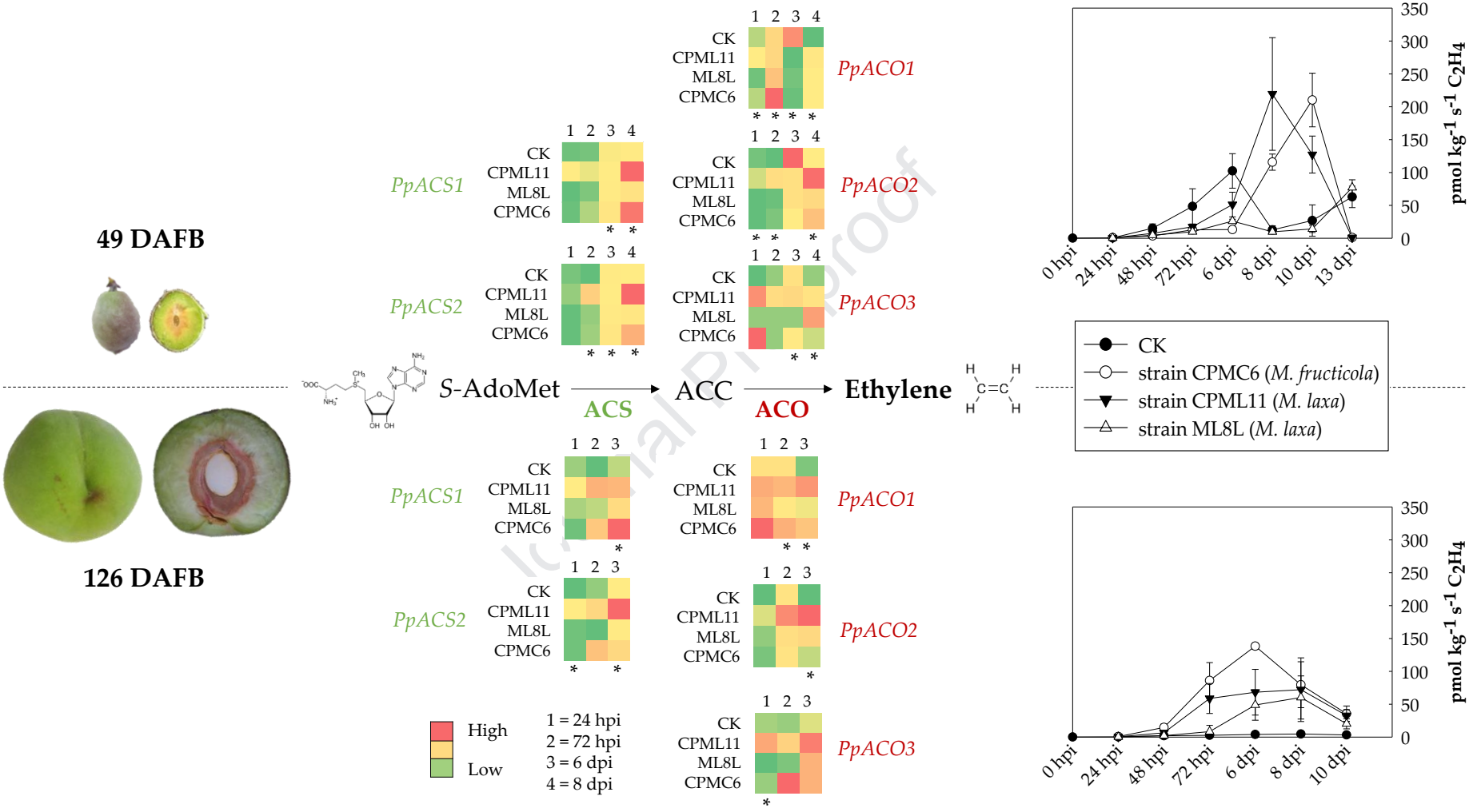

Double-sided battle: the role of ethylene during *Monilinia* spp. infection in peach at different phenological stages

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Abstract

Controversy exists on whether ethylene is involved in determining fruit resistance or susceptibility against biotic stress. In this work, the hypothesis that ethylene biosynthesis in peaches at different phenological stages may be modulated by *Monilinia* spp. was tested. To achieve this, at 49 and 126 d after full bloom (DAFB), ethylene biosynthesis of healthy and infected 'Merryl O'Henry' peaches with three strains of *Monilinia* spp. (*M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L) that differ in terms of aggressiveness) was analysed at the biochemical and molecular level along the course of infection in fruit stored at 20 °C. At 49 DAFB, results evidenced that infected fruit showed inhibition of ethylene production in comparison with non-inoculated fruit, suggesting that the three *Monilinia* strains were somehow suppressing ethylene biosynthesis to modify fruit defences to successfully infect the host. On the contrary, at 126 DAFB ethylene production increased concomitantly with brown rot spread, and values for non-inoculated fruit were almost undetectable throughout storage at 20 °C. The expression of several target genes involved in the ethylene biosynthetic pathway confirmed that they were differentially expressed upon *Monilinia* infection, pointing to a strain-dependent regulation. Notably, *Prunus persica* 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (*PpACS*) family was the most over-expressed over time, demonstrating a positive ethylene regulation, especially at 126 DAFB. At this phenological stage it was demonstrated the ability of *Monilinia* spp. to alter ethylene biosynthesis through *PpACS1* and benefit from the consequences of an ethylene burst likely on cell wall softening. Overall, our results put forward that infection not only among different strains but also at each stage is achieved by different mechanisms, with ethylene being a key factor in determining peach resistance or susceptibility to brown rot.

Keywords: 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), brown rot, gene expression analysis, host-pathogen interaction, *Prunus persica*.

1. Introduction

Brown rot caused by *Monilinia* spp. have attained great importance worldwide as the pathogen have been disseminated and is responsible of enormous economic losses in postharvest of stone fruit. Additionally, the management of this disease is facing obstacles due to the emerging fungicide resistance and the growing public concerns over fungicide usage. In this context, the irruption of "omics" has prompted a renewed interest in molecular genetic approaches to study fruit-pathogen interactions from a global point of view which, in turn, resulted in important advances towards searching new control strategies (Tian et al., 2016). In particular, for brown rot, both the host (peach) (Verde et al., 2013) and the pathogen (*Monilinia* spp.) (Landi et al., 2018; Naranjo-Ortíz et al., 2018; Rivera et al., 2018) genomes are currently available. As a result, the process of understanding the pathogen's virulence factors and the fruit resistance/susceptibility mechanisms is now becoming more feasible.

Using functional genomics, many research groups are highlighting the potential that studying the host immune system can have in disease protection (reviewed in Pétriacq et al., 2018). Plants are in continuous exposure to various forms of biotic stresses such as insects and pathogens. In response, they express numerous constitutive and induced defence mechanisms (reviewed in Pandey et al., 2016). Once constitutive mechanisms (i.e., structural or physical barriers) have been trespassed by the pathogen, inducible

defence mechanisms become responsible for halting pathogen progress. These mechanisms involve responses that rely on a network of cross-communicating signalling pathways of which salicylic acid, jasmonic acid and ethylene are the principal mediators in plants (De Vos et al., 2005). Besides, jasmonic acid and ethylene are considered to play pivotal roles in regulating the plant response towards necrotrophic fungal infection (Glazebrook, 2005; Pandey et al., 2016). Specifically, for *M. laxa* further evidence was provided from the dramatic changes in the expression of phenylpropanoid and jasmonate-related genes obtained by microarray analysis of susceptible (two weeks before pit hardening) and resistant (pit hardening) phases (Guidarelli et al., 2014). Both the phenylpropanoid and jasmonate pathways are ethylene-dependent (Broekgaarden et al., 2015; Ecker and Davis, 1987; Wang et al., 2002). Ethylene is a simple gaseous hydrocarbon first discovered for its role in fruit maturation, senescence, germination and flowering (Bleecker and Kende, 2000; Payton et al., 1996), but it was later shown to also function as a modulator of the plant immune signalling network (reviewed in van Loon et al., 2006).

