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 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[7] 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[7], the mechanism related to the etiological cause leading to the scald development 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 superficial scald onset 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 (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 specific 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 g^{-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 work 2011(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 an ultra-high-performance liquid chromatography (UHPLC) Dionex 3000 (Thermo Fischer

Scientific Germany), with a RP Ascentis Express column (15 cm 9 2.1 mm; 2.7 lm C18) applying 30-min of multistep linear gradient. The UHPL chromatographic system was coupled to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an ESI source. Lipids were identified based on reference standards and retention time, and further quantified as µg/g of fresh weight.

2.5 Profiling of phenolic compounds

The analysis of phenols followed the protocol described in Vrhovsek et al. (Vrhovsek et al., 2012) with a simplified sample extraction (Giné-Bordonaba et al., 2019), and using three biological replicates. For this assessment a Waters Acquity UPLC system (Milford, MA, USA) coupled to a Waters Xevo TQMS mass spectrometer (Milford, MA, USA) was employed. The capillary voltage was 3.5 kV in the positive mode and −2.5 kV in the negative mode. Each compound was analyzed under the optimized MRM conditions (precursor and product ions, quantifiers and qualifiers, collision energies, and cone voltages) as described (Vrhovsek et al., 2012). Waters MassLynx 4.1 and TargetLynx software were used to process the phenolic data and each phenolic compound was characterized on the base of reference compounds and expressed as mg/kg of fresh weight.

2.5 Gene expression profiling by RT-qPCR

The peel from 5 fruit per replicate and per each treatment and sampling point was isolated, immediately frozen with liquid nitrogen, grinded into a fine powder, and finally stored at -80°C until processing. RNA extraction was carried out using Spectrum Plant total RNA kit (Sigma-Aldrich Co., St Luis, MO, USA). The RNA, extracted by two biological replicates (of five fruit each), was quantified and assessed with a NanoDrop ND-8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For each sample, 1 µg of total RNA was treated with 1 Unit of 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 realized 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 (α = 0.05) has been performed using the software R in order to indicate significative differences between treatments and genotypes for each specific sampling.

3.0 RESULTS

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

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

In order to verify the impact of the different treatments on fruit quality and ripening progression, fruit firmness (Fig. 1b), titratable acidity (TTA - Suppl. Table2) and soluble solid content (SSC – Suppl. Table2) were measured. TTA and SSC did not show any significative variation among treatments for any of the cultivars investigated. On the contrary, a completely different behavior was observed for the fruit firmness~~ when comparing ‘Blanquilla’ and ‘Conference’ pears~~. In ‘Blanquilla’ an important firmness loss occurred in all samples, except for 1-MCP treated fruit during (1.76-fold) and after (3.52-fold) cold-storage. ~~1-MCP treated ‘Blanquilla’ pears remained firm even after 5 days of shelf-life and reached firmness for consumption only after 10 days of shelf-life (data not shown).~~ In contrast, ‘Conference’ pears did not ~~experience~~ show any firmness loss during cold storage, but it~~firmness~~ sharply declined~~, for all treatments,~~ as the fruit were moved ~~ripened~~ at 20ºC shelf-life (80% of firmness loss; Fig. 1b). Slightly yet significantly higher firmness values were observed for 1-MCP treated ‘Conference’ pears after 4 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 ~~time~~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 ~~dramatic~~ effect on the fruit ethylene production ~~pattern~~ (Fig. 1c)~~. The production of ethylene changed only slightly in fruit treated with the exogenous ethylene, when compared to control~~.

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~~ (*ACS, ACO, ERS1, ERS2, ERF1* and *ERF2*)~~, auxin signaling~~ (*AUX/IAA*,)~~, polyphenol biosynthesis and oxidation~~ (*PAL, PPO*)~~, volatile biosynthesis~~ (*AAT, HPL, LOX, HMGR* and *AFS1*)~~, ROS scavenging ~~(*APX, DHAR* and *MDHAR*)~~ and cell wall disassembling ~~(*PG1*)~~ (Suppl. Table1). ~~This set of genes was selected according to previous work describing the physiological changes occurring during ‘Abate Fetel’ pears ripening (Busatto et al., 2019) and during the superficial scald onset in pear (Giné-Bordonaba et al., 2020; Lindo-García et al., 2020a).~~

