Ethylene biosynthesis and response factors are differentially modulated during the interaction of peach petals with *Monilinia laxa* or *Monilinia fructicola*

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Abstract

Monilinia spp. may infect stone fruit at any growth stage, although susceptibility to brown rot depends on both host properties and climatological conditions. This said, no studies deciphering the host response in the interaction between peach blossoms and *Monilinia* spp. are yet available. This study presents an in-depth characterization of the role of ethylene in the interaction of 'Merrill O'Henry' peach petals (*Prunus persica* (L.) Batch) with *Monilinia laxa* and *M. fructicola*. We investigated the physiological responses of the host and the fungi to the application of ethylene and 1-methylcyclopropene (1-MCP) as well as the molecular patterns associated with the biosynthetic and ethylene-dependent responses during the interaction of both *Monilinia* species with the host. The incidence of both species was differentially affected by 1-MCP and ethylene; *M. laxa* was favoured by the enhanced host ethylene production associated with the treatments whereas *M. fructicola* reduced its infection capacity. Such differences were host-dependent as treatments did not affect growth or colony morphology of *Monilinia* spp. Besides, host ethylene production was altered in *M. laxa* inoculated petals, either by the fungus or the host itself. Molecular analysis revealed some important ERFs that could be involved in the different ability of both species to activate a cascade response of peach petals against these pathogens.

Keywords: Brown rot, *Prunus persica*, 1-Methylcyclopropene (1-MCP), 1-Aminocyclopropane-1-carboxylic acid synthase (ACS), ACC oxidase (ACO), Ethylene response factor (ERF).

1. Introduction

Monilinia spp. causing brown rot in stone fruit is responsible of important economic losses both in the field and during the postharvest supply chain. The infection incidence can largely vary among years mainly due to diverse climatic conditions [1,2]. In Spain, the main causal agents of brown rot are, since 2006, *M. laxa*, *M. fructicola*, and *M. fructigena*, although the latter is more common in pome than in stone fruit [3]. To a lesser extent, *M. polystroma* has also been reported in some countries [4], also mainly affecting pome fruit. The onset of brown rot infections in the field is occasionally due to the presence of primary inoculum in the orchard, being mummied fruit the main source of overwintering