The biosynthesis of ethylene consists of two enzymatic steps: a first level of regulation occurs by the action of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), followed by the oxidative cleavage of ACC by ACC oxidase (ACO) forming ethylene (Wang et al., 2002). In most instances, ACS may act as the rate-limiting step in ethylene biosynthesis, however, in conditions of high ethylene production, such as in ripening fruit, ACO is often the limiting factor (Argueso et al., 2007). Both ACS and ACO are encoded by multigene families, which are differentially expressed during fruit development and ripening (Wang et al., 2002). To date, many studies have focused on ethylene biosynthesis in peach, gaining insight into the regulation of peach ripening and the elements related to ethylene signal transduction

(Basset et al., 2002; Hayama et al., 2006; Rasori et al., 2002; Tadiello et al., 2016; Wang et al., 2017). However, no studies have tried to explore whether the different genes coding for the two enzymes involved in the conversion of *S*-adenosyl-methionine (*S*-AdoMet) to ethylene show a specific expression profile upon infection in the *Monilinia* spp.-stone fruit pathosystem. Noteworthy, studies aimed to elucidate the role of ethylene in determining the outcome of plant-pathogen interactions in other pathosystems (i.e., *Botrytis cinerea*-tomato (Blanco-Ulate et al., 2013); *Penicillium digitatum*-citrus (Ballester et al., 2011; Marcos et al., 2005); *Penicillium* spp.-apples (Vilanova et al., 2017)), have provided evidence on the dual role that this hormone can play on the fruit-pathogen interactions. So far, a work recently conducted by Baró-Montel et al. (unpublished data), pointed out the importance of ethylene in determining the peach susceptibility to brown rot at different phenological stages, as well as the differential ability of three strains of *Monilinia* spp. to infect non-wounded peaches. Accordingly, the aim of this study was to further investigate whether peach ethylene biosynthesis, at the molecular level, was affected in response to *M. fructicola* and *M. laxa* infection at 49 and 126 d after full bloom (DAFB), phenological stages with outstanding differences in terms of susceptibility to *Monilinia* infection. To achieve this, evolution of ethylene production and expression pattern of genes coding for *PpACS* and *PpACO* families were analysed over time upon infection.

2. Material and methods

2.1.Plant material

Experiments were conducted with 'Merryl O'Henry' peaches (*Prunus persica* (L.) Batch) obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain). Fruit free of physical injuries and rot were picked at 49 and 126 DAFB, being full

bloom the stage when at least 50 % of flowers were opened, and framed in the BBCH scale (Meier et al., 1994) as follows: 49 (BBCH = 72) and 126 (BBCH = 81). After each harvest, peaches were immediately transported to IRTA facilities under acclimatised conditions (20 °C).

2.2.Pathogen and inoculum preparation

In this study three single-spore strains of *Monilinia* spp. were used: *M. fructicola* (CPMC6) and *M. laxa* (CPML11 and ML8L), being different in terms of aggressiveness and coming from different sources. The strain CPML11 belong to the collection of the Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain). CPML11 was isolated from an infected peach fruit from a commercial orchard in Sudanell (Lleida, Spain) in 2009, and identified by the Department of Plant Protection, INIA (Madrid, Spain). The strains CPMC6 and ML8L were isolated from a latent infection of a peach fruit from a commercial orchard in Alfarràs (Lleida, Spain) in 2010, and from a mummified 'Sungold' plum fruit from a commercial orchard in Lagunilla (Salamanca, Spain) in 2015, respectively, and are deposited in the Spanish Culture Type Collection (CECT 21105 and CECT 21100, respectively). All strains were maintained in 20 % glycerol (*w/v*) at -80 ºC for long-term storage and subcultured periodically on Petri dishes containing potato dextrose agar (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25 % tomato pulp and incubated under 12-h photoperiod at 25 °C / 18 °C for 7 d.

Conidial suspensions of the fungal cultures were prepared by adding 10 mL of sterile water with 0.01 % Tween-80 (*w/v*) as a wetting agent over the surface of 7-day-old cultures grown on PDA supplemented with 25 % of tomato pulp and scraping the surface of the agar with a sterile glass rod. The inoculum was filtered through two layers of sterile cheesecloth

to minimize the presence of mycelial fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration (10^5 conidia mL^{-1}).

2.3.Fruit inoculation and experimental design

'Merryl O'Henry' peaches were disinfected with 0.5 % (*v/v*) sodium hypochlorite (NaClO) for 180 s and rinsed five times with tap water. Once dried, fruit were separated into four sets according to the treatment being applied. Then, non-wounded fruit were immersed for 60 s in a tank of running tap water containing a concentration of 10^5 conidia mL^{-1} of strain CPMC6, CPML11 or ML8L. The remaining set was immersed in a tank containing only water, and thus serve as a control (CK). After that, fruit were placed on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample) and separated into three different batches depending on whether they were used for: i) assessment of brown rot susceptibility, ii) determination of ethylene production and respiration rate, and iii) gene expression analysis. All the fruit was incubated in a chamber for a maximum of 14 d at 20 °C.

2.3.1. Assessment of brown rot susceptibility

Fruit were inspected daily to know when disease symptoms initiated, but the number of brown rot infected fruit was recorded only after 7 and 14 d of incubation. Experiments were conducted with 4 replicates of 10 fruit each, thereby assessing 40 fruit per each phenological growth stage and pathogen.

2.3.2. Determination of ethylene production and respiration rate

Fruit ethylene production was measured at 24 h, 48 h, 72 h, 6 d, 8 d, 10 d and 13 d post-inoculation. At each sampling point, fruit were placed in 2 L sealed flasks, in an

acclimatised chamber at 20 °C, equipped with a silicon septum for sampling the gas of the headspace after 2 h incubation. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) using the methodology described elsewhere (Giné-Bordonaba et al., 2017). Results were expressed on a standard weight basis (pmol kg^{-1} s^{-1} C_2H_4). Experiments were conducted with 4 replicates of 5 fruit each, thereby assessing 20 fruit per each phenological growth stage and pathogen.

Fruit respiration was determined from the same flasks used for ethylene measurements. After 2 h incubation at 20 °C, the headspace gas composition was quantified using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Ringsted, Denmark). Results were expressed on a standard weight basis (nmol kg^{-1} s^{-1} CO_2). The fruit respiratory quotient (RQ) was determined by the ratio of the amount of carbon dioxide produced divided by the amount of oxygen consumed after the 2 h incubation period. Experiments were conducted with 4 replicates of 5 fruit each, thereby assessing 20 fruit per each phenological growth stage and pathogen.

2.3.3. Gene expression analysis

At 24 h, 72 h, 6 d and 8 d post-inoculation, samples of peel and pulp tissue (10 mm diameter and 5 mm deep) encompassing all the surface of the fruit were collected using a cork borer and immediately frozen with liquid nitrogen. Afterwards, samples were lyophilised in a freeze-dryer (Cryodos, Telstar S.A., Terrassa, Spain) operating at 1 Pa and -50 °C for 5 d and grounded prior to being kept at -80 °C until further molecular analysis.

