
760 panel **b** and **c**, respectively. Different letters above each column indicate significative differences
761 between treatments and cultivars for each specific sampling. Error bars depict the standard
762 deviation.

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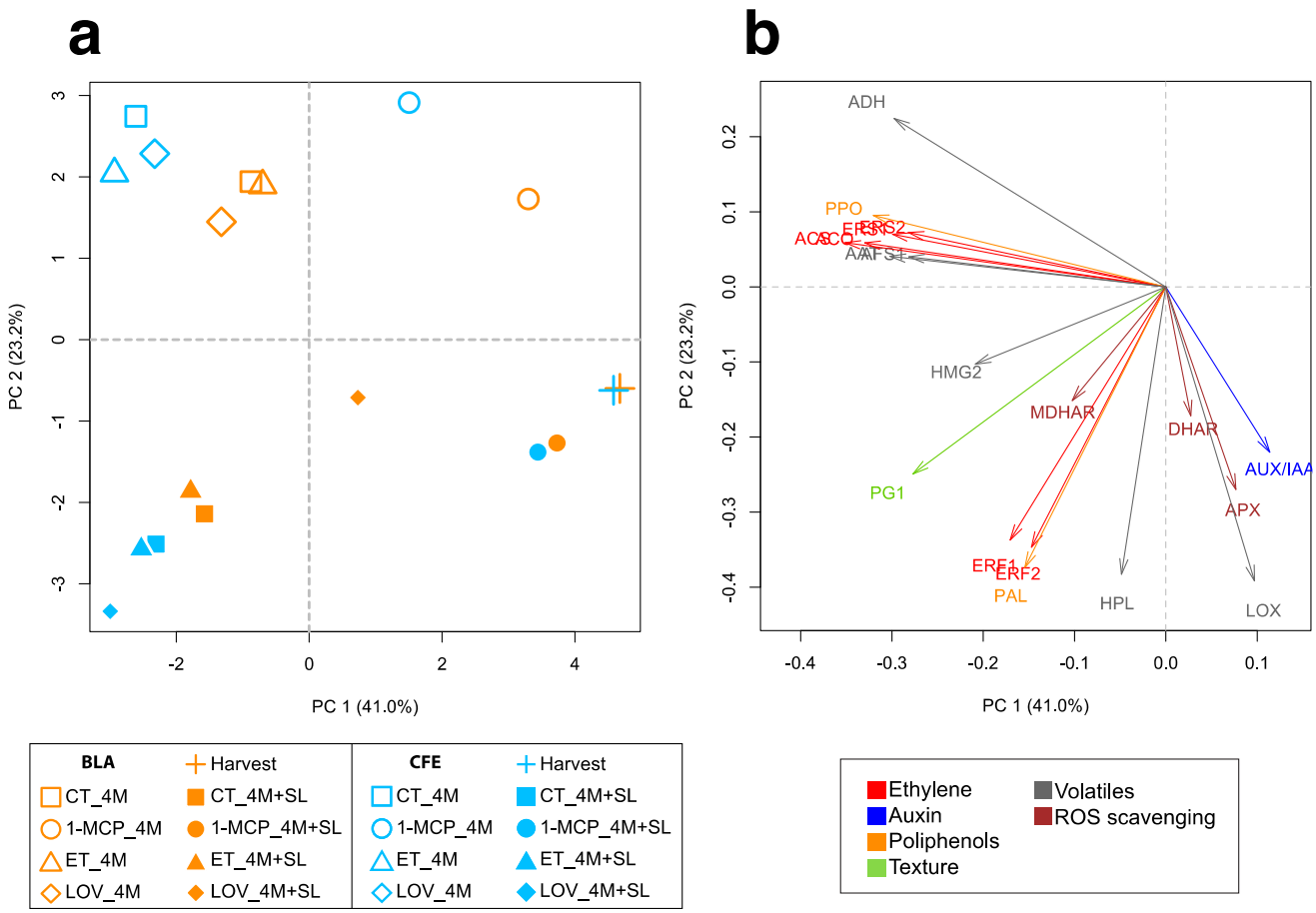