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 (~~after 4 months of~~ cold-storage and ~~further~~ shelf-life). The different treatments ~~applied~~ 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 ~~biosynthesis and perception~~ 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 ~~On the other hand,~~ the gene regulation observed in the samples treated with ethylene or lovastatin was similar to that observed in untreated fruit, ~~. Nevertheless,~~ lovastatin had a significant effect on repressing the ~~a reduced set of~~ genes ~~known to be~~ 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. ~~The different transcriptional response to the postharvest treatments between ‘Blanquilla’ and ‘Conference’ was illustrated in the hierarchical clustering shown in Fig. 3 (a and b), showing that the effect of each compound, although being visible already after 4 months of cold storage, was further magnified during shelf-life.~~

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

~~The effect of 1-MCP, ethylene and lovastatin application on the pear volatilome after cold storage and further shelf-life was assessed by using a PTR-MS-TOF instrument.~~ The detection of 139 VOC mass peaks enabled a clear distinction of the samples over the 2D-PCA space (Fig. 4a)~~ defined by the first two principal components PC1 and PC2, accounting together for 69% of the total variance~~. ~~The role of the genetic background in determining pear VOC profile was clearly evident.~~ 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 acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate, ethyl acetate, butanoic acid hexyl ester and alcohols (hexanol, 1-butanol, ethanol) (Suppl. Table 2).

~~On the other hand,~~The PC2 values efficiently depicted the influence of the different treatments and storage stages for both cultivars. Among the most relevant loadings, ~~characterizing PC2~~ it is worthwhile to mention α-farnesene together with some aldehydes, such as nonenal, 2-heptenal, octanal, 2,4-hexadienal, heptanal, heptadienal, butenal, hexenal and 2-methyl butanal (Fig. 4b). Within the distribution of the samples based on the volatilome variability, it is interesting also to note that the harvest samples for the two varieties were closely plotted, ~~over the PCA plot~~ and the distinction between cultivars based on their volatile profile only occurred after postharvest storage. Samples from ‘Blanquilla’ collected at shelf-life were characterized by the highest VOC production. The volatilome was also ethylene-related. Treatment with 1-MCP lower down the production of VOCs, while ~~Moreover, samples distinguished by a high production of ethylene (control and ethylene treated) also showed a more important production of VOCs. In contrast,~~ samples treated with lovastatin showed an intermediate production of aromatic compounds.~~were positioned between the control/ethylene treated samples and 1-MCP treated samples the latter being characterized by the lowest production of VOCs.~~ For aldehydes, a general decreased after harvest was observed for both cvs (Fig.5a), with a slightly higher accumulation in ‘Blanquilla’ than in ‘Conference’ and showing ~~unnoticeable~~ imperceptible changes in response to the different treatments. Also, for alcohols and esters, the accumulation was higher in ‘Blanquilla’ than in ‘Conference’, which showed a significant higher accumulation in control and ethylene treated samples after shelf-life (Fig. 5b and 5c). Particularly interesting was the accumulation of ethanol in lovastatin-treated ‘Blanquilla’ pears, showing 1,8-fold higher values than untreated fruit. α-farnesene content was greater in control and ethylene treated samples and strongly inhibited by both1-MCP and lovastatin in both cultivars (Fig. 5d). Likewise, the accumulation of 6-MHO was higher in control and ethylene treated ‘Blanquilla’ samples and severely reduced by 1-MCP or to a lesser extent also by lovastatin (Fig. 5e). Especially for the control and ethylene treated samples, the ~~cultivar differential~~ accumulation of 6-MHO in ~~after a shelf-life period was evident, with~~ ‘Blanquilla’ was ~~showing higher (~~2,56-fold (in average) higher ~~amount of this volatile compound~~ than ‘Conference’.

3.4 Changes in the phenolic compounds and lipids induced by treatments with lovastatin, 1-MCP 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 ~~(accounting for 49.6% of the total variability)~~, whereas 'Conference' fruit were characterized by positive PC1 values. The effect of shelf life and treatments was instead ~~more accurately~~ represented by the PC2 (16.5% of the total variability). ~~The distinction of the two varieties was related to a specific accumulation of different phenolic compounds through cold storage and further shelf life, as depicted in the variable projection plot given in Fig. 6b.~~