Experiments were conducted with 3 replicates of 5 fruit each, thereby assessing 15 fruit per each phenological growth stage, pathogen and sampling point.

2.3.3.1.RNA extraction

Total RNA corresponding to the healthy or infected fruit at each sampling point was extracted following the protocol described by Ballester et al. (2006) with some modifications. Briefly, 1 g of peach tissue (pulp and peel) was added to a preheated mixture of 5 mL phenol and 10 mL extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 50 mM EDTA pH 8.0, 2 % *L*-lauroylsarcosine sodium salt (w/v), 1 % polyvinylpyrrolidone 40 (w/v), 1 % β-mercaptoethanol). The extract was incubated for 15 minutes at 65 ºC and cooled before 5 mL of chloroform-isoamyl alcohol (24:1, v/v) were added. The homogenate was centrifuged at 2,200 *g* during 20 minutes at 4 ºC. The aqueous phase was recovered, re-extracted with 10 mL phenol-chloroform-isoamyl alcohol (25:24:21, v/v/v) and centrifuged at 2,200 *g* for 20 minutes at 4 ºC. The aqueous phase was transferred to a new tub and centrifuged again at 24,600 *g* for 15 minutes at 4 ºC. The supernatant was recovered and precipitated overnight at – 20 ºC by adding one third volume of 12 M lithium chloride. 1 mL of 3 M sodium acetate was added to the pellet obtained after centrifugation at 24,600 *g* for 45 minutes at 4 ºC and centrifuged again at 13,900 *g* for 5 minutes at room temperature. The pellet obtained was washed in 70 % ethanol and centrifuged as before. The pellet was finally dissolved in 50 µL of water, incubated at 65 ºC for 10 minutes and centrifuged at 13,900 *g* for 5 minutes at room temperature. The supernatant was recovered and transferred to a new tube. RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). Contaminant DNA was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the

208 manufacturer's recommendations. Both RNA integrity and the absence of DNA were
209 assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid
210 Gel Stain (Biotium, Hayward, CA, USA). First-strand cDNA synthesis was performed
211 on 3 µg of DNase-treated RNA using the SuperScript IV First-Strand Synthesis System
212 (Invitrogen, Carlsbad, CA, USA).

213 *2.3.3.2.Primers design and validation*

214 The primers used for quantitative real-time polymerase chain reaction (RT-qPCR)
215 analysis (Table S1) were adopted from the literature (Tadiello et al., 2016). Among the
216 members of ACS and ACO families reported in the cited study, the genes *PpACS1*,
217 *PpACS2*, *PpACO1*, *PpACO2* and *PpACO3* were selected based on their relative
218 expression profiles in fruit at different stages of development, specifically at 49 and 126
219 DAFB. Genes encoding for translation elongation factor 2 (*TEF2*) and RNA polymerase
220 II (*RPII*) were used as independent reference genes in all the experiments due to its high
221 statistical reliability (Tong et al., 2009). Annealing temperature conditions for each pair
222 of primers of both target and reference genes were optimised in the annealing
223 temperature range of 58-62 ºC using the Verity Thermal Cycler 96-wells Fast (Applied
224 Biosystems, Foster City, CA). Additionally, non-amplification of the cDNA derived
225 from the fungi was also verified. Primer efficiency was determined by the serial dilution
226 method, using a mix of all cDNA samples as a template (Table S1).

227 *2.3.3.3.Relative quantification by RT-qPCR*

228 RT-qPCR was performed on a 7500 Real Time PCR System (Applied Biosystems). The
229 reaction mix consisted of KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems,
230 Inc., Wilmington, USA), 100 nM of each primer and the amount of diluted cDNA,

according to standard curves. Thermal conditions applied were as follows: i) initial denaturation at 95 ºC for 10 min, ii) 40 cycles of denaturation at 95 ºC for 15 s, and iii) annealing/extension at 60 ºC for 1 min. To determine the melting curve, a final amplification cycle at 95 ºC for 15 s, 60 ºC for 1 min, 95 ºC for 30 s and 60 ºC for 15 s was applied. In all cases, a non-template control (NTC) was included using DNAse free water instead of DNA. The standard Cq method (Pfaffl, 2001) was used to calculate the relative transcript abundance of target genes relative to 0 hpi condition and normalized to the geometrical mean of both reference genes. Three technical replicates were analysed for each biological replicate for both the target and the reference genes.

2.4.Statistical analysis

Data were collated and statistically analysed with JMP® software version 13.1.0 (SAS Institute Inc., Cary, NC, USA). Means were analysed by analysis of variance (ANOVA) of data expressed on a standard fresh weight basis. When the analysis was statistically significant, the Tukey's HSD test at the level $p < 0.05$ was performed for comparison of means, while comparisons between phenological stages (49 *vs.* 126 DAFB) for each pathogen at specific time was done by least significance difference value test (LSD; $p< 0.05$) using critical values of t for two-tailed tests. Significance of correlations between traits was checked by Spearman's rank correlation.

3. Results and discussion

3.1.Effect of strain on the fruit susceptibility to brown rot

The three single-spore strains of *Monilinia* spp. used in this study are phenotypically different when grown under *in vitro* conditions (Fig. 1A) (i.e., colour, concentric rings, morphology, spore density), but such differences were strongly confirmed with the two

in vivo approaches performed (Fig. 1B and C). In detail, the first visual infection symptoms at 49 DAFB were evident at 7 d post-inoculation (dpi) for CPMC6 and CPML11, and at 13 dpi for ML8L, whereas at 126 DAFB visual infection symptoms were evident much earlier, at 3 dpi for CPMC6 and CPML11, and at 5 dpi for ML8L. Moreover, such dissimilarities were not only visual, but also numerical since significant differences regarding its infection capacity at 7 dpi were recorded between strains CPMC6 (100 % incidence at 49 and 126 DAFB) and CPML11 (100 % and 90 % incidence at 49 and 126 DAFB, respectively), and ML8L (40 % and 23 % incidence at 49 and 126 DAFB, respectively) (data not shown). Remarkably, although the time interval between infection inoculation and the onset of symptom from that infection (incubation period) for strains CPMC6 and CPML11 was the same, CPMC6 decay area was fully covered by spores, contrary to what was observed for CPML11 that mainly developed mycelium. Hence, it seemed that each strain had specific mechanisms to overwhelm peach defences, yet information regarding virulence factors of these strains is currently not available in the literature.

3.2.Analysis of ethylene production of 'Merryl O'Henry' peaches inoculated with different strains of Monilinia *spp.*

The ethylene production and respiration rate were monitored in healthy and infected peaches covering the different fruit infection stages as depicted in Fig. 2. As regards to ethylene production at 49 DAFB, when the fruit showed low resistance to most *Monilinia* strains, significant differences were found at all sampling points, except at 24 h post-inoculation (hpi) (Fig. 2A). From 24 hpi to 6 dpi values varied widely between infected and healthy peaches. In non-inoculated fruit, ethylene production increased constantly up to 102 pmol kg^{-1} s^{-1} at 6 dpi and declined thereafter. To the best of our

knowledge no other studies have previously shown that fruit harvested at 49 DAFB is capable of showing a climacteric-like behavior in terms of ethylene production. Thus said, such climacteric-like ethylene production pattern was not translated into fruit softening or ripening as observed in fully mature fruit. Infected samples displayed a significant delay in the ethylene production if compared to the CK, and the ethylene peak, being higher than in non-inoculated fruit, was observed at 10 (210 $pmolkg^{-1}$ s^{-1}) and 8 dpi (219 $pmolkg^{-1}$ s^{-1}) in fruit inoculated with strains CPMC6 and CPML11, respectively. For ML8L, values remained low and did not fluctuate until 13 dpi, when a 5-fold increase (77 pmol kg^{-1} s^{-1}) was observed. Thus, at early stages of infection the three strains seemed to suppress the ethylene production observed in non-inoculated fruit. Besides, in inoculated samples, ethylene starts to rise when disease symptoms started to be visible, which is likely related to senescence due to the maceration of the tissue in response to infection.