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770 **Fig.2** 2D-PCA plot depicting the whole variance among the different treatments based on their
771 transcriptomic profiles. On the left panel **(a)** each element represents a different batch of
772 'Blanquilla' (BLA, orange) and 'Conference' (CFE, blue) fruit treated with 1-methylcyclopropene
773 (1-MCP), lovastatin (LOV), ethylene (ET) or left untreated (CT) at harvest (H), after 4 months of
774 cold storage (4M) and after 4 months of cold storage plus 5 days of shelf life (4M+SL). On the right
775 panel **(b)** the corresponding loading plot where the variables employed for describing the total
776 variability are depicted. The profiled genes were groped in six different classes according to their
777 metabolic pathway, as shown in the inserted legend.

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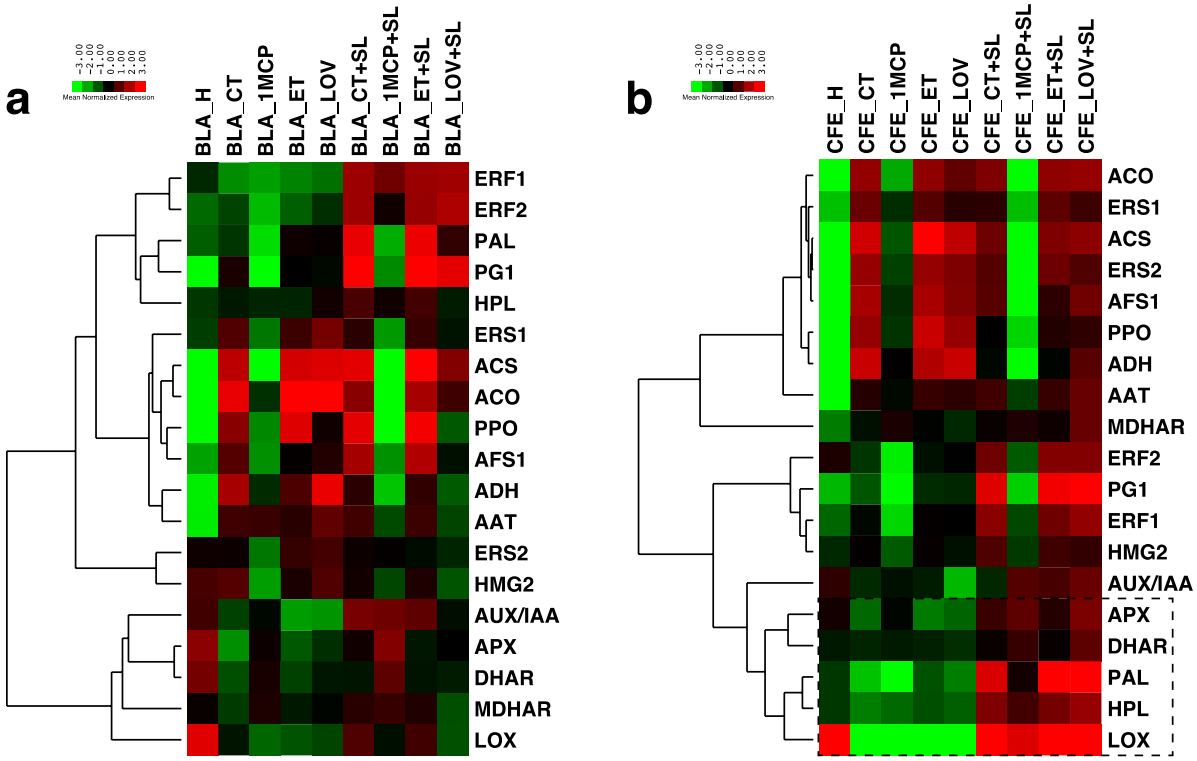


Fig.3 Hierarchical heat-map representing the gene expression level of each gene with regards to the effect of the 1 MCP, ethylene and lovastatin treatments in the two cultivars: 'Blanquilla' -BLA- (panel **a**) and 'Conference' -CFE- (panel **b**). The color pattern indicates the level of the Mean Normalized Gene Expression with green and red for low and high values, respectively. The dashed frame highlights the genes specifically modulated in 'Conference'. The description of each gene can be found in the Suppl. Table1.