'Conference' showed a higher accumulation of polyphenols in all the conditions analyzed~~ in this work~~, 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 ~~Similarly, to that done for phenolic compounds, a 2D PCA plot~~ (~~accounting for 68% of the total variability,~~ Fig. 8a)~~ was also used to elucidate the differentiation among samples based on their lipid profile~~. The two pear cultivars were distinguished along the PC2 axes~~ (accounting for 30.2% of the captured variability)~~, 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 ~~(accounting for the 37.8% of the total variance)~~ 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 temperatures. 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* [35]. 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 unclear. Not only flavonoids but the total amount of phenolic compounds was overall greater in ‘Conference’ than in ‘Blanquilla’ (Fig. 7a), and especially for chlorogenic acid (Fig. 7b). Previous studies have shown that the accumulation of chlorogenic acid is correlated to the superficial scald onset (Busatto et al., 2014), a result that cannot be confirmed in our study since ‘Conference’ pears own higher content of this compound but displayed very limited scald symptoms. Discrepancies between this and previous studies[8] might be explained by the different expression of the *PPO* gene deputed to encode for a protein responsible for the oxidation of this hydroxycinnamic acid finally leading to the peel browning characteristics of superficial scald. While in ‘Blanquilla’ PPO was highly expressed during the stage where superficial scald was boosted (shelf-life after postharvest cold storage), in ‘Conference’ the expression of this gene was severely down-regulated (Fig. 3a and Supp. Fig. 1). This result suggested a different genetic regulation of the *PPO* gene among pear cultivars that warrants further investigation.

Besides phenolic compounds, the role of cis-vaccenic acid in enhancing cold resistance has been demonstrated in several plant species, as for example in *Solanum lycopersicum* transgenic lines, where the overexpression of cis-vaccenic acid induced an improved tolerance to freezing temperatures (Badea and Basu, 2009; De Palma et al., 2008). The cold tolerance mechanism is also regulated by the integrity of the internal lipidic membrane that during cold tolerance can progressively loose permeability, with a consequent ion leaking coupled to the production of reactive oxygen species. ROS can contribute to the peroxidation of lipids(Marangoni et al., 1996), causing a loss of unsaturated fatty acids with an increased membrane rigidity due to the formation of covalent bonds among lipid radicals(Alonso et al., 1997; Hara et al., 2003). The increase of the unsaturated/saturated fatty acid ratio acid represents one of the key factor determining the temperature at which the internal membrane changes from gel to liquid crystalline phase (Badea and Basu, 2009; Browse, 2010; Marangoni et al., 1996). Interestingly, ‘Conference’ accumulated linolenic acid (C18:3), a trienoic fatty acids having three cis double bonds, which abundancy is frequently correlated to the growth at low temperatures, 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), showing a profile that was also detected in ‘Blanquilla’, although less clearly. 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.

In addition, 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.

4.2 Lovastatin and 1-MCP treatments have a different effect on the superficial scald onset and α-farnesene production in ‘Blanquilla’ and ‘Conference’.

Lovastatin effectively prevented the scald development in ‘Blanquilla’, promoting the accumulation of ethanol during the shelf life period (Fig. 5f). Normally ethanol production is associated with fermentation processes ongoing when fruit is stored under low-oxygen conditions (Geigenberger, 2003) but it is also considered and efficient control agent of superficial scald in apple (Ghahramani and Scott, 1998; Wang and Dilley, 2019, 2000) and pear (Larrigaudière et al., 2019) as well as responsible for the induction of freezing tolerance in *Cucumis sativus* seedlings (Frenkel and Erez, 1996) .

Moreover, the treatment with lovastatin was effective in reducing the superficial scald incidence in ‘Blanquilla’, albeit its efficacy was slightly lower than that observed for 1-MCP (Fig. 1a). In ‘Conference’, 1-MCP as well as lovastatin, was not capable to totally inhibit scald symptoms, even though this cultivar generally displayed a much lower scald susceptibility (Fig, 1b). This response to lovastatin was also observed at transcriptional level in the regulation of *HMG2,* one of the rate limiting steps of the cytosolic mevalonate pathway for isopentenyl diphosphate synthesis (Hedl and Rodwell, 2004), a compound involved in the synthesis of α-farnesene (Liao et al., 2016). In ‘Blanquilla’ HMG2 was repressed both in 1-MCP and LOV samples. On the contrary, in ‘Conference’ the expression level of *HMGR2* was lowered only in the 1-MCP treated samples and not by lovastatin (Fig. 3b, Suppl Fig1). The activity of *AFS1*, the last committed step devoted to the production of α-farnesene (Lurie et al., 2005), exhibited a transcriptional pattern similar to *HMGR2*, in both cultivars. Moreover, the final quantification of α-farnesene and 6-MHO production (Fig. 5e) showed a substantial decrease in both cultivars. The discrepancy observed between the transcript profile and α-farnesene accumulation can be explained by the mode of action of lovastatin. This compound physically bounds to the enzymes belonging to the HMG-CoA reductase class (Hedl and Rodwell, 2004) regulating its activity at the protein level and reducing the amount of available substrate used by *AFS1* for the synthesis of α-farnesene. Therefore, the different transcriptional regulation of *HMG2* and *AFS1* in the two cultivars could be explained by the complex tuning of the mevalonate pathway existing both in plants and animals (Goldstein and Brown, 1990). For example, in cultured animal cells, an eightfold increase in reductase mRNA has been reported after treatment with compactin, a lovastatin analog, with a reduction of the enzyme accumulation attributable to the decline in translation of the mRNA (Goldstein and Brown, 1990; Nakanishi et al., 1988). These findings suggested that the inhibition of the functional HMGR2 protein could not be followed by a subsequent negative feedback in the regulation of the gene expression, but instead by the continuation of the transcription in the attempt to restore a more physiological condition in a cultivar specific manner.