Unlike to what occurred at 49 DAFB, at 126 DAFB non-inoculated fruit did not exhibit a peak in ethylene production and levels were almost undetectable (between 0.20 and 4.81 pmol kg^{-1} s^{-1}) (Fig. 2B). This data is in agreement with the results reported in the literature, and attributed to the low capability of converting ACC to ethylene in fruit harvested at earlier maturity stages (Yang and Hoffmann, 1984). In contrast, infected samples showed a progressive increase of ethylene production before peaking at 6 dpi for CPMC6 (138 pmol kg^{-1} s^{-1}) and at 8 dpi for CPML11 (72 pmol kg^{-1} s^{-1}) and ML8L (60 pmol kg^{-1} s^{-1}) strains. Notably, the behavior of ML8L was identical to that of the control until 72 hpi, and as a result, both CPMC6 and CPML11 caused faster disease development and higher incidence than ML8L. In this phenological stage, the extent of the increased ethylene production in response to the inoculation was in parallel with the disease spread, and proportional to the incidence. For instance, peaches infected with

CPMC6 showed significantly higher ethylene production at all post-inoculation times, with the exception of 8 and 10 dpi, which may be in turn related to the more aggressiveness of this strain. Indeed, concomitantly with the increase in ethylene production, there were increments in the respiration patterns of 'Merryl O'Henry' peaches infected with CPMC6 and CPML11 strains (Fig. 2D). These results would fit with those of Hall (1967), which observed an acceleration of the respiratory activity and ethylene production in peaches inoculated with *M. fructicola.* Furthermore, at this phenological stage respiration significantly correlated with ethylene production (R^2 = 0.74; $p < 0.0001$), confirming that biotic stress stimulates the respiration rate of peaches. The relationship between increased ethylene levels and aggressiveness observed at this phenological stage may reflect either the fruit response to the infection or a greater capability of CPMC6 to alter ethylene production with the aim to infect its host. In accordance with this latter line, there are numerous examples, including insects (Zhu et al., 2018), viruses (Zhao et al., 2017) and fungi (Di et al., 2017) in which it has been described the ability of the pathogen to modulate the ethylene biosynthetic pathway in order to increase host susceptibility to their infection, but to date no other studies have tried to elucidate how ethylene biosynthesis in peach may be altered in response to *Monilinia* spp. infection.

Overall, this first approach at the physiological level pointed out that *Monilinia* strains might use two distinct mechanisms to infect peaches depending on the fruit maturity stage. Thus, while at 49 DAFB it seemed that the fungi tried to suppress the ethylene biosynthetic pathway with the ultimate goal of inhibiting fruit defence responses, at 126 DAFB, when the fruit by itself is not capable of producing ethylene, the infected fruit displayed normal defence reactions, which included ethylene synthesis and increased respiration. To further investigate if physiological responses were correlated at the

molecular level, and also to check if the different strains of *Monilinia* were able to differentially regulate or alter the ethylene biosynthetic pathway, transcriptional responses of some *PpACO* and *PpACS* of both healthy and infected samples were analysed by qRT-PCR.

3.3. Gene expression analysis of 'Merryl O'Henry' peaches inoculated with different strains of Monilinia *spp.*

In detail, 8 genes encoding *ACS* and 5 genes encoding *ACO* have been described (Mathooko et al., 2001; Ruperti et al., 2001), and reported to be differentially expressed during both fruit development and ripening (Tadiello et al., 2016). However, the study presented herein was only focused on 2 genes encoding ACSs (*PpACS1* and *PpACS2*), and 3 genes encoding ACOs (*PpACO1*, *PpACO2* and *PpACO3*), chosen based on their relative expression profile in fruit at 49 and 126 DAFB (Tadiello et al., 2016). For instance, *PpACS1* is dramatically induced by ripening (Trainotti et al., 2007), and *PpACS2* expression is relatively abundant in fully developed leaves, but it is very low in fruit, with a peak at the beginning of development (40 DAFB) and a maximum in senescence (120 DAFB) (Tadiello et al., 2016). As regards to ACOs, *PpACO1* expression is induced by ethylene, *PpACO2* expression is almost constitutive, whereas *PpACO3* is the less expressed but with a maximum at 115 DAFB (Ruperti et al., 2001; Tadiello et al., 2016).

In the present study, at 49 DAFB, the *ACS* family was expressed at different levels depending on the strain inoculated and time condition (Fig. 3). As a role, the amount of *PpACS1* transcripts increased over time, confirming the role of this gene on the ripening process (Tatsuki et al., 2006), or at least its tight correlation with the fruit ethylene production. Significant differences among treatments were found at 6 and 8 dpi. At 24

hpi, expression levels of *PpACS1* rose up 28-fold, 1,348-fold, 119-fold and 1,188-fold for CK, CPML11, ML8L and CPMC6, respectively (Fig. 3A). Regarding *PpACS2*, results showed two distinct expression profiles (Fig. 3B). *PpACS2* has been described to be induced by abiotic stressors such as wounding (Tatsuki et al., 2006), and negatively regulated by ethylene in citrus (Marcos et al., 2005). Our results showed a positive ethylene regulation and hence are not in accordance with data from Marcos et al. (2005), most likely because we are working on a typical climacteric specie while they did in a non-climacteric fruit such as citrus. In fact, results from the present study showed that expression levels of *PpACS2* for both CK and ML8L treatments were very low and only slightly induced (1.4-fold and 1.6-fold, respectively) at 6 dpi. However, for the fruit infected with CPML11, an enhanced production at 8 dpi which correlated with the increased ethylene production was observed. Our results also shown that both *PpACS1* and *PpACS2* were over-expressed during pathogen-induced senescence. Enhanced ethylene production is frequently observed during plant–pathogen interactions, acting as a signalling molecule in response to biotic attacks and hence, contributing to the induction of the plant response. Such recognition by the plant immune system elicit host defences, resulting in rapid responses that are triggered by pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Hence, to establish proliferation, fungi must avoid eliciting PAMP-triggered immunity (PTI) first line of defence reactions, or either cope with or suppress it. Another measure for controlling the defences of the whole plant against infections by pathogens is through the systemic acquired resistance (SAR) in which ethylene has also been implicated (Ryals et al., 1996). In agreement to the above mentioned, the fact that these defence mechanisms might been activated after the onset of brown rot symptoms reinforce the hypothesis of the suppression of the natural ethylene production pattern as a strategy of

the fungus to inhibit SAR, jasmonic acid signalling cascades and thereby phenylalanine ammonia-lyase (PAL) biosynthesis, and hence facilitate colonisation.