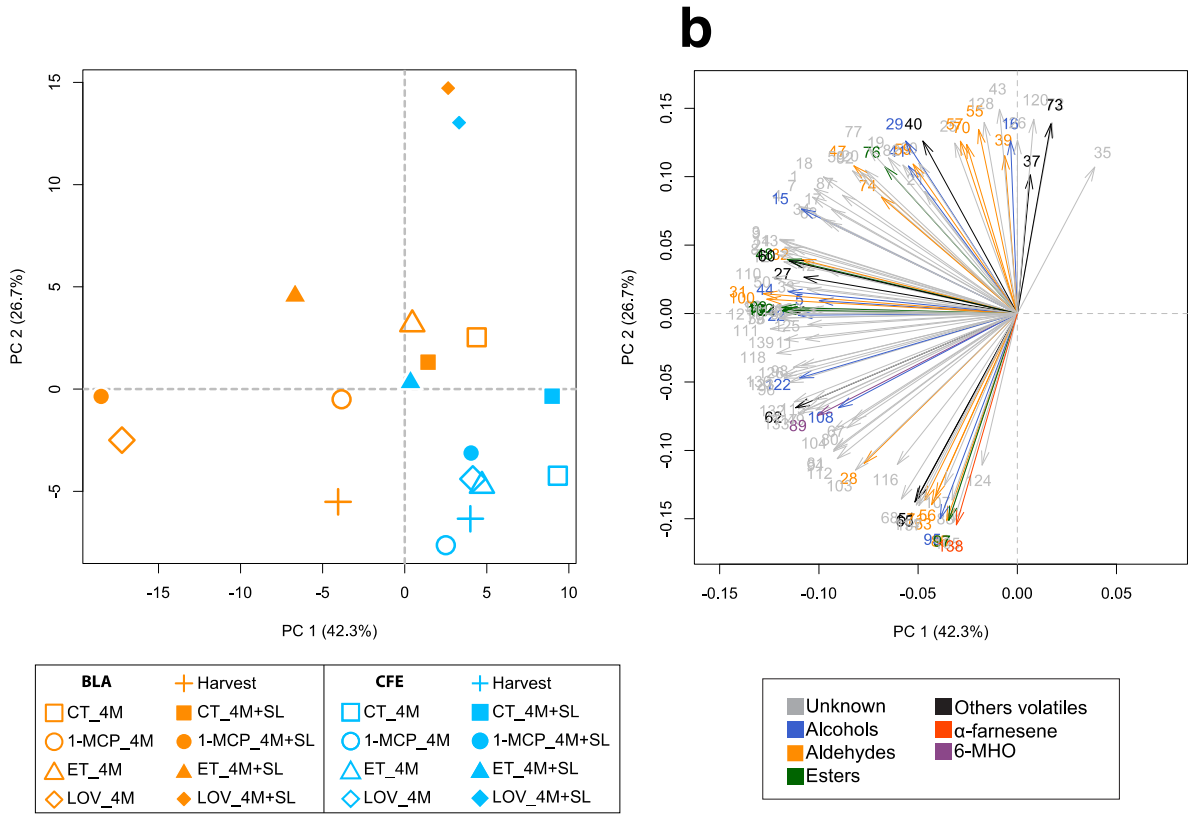


Fig. 4 2D-PCA plot depicting the whole variance among the different treatments based on the volatile production. On the left panel **(a)** each element represents a different batch of 'Blanquilla' (BLA, orange) and 'Conference' (CFE, blue) fruit treated with 1-methylcyclopropene (1-MCP), lovastatin (LOV), ethylene (ET) or left untreated (CT) at harvest (H), after 4 months of cold storage (4M) and after 4 months of cold storage plus 5 days of shelf life (4M+SL). On the right panel **(b)** the corresponding loading plot where is visualized the variables employed for describing the total variability showed in the panel a. The profiled volatiles were grouped in seven different classes, as shown in the inserted figure legend.