4.3 Fruit ripening process and quality are not impaired by lovastatin treatment.

The residual effect of 1-MCP on the ripening recovery after cold storage (Chiriboga et al., 2011, 2013) is one of the major problems related to the use of this ethylene inhibitor when attempting to increase the storability of pears (Busatto et al., 2017; Watkins, 2006).

The production of ethylene and the expression profiles of genes belonging to the ethylene domain (*ACS*, *ACO*, *ERS1*, *ERS2*, *ERF1* and *ERF2*) were severely downregulated by the treatment with 1-MCP in both cultivars (Fig 3a, 3b, Suppl. Fig. 1), leading to some extent to an impaired ripening, as depicted by the transcription suppression of the group of genes related to fruit firmness and aroma production (*PG1*, *ADH*, *AAT* and *HPL*). The impact of lovastatin on the aroma of the two cultivars was much less dramatic then 1-MCP (Fig. 5a, 5b, 5c), enabling the production of aldehydes, esters and alcohols, essential components of the aroma in pears (Busatto et al., 2019; El Hadi et al., 2013).

Likewise, the impact of lovastatin on ethylene and texture related genes was negligible and did not interfere with the ripening progression (Fig. 3a, Suppl. Fig. 1b). Especially in ‘Blanquilla’, the firmness values in the LOV samples were similar to the control, without any relevant difference, as also demonstrated by the expression profile of *PG1*, one of the key gene involved in the softening process in European pears (Hiwasa et al., 2004).

Among all the genes analyzed in this work, the auxin-regulated gene *AUX/IAA* was induced by all the treatments (1-MCP, ET, LOV) during the shelf life in both cultivars. In apple this gene normally decreases during late ripening (Busatto et al., 2017, 2016; Schaffer et al., 2013), but an increased expression was observed after treatment with 1-MCP. Surprisingly also in pear, 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 ‘Conferece’ highlighted the molecular basis contributing to the specific scald susceptibility that characterizes these two 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.

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FIGURE LEGENDS

Fig.1 Scald incidence (panel **a**) (% of affected fruit) in Blanquilla (BLA) and Conference (CFE) pears, at harvest (H), and treated with 300 nL L^{-1} of the ethylene inhibitor 1-methylcyclopropene (1-MCP), 1.25 mmol/L of the HMGR inhibitor lovastatin (LOV) or with 200 nL L^{-1} of exogenous ethylene (ET), after 4 months of cold storage and after 4 months of cold storage (4M) plus 5 days of ripening at 20ºC (SL). Change in firmness and ethylene production are instead depicted in panel **b** and **c**, respectively. Different letters above each column indicate significative differences between treatments and cultivars for each specific sample. Standard deviations are represented with vertical lines for each value (N=6).

Fig.2 2D-PCA plot depicting the whole variance among the different treatments based on their transcriptomic profiles. 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 5 days of shelf life (4M+SL). On the right panel **(b)** the corresponding loading plot where the variables employed for describing the total variability are depicted. The profiled genes were groped in six different classes according to their metabolic pathway, as shown in the legend.

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 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 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 gray and white, respectively. Different letters above each column indicate significative differences between treatments and genotypes for each specific. Standard deviations are represented for each value (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) 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.

Fig.7 Total phenol content **(a)**, chlorogenic acid **(b)** and flavonols profile **(c)** in ‘Blanquilla’ (BLA) and ‘Conference’ (CFE), gray and white bars, respectively, across all the samples included in the experimental design. Different letters above each column indicate significative differences. Standard deviations are represented for each value (N=3).

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 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, categorized according the level of unsaturation.

Fig.9 Accumulation profiles of linolenic acid **(a)** and oleic acid + cis-vaccenic acid **(b)** in ‘Blanquilla’ (BLA) and ‘Conference’ (CFE) depicted with gray and white bars, respectively, across all the samples included in the experimental design. Different letters above each column indicate significative differences between treatments and genotypes for each specific sampling. Standard deviations are represented for each value (N=3).