As refers to the *ACO* family at 49 DAFB, a complex expression pattern was obtained, and remarkably, expression levels of *PpACO1* were considerably higher than those of both *PpACO2* and *PpACO3* (Fig. 4), in agreement with the studies already published (Tadiello et al., 2016). For *PpACO1* significant differences were found depending on the strain inoculated (Fig. 4A). In detail, at 72 hpi it was detected a transient increase up to 230-fold, 101-fold and 135.5-fold for CPMC6, CPML11 and ML8L, respectively. At 6 dpi, a decrease was monitored in all the treatments, except for the control that reached its maximum expression level (190-fold). The results obtained for the control were in agreement with previous studies (Tonutti et al., 1997), which demonstrate an increase in ethylene production enhanced by the up-regulation of *PpACO1*. At 8 dpi, the expression profile was the opposite; while the levels of the control fruit decreased with respect to 6 dpi, the infected fruit experienced and up-regulation of *PpACO1* levels irrespective of the fungus, and this could be likely related to senescence. As observed for *PpACO1*, an up-regulation at 6 dpi was also obtained for *PpACO2* for the CK sample, coinciding with the maximum ethylene production. However, levels were very low if compared to *PpACO1* and are somehow confirming that this isogene is not strictly involved with the climacteric system II (Tadiello et al., 2010). Regarding *PpACO3*, a tendency to the up-regulation was observed at 24 hpi for both CPML11 and CPMC6, being in line with *PpACO2* at 24 hpi. These findings also coincide with the ones observed in apple-*P. expansum* interaction, in which a massive induction of *MdACO3* expression was observed after the inoculation with the compatible pathogen (Vilanova et al., 2017). In other climacteric fruits such as apple ACO has been related in the transition from system I to system II, being negatively regulated by ethylene (Bulens et al., 2014),

which correlates with the results presented herein since the peaks of ethylene production took place when expression levels of this transcript were reduced. The strain ML8L triggered an induction of this gene but only at 8 dpi (4-fold) (Fig. 4C). Overall, our results suggest that the inhibition of the fruit ethylene production by the *Monilinia* spp. short after inoculation was not strictly regulated at the molecular level of the ethylene biosynthetic pathway. It is therefore likely that other mechanisms are used by the fungi at this developmental stage to inhibit the ethylene burst occurred and hence suppress SAR. In other fruit-pathosystems, polyamines have been shown to play a pivotal role in determining the fruit susceptibility to pathogen infection (Nambeesan et al., 2012). Accordingly, it is acknowledged that biosynthesis of both polyamines and ethylene share *S*-AdoMet as a common precursor (Pandey et al., 2000). In fact, peach fruit treated with polyamines putrescine and spermidine has demonstrated to inhibit ethylene production, interfering at both biochemical and molecular level (Ziosi et al., 2006). Besides, transgenic tomato lines overexpressing an enzyme involved in polyamine biosynthesis were more susceptible to *B. cinerea* (Nambeesan et al., 2012). Thus, during *Monilinia* infection, enhanced secretion of fungi polyamines may explain the down-regulation of genes involved in ethylene biosynthesis, which in turn could also lowered the defence responses resulting in higher brown rot incidence. Furthermore, the suppression of ethylene observed at 49 DAFB, but not at 126 DAFB, is in line with Apelbaum et al. (1981), who reported that polyamines are more effective in inhibiting ethylene at earlier fruit developmental stages. Another explanation may relate to fungal secretion of effectors that suppress the host immune response or manipulate host cell physiology (reviewed in Lo Presti et al., 2015). Nonetheless, further studies are warrant to decipher the mode of action for *Monilinia* spp. to infect stone fruit at earlier developmental stages.

Analogous to what occurred at 49 DAFB for *ACS* family, at 126 DAFB, expression levels were larger than those observed for the *ACO* family. Notably, both *PpACS1* (Fig. 5A) and *PpACS2* (Fig. 5B) followed the same pattern and precede or parallel the ethylene peak, demonstrating a positive ethylene regulation. Besides, significant differences were found depending on the strain inoculated, especially at 6 dpi (Fig. 5). At this sampling point, CPMC6 induced the largest expression (767-fold) for *PpACS1*, followed by CPML11 (330-fold), and ML8L (25-fold) and CK (2-fold) (Fig. 5A). Again, the increased expression levels coincided with the major ethylene production, confirming the positive role of this gene on the ethylene biosynthesis and pointing out the capacity of these fungi to alter gene expression to ultimately induce ethylene production. By the moment, no data regarding ethylene production by *Monilinia* has been described and preliminary results pointed out that this fungus is not able to produce ethylene by itself unless grown in very specific conditions (unpublished data). Hence, it is feasible to attribute the higher ethylene production to the up-regulation of *PpACS1*. At this phenological stage, it seems that increased ethylene production is not parallel by an action of SAR, or at least that the three strains, and especially CPMC6 and CPML11, were likely capable of coping with it and hence benefit from it. For instance, the increased ethylene synthesis due to *PpACS1* induction may lead to the autocatalytic ethylene evolution characteristic of system 2 ethylene (Mathooko et al., 2001; Tatsuki et al., 2006), which, in turn, could trigger polygalacturonase (PG) and pectin methyl esterase (PME) actions (Hayama et al., 2006). It is known that both enzymes contribute to the weakening of peach tissue following cell wall degradation (Brummell et al., 2004), and thus their action could facilitate penetration. For *PpACS2*, CPML11 induced the highest expression levels at 24 hpi (4.2-fold) and 6 dpi (709-fold), while no significant differences were found among CPMC6, ML8L and CK (Fig. 5B).

In the control fruit, and as described before for this development stage (Tadiello et al., 2016), very low levels were detected during the time course of the experiment. Taking all together, these results demonstrate the capability of *Monilinia* spp. to alter the expression of genes related to ethylene biosynthesis and, consequently, ethylene production before initiation of decay.

In contrast to that described above, *ACO* family was poorly expressed (Fig. 6), especially if compared to 49 DAFB. This trend is likely related to the fact that at this phenological stage we did not observed ethylene production in the control fruit. Hence, the expression levels of *PpACO* were very low and in line with the lower ethylene capacity of the non-inoculated fruit. Briefly, for *PpACO1* significant differences were found between strains CPML11 and ML8L at 72 and 6 dpi (Fig. 6A), displaying the different capability of this two strains to modulate the expression of this gene. For *PpACO2* significant differences among strains were only found at 6 dpi, when CPML11 enhanced the induction of the transcript levels of this gene by 3.4-fold (Fig. 6B). At the other time points, none of the infected samples changed significantly the expression levels of this transcript, being almost constitutive as reported earlier (Tadiello et al., 2016). On the other hand, for *PpACO3* significant differences were found earlier, especially at 24 hpi, when a significant increase of 2.6-fold was monitored for CPML11 (Fig. 6C). This up-regulation concurred with the moment when ethylene levels were almost null, which correlates with its implication with system I reported in previous works (Vilanova et al., 2017). In general, the low expression levels in this family could explain the nearly constant ethylene production pattern observed in the control fruit at this development stage compared to 49 DAFB, although no increase in genes involved in system I, such as *PpACO3* is demonstrated. Moreover, these findings explain that the

increase in ethylene production of the infected fruit, at least, is not the result of *PpACO3* alteration.