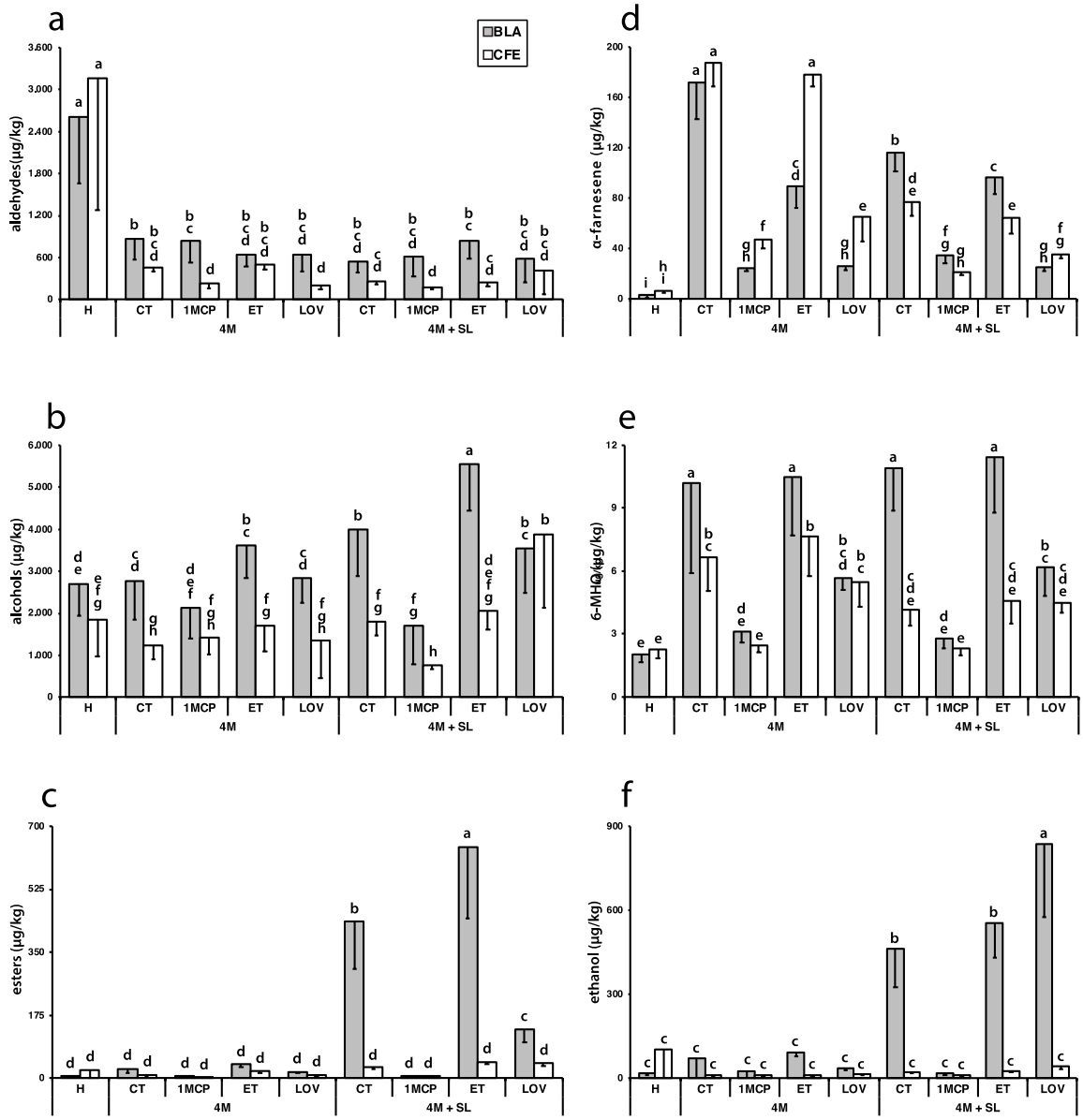


Fig.5 Accumulation of aldehydes **(a)**, alcohols **(b)** and esters **(c)** (as categorized in Suppl. Table 2), *α*-farnesene **(d)**, 6-methyl-5-hepten-2-one (6-MHO) **(e)** and ethanol **(f)** in 'Blanquilla' (BLA) and 'Conference' (CFE), in grey and white, respectively. Different letters above each column indicate significative differences between treatments and genotypes for each specific. Error bars depict the standard deviations (N=6).