4. Conclusions

Collectively, it could be observed that the strains of *Monilinia*, through different mechanisms that depend on the fruit developmental stage, succeed in infecting peaches. At 49 DAFB, in which we have demonstrated a climacteric-like behaviour, the infected fruit failed to display normal defence reactions, which included ethylene synthesis and increased respiration until, at least, 6 dpi, when a clear development of the decay was already observed. Besides, such inhibition of the ethylene production by *Monilinia* spp. to avoid SAR responses and facilitate colonisation was not mediated at the molecular level, pointing out that other pathways, including the production of polyamines, could have been implicated. On the other hand, at 126 DAFB ethylene production precede the symptoms of decay development, likely enhancing the capability of *Monilinia* spp. to successfully infect stone fruit through the putative activation of pectin-degrading enzymes that accelerate the rate of softening. Finally, by looking at the control for both phenological stages, we have demonstrated that *PpACS1* is the key gene involved in the ethylene biosynthetic pathway, and at 126 DAFB, in which a non-climacteric behaviour was observed, also a suitable target that *Monilinia* spp. tend to up-regulate to induce changes associated with increasing susceptibility to infection. Such knowledge is critical for understanding the host (peach) and the pathogen (*Monilinia* spp.) factors important for the rapid spread and dramatic impact of brown rot and may open new paths for the control of this disease.

5. Contributions

JGB, NBM and RT conceived and designed the experiment. NBM, NV and JGB analysed all the data. NBM, JGB and NV were responsible for the ethylene and respiration rate measurements. NBM, NV and SS were responsible for the gene expression analysis. NT and JU led the fruit inoculation and pathogenicity studies. NBM, NV, JGB and RT wrote the article and all remaining authors contributed in improving the final version of the manuscript.

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690

Table 1. Brown rot incidence (%) of 'Merryl O'Henry' peach fruit inoculated with different strains of *Monilinia* spp. at 49 and 126 d after full bloom (DAFB). Fruit were inoculated by immersion for 60 s in a conidial suspension containing 10^5 conidia mL^{-1} of strain CPMC6 of *M. fructicola* (■) or strains CPML11 (■) and ML8L (■) of *M. laxa*, and incubated for 7 d at 20 °C and 100 % relative humidity. Data represent the mean (n = 40) ± S.D. Mean values with the same uppercase letter within the same strain or mean values with the same lowercase letter within the same phenological stage are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

Phenological stage (DAFB)	**Strain**		
	CPMC6	**CPML11**	**ML8L**
49	100 ± 0.0	100 ± 0.0	40 ± 8.2
126	100 ± 0.0	90 ± 8.2	22.5 ± 12.6

Fig. 1. Images of *in vitro* (A) and *in vivo* (B and C) phenotypic differences among three strains of *Monilinia* spp.: *M. fructicola* (CPMC6) and *M. laxa* (ML8L and CPML11). Images A and C were captured 7 d after the fungal inoculation, whereas image B was captured 14 d after the fungal inoculation.

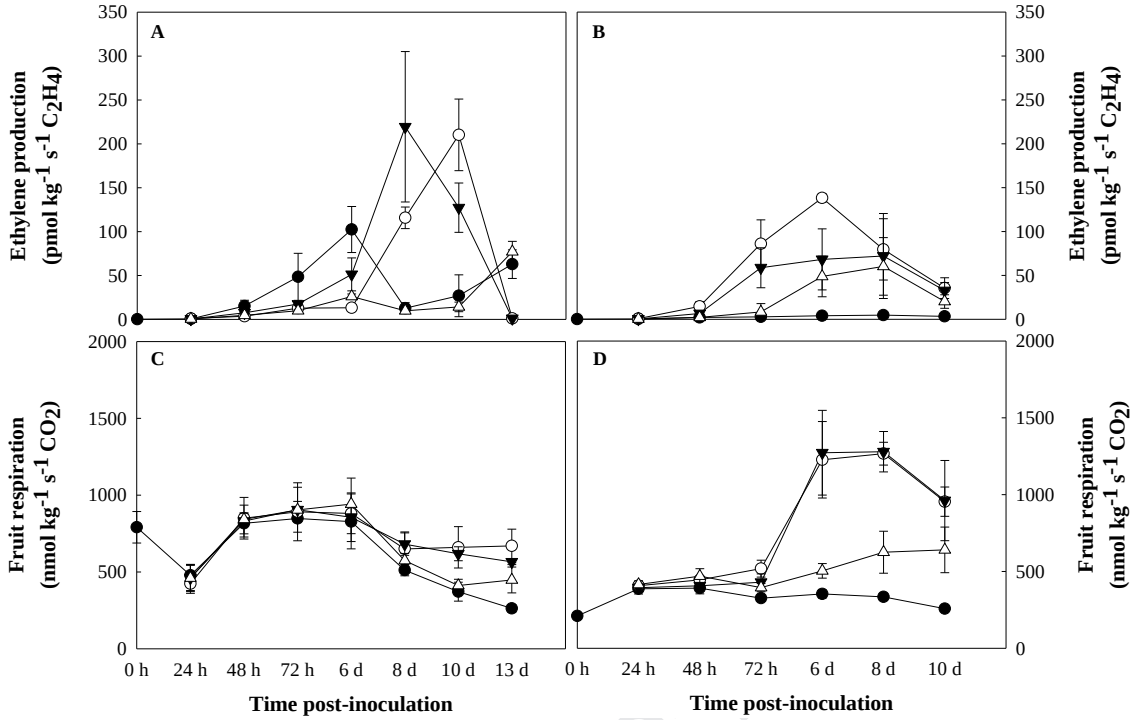

Fig. 2. Changes in ethylene production (pmol kg^{-1} s^{-1} C_2H_4) and fruit respiration (nmol kg^{-1} s^{-1} CO_2) on a standard fresh weight basis of 'Merryl O'Henry' peach fruit control (●) and inoculated with different strains of *Monilinia* spp. (strain CPMC6 of *M. fructicola* (○) or strains CPML11 (▼) and ML8L (△) of *M. laxa*) at 49 (A and C) and 126 (B and D) d after full bloom (DAFB). Fruit was incubated at 20 °C and 100 % relative humidity until the time of sampling. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 4).

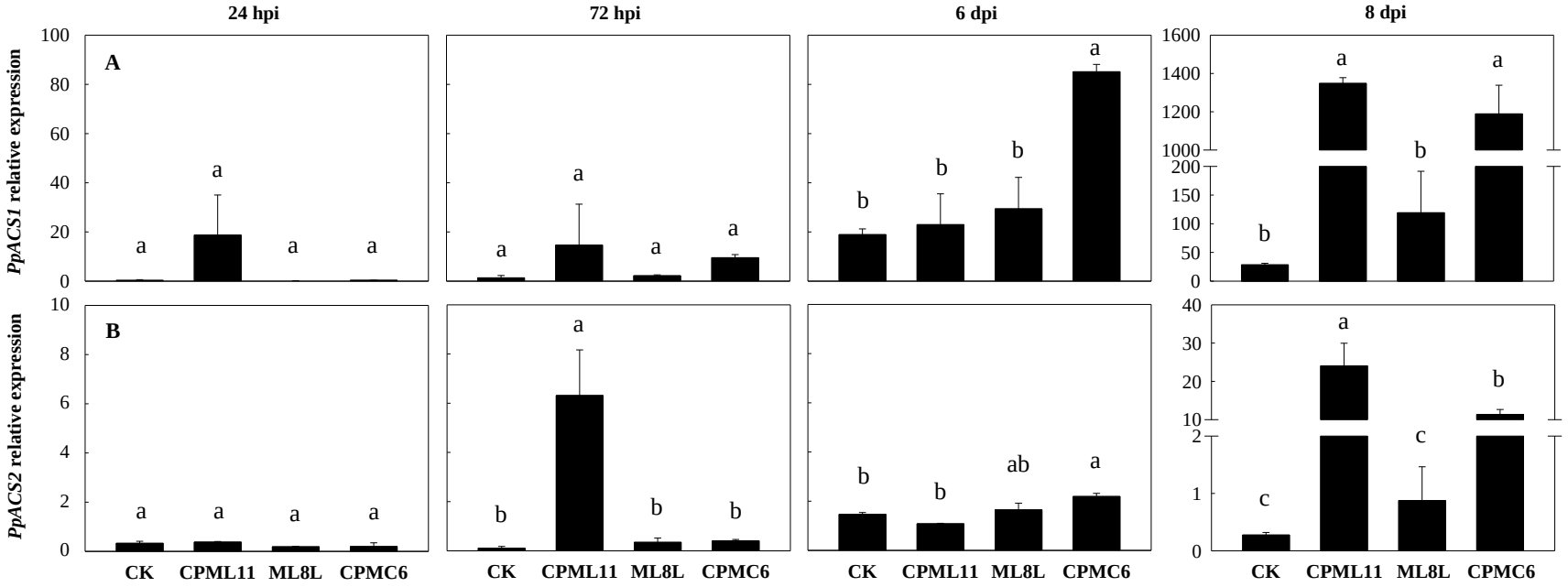

Fig. 3. Changes in *in vivo* gene expression levels of *PpACS* family (*PpACS1* (A) and *PpACS2* (B)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 49 d after full bloom (DAFB). Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 and 8 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

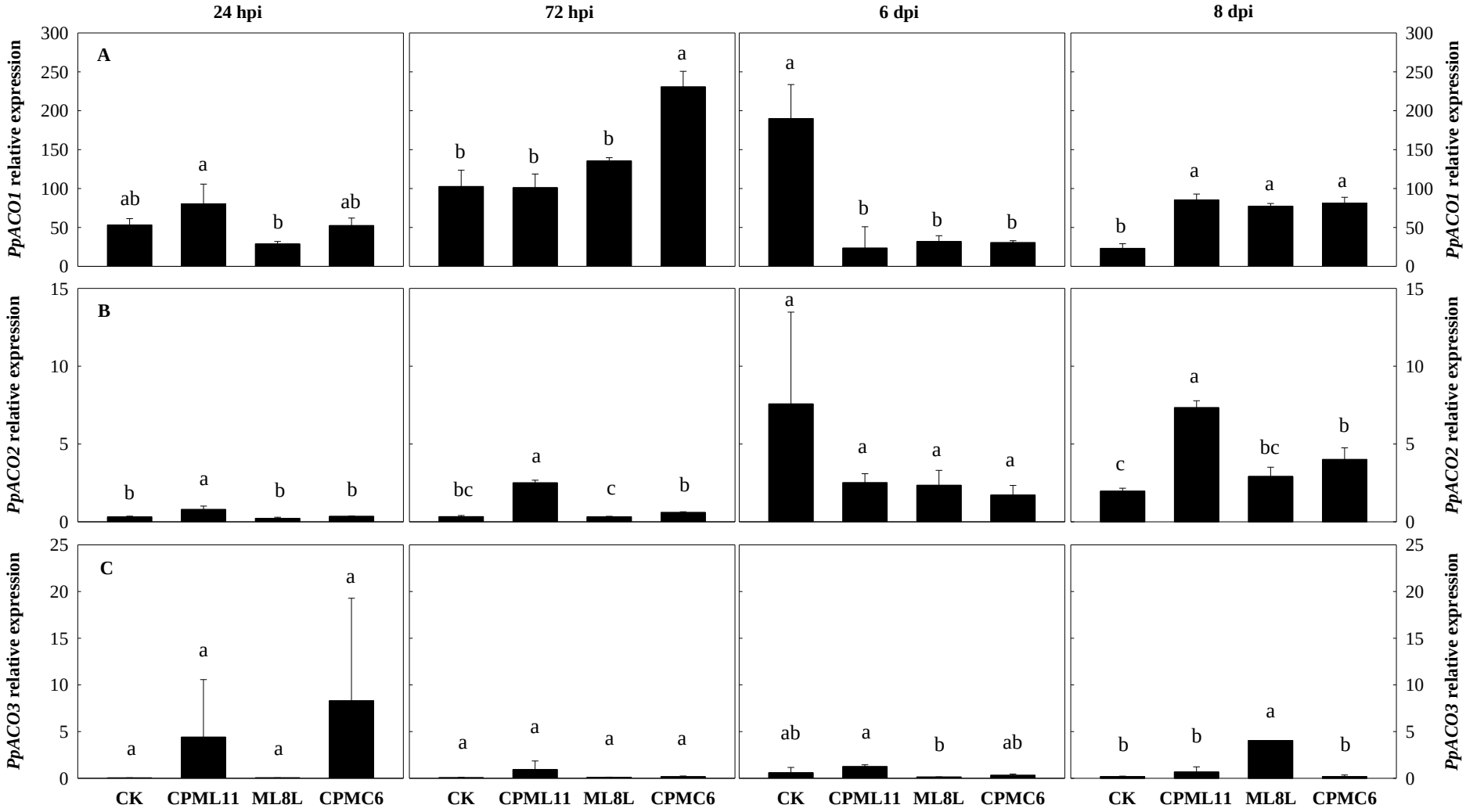
24 hpi
72 hpi
6 dpi
8 dpi
A
B
C
PpACO1 relative expression
PpACO2 relative expression
PpACO3 relative expression
CK
CPML11
ML8L
CPMC6

Fig. 4. Changes in *in vivo* gene expression levels of *PpACO* family (*PpACO1* (A), *PpACO2* (B) and *PpACO3* (C)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 49 d after full bloom (DAFB). Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 and 8 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