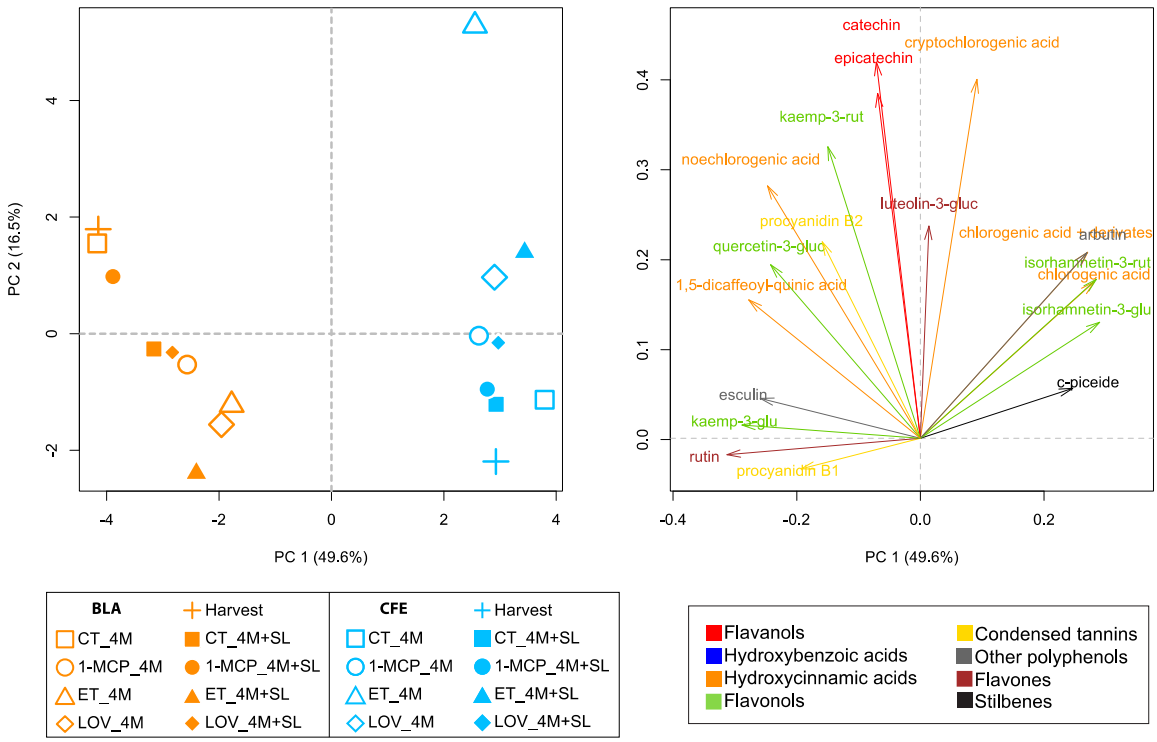
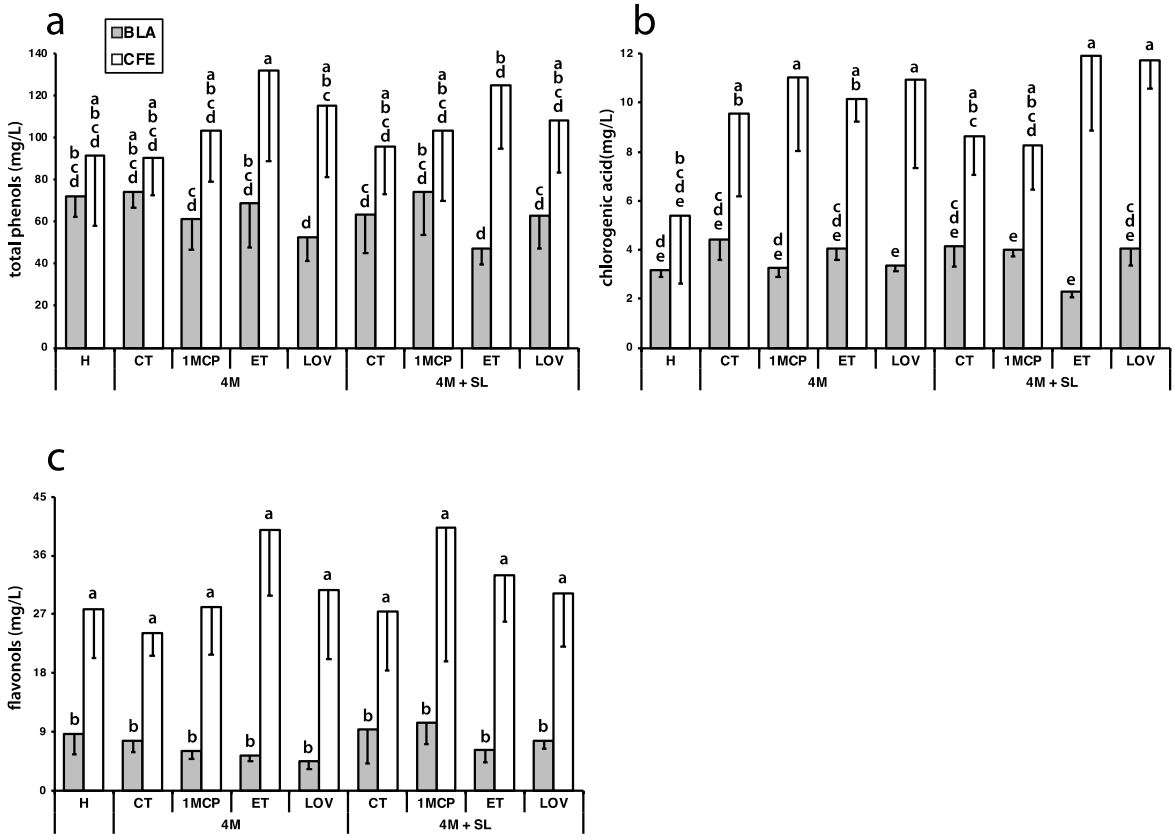