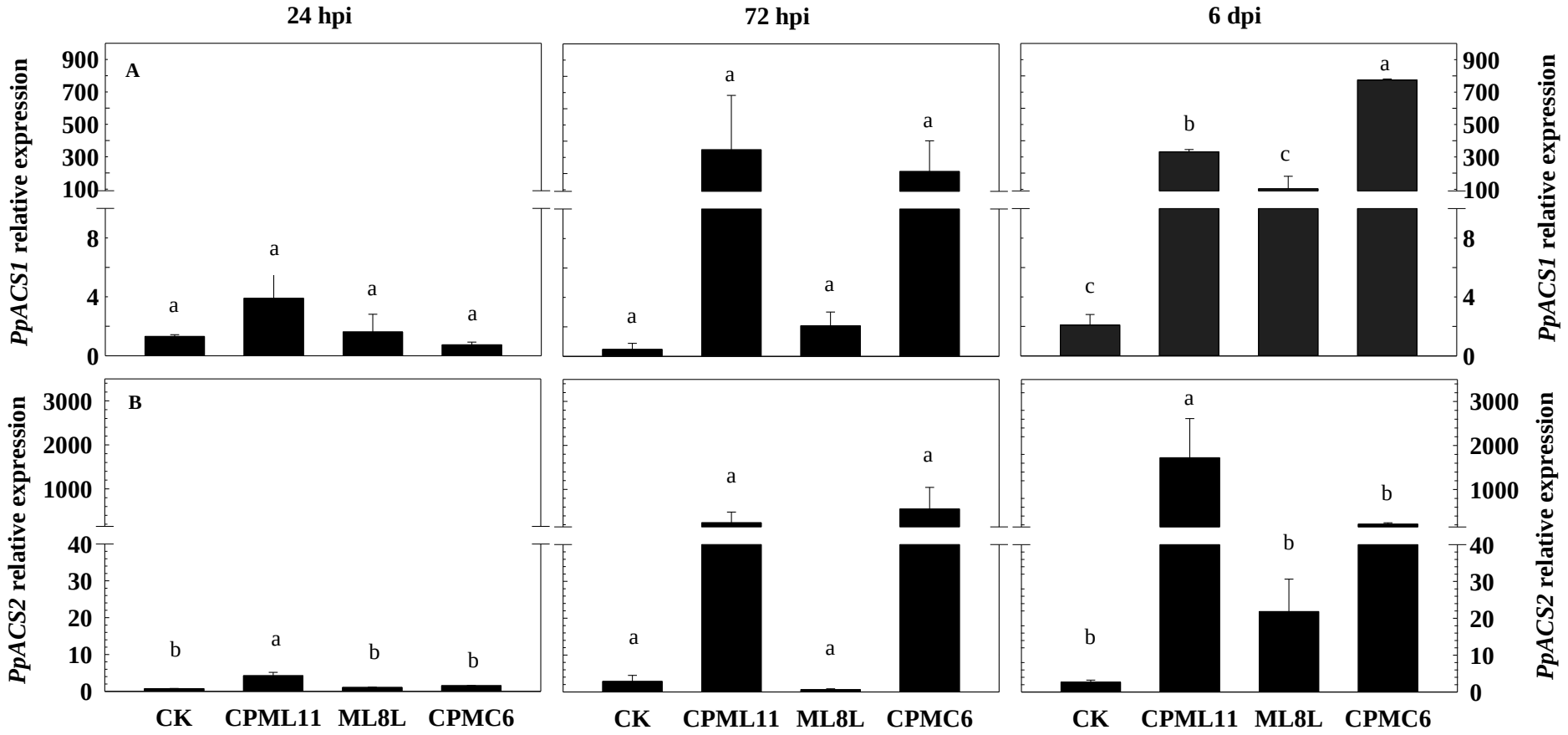

Fig. 5. Changes in *in vivo* gene expression levels of *PpACS* family (*PpACS1* (A) and *PpACS2* (B)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 126 d after full bloom (DAFB).

Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

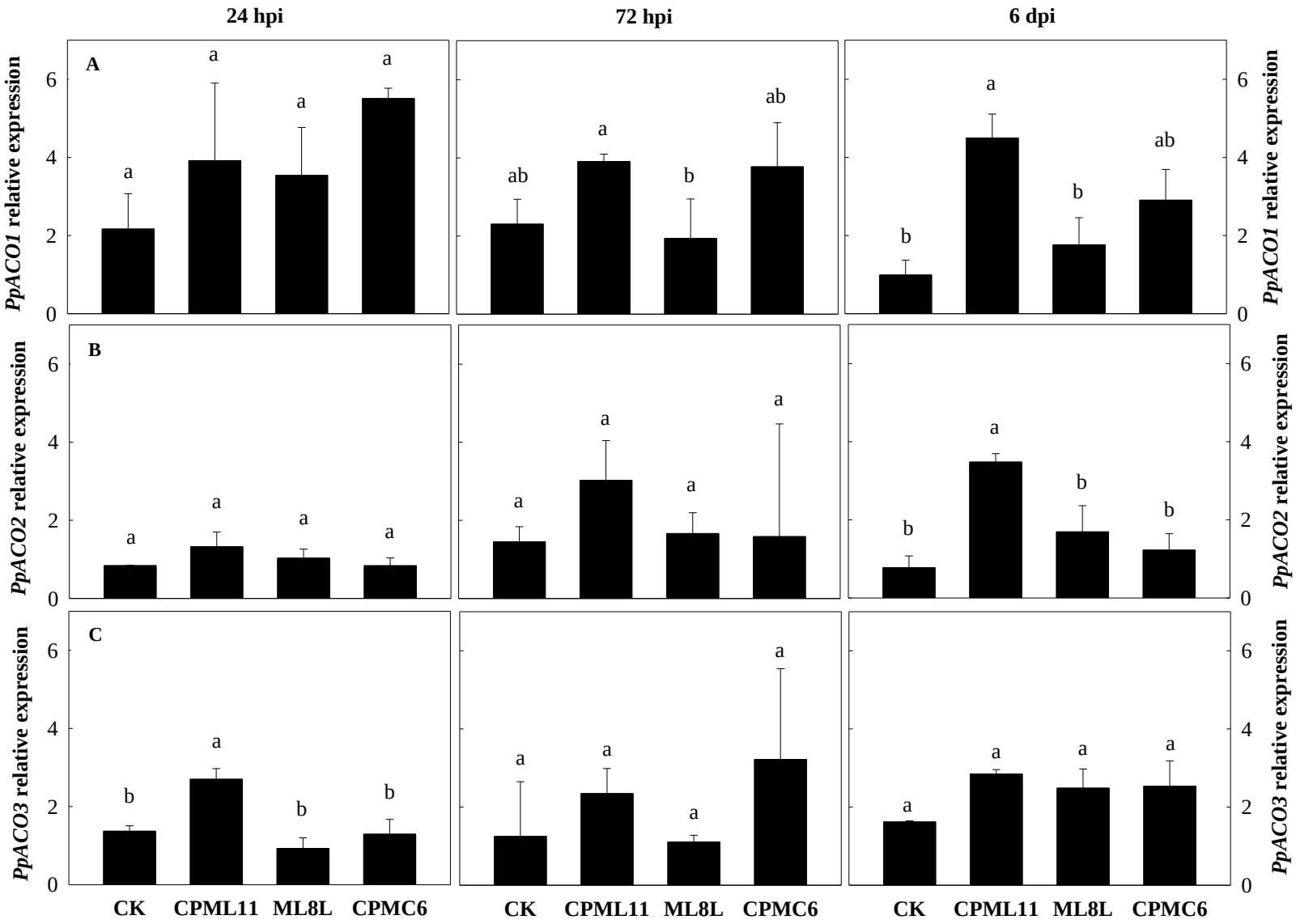
24 hpi
72 hpi
6 dpi
A
B
C
PpACO1 relative expression
PpACO2 relative expression
PpACO3 relative expression
0
2
4
6
a
ab
b
CK
CPML11
ML8L
CPMC6

Fig. 6. Changes in *in vivo* gene expression levels of *PpACO* family (*PpACO1* (A), *PpACO2* (B) and *PpACO3* (C)) of 'Merryl O'Henry' peach fruit non-inoculated (CK) and inoculated with strains CPML11 and ML8L of *Monilinia laxa* or CPMC6 of *M. fructicola* at 126 d after full bloom (DAFB). Each column represents the mean of three biological replicates after 24 and 72 hours post-inoculation (hpi), and 6 d post-inoculation (dpi). At each sampling point, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

Highlights:

- Ethylene is a key player with a dual role in determining brown rot susceptibility.
- *Monilinia* infection mechanisms in peach depend on the fruit developmental stage.
- Impairing the ethylene biosynthetic pathway is a putative mechanisms by which *Monilinia* spp. is able to infect peach fruit.
- *PpACS1* may be considered as a key gene in the peach-*Monilinia* spp. interactions.

Author's contributions

JGB, NBM and RT conceived and designed the experiment. NBM, NV and JGB analysed all the data. NBM, JGB and NV were responsible for the ethylene and respiration rate measurements. NBM, NV and SS were responsible for the gene expression analysis. NT and JU led the fruit inoculation and pathogenicity studies. NBM, NV, JGB and RT wrote the article and all remaining authors contributed in improving the final version of the manuscript.