Fig. 6 2D-PCA plot depicting the whole variance among the different conditions and based on the polyphenol accumulation. On the left panel **(a)** each element represents a different batch of 'Blanquilla' (BLA, orange) and 'Conference' (CFE, blue) fruit treated with 1-methylcyclopropene (1-MCP), lovastatin (LOV), ethylene (ET) or left untreated (CT) at harvest (H), after 4 months of cold storage (4M) after 4 months of cold storage plus and 5 days of shelf life (4M+SL). On the right panel **(b)** the corresponding loading plot where is visualized the variables employed for describing the total variability showed in the left panel is shown.



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836 **Fig.7** Total phenol content **(a)**, chlorogenic acid **(b)** and flavonols profile **(c)** in 'Blanquilla' (BLA)
837 and 'Conference' (CFE), gray and white bars, respectively, across all the samples included in the
838 experimental design. Different letters above each column indicate significative differences. Error
839 bars depict the standard deviations (N=3).

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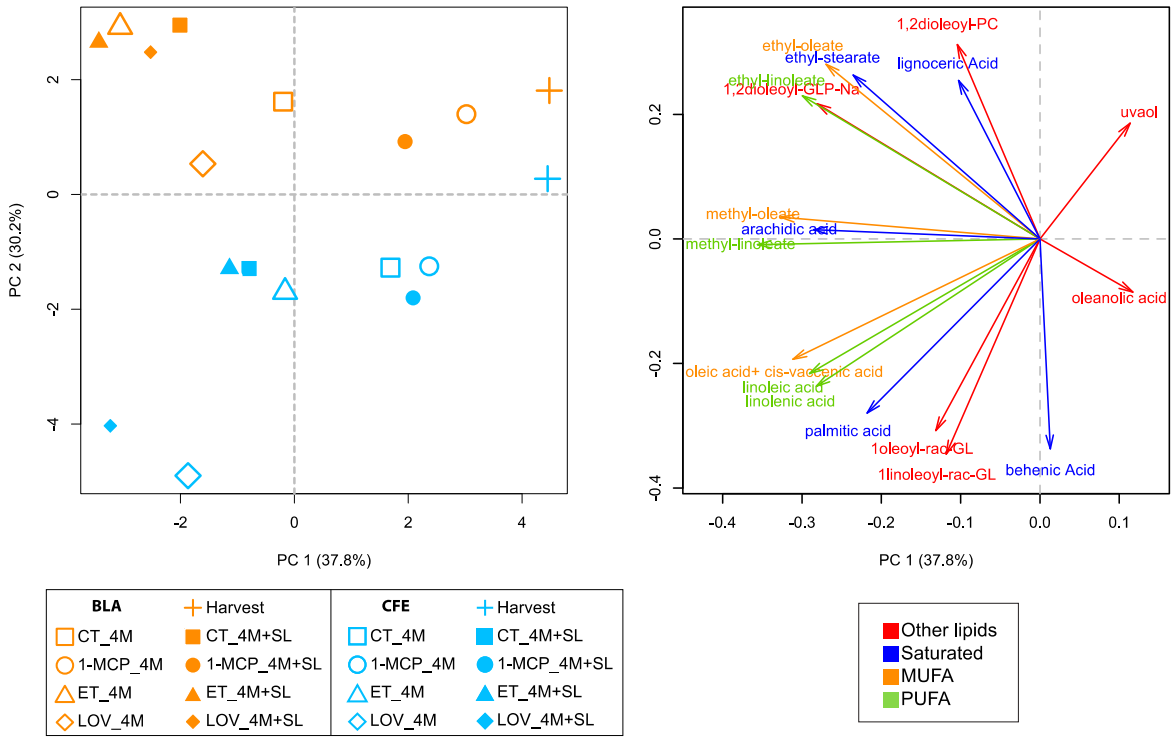
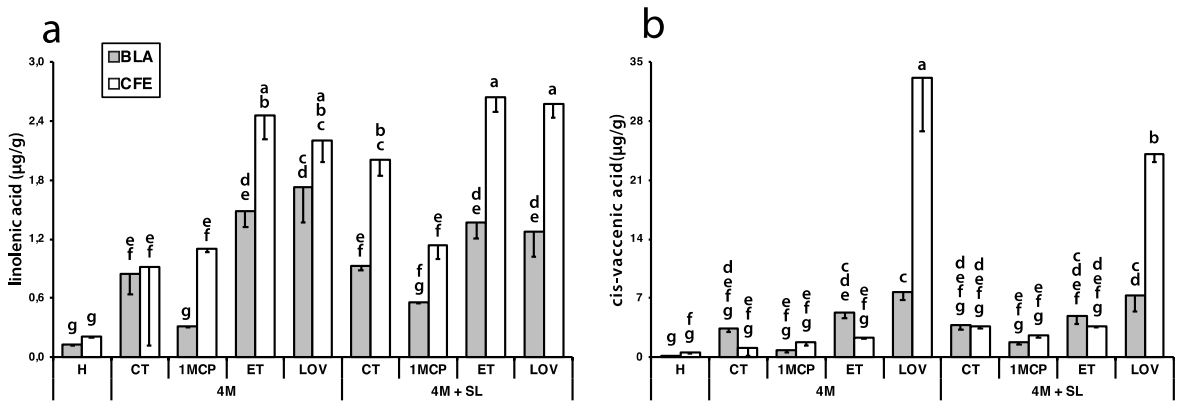


Fig. 8 2D-PCA plot depicting the whole variance among treatments and genotypes, based on the lipid profiling. On the left panel **(a)** each element represents a different batch of 'Blanquilla' (BLA, orange) and 'Conference' (CFE, blue) fruit treated with 1-methylcyclopropene (1-MCP), lovastatin (LOV), ethylene (ET) or untreated (CT) at harvest (H), after 4 months of cold storage (4M) and after 4 months of cold storage plus 5 days of shelf life (4M+SL). On the right panel **(b)** the corresponding loading plot where is visualized the variables employed for describing the total variability showed in the left panel is shown, in this case categorized according the level of unsaturation.



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863 **Fig.9** Accumulation profiles of linolenic acid **(a)** and oleic acid + cis-vaccenic acid **(b)** in
864 'Blanquilla' (BLA) and 'Conference' (CFE) depicted with gray and white bars, respectively, across
865 all the samples included in the experimental design. Different letters above each column indicate
866 significative differences between treatments and genotypes for each specific sampling. Error bars
867 depict the standard deviations (N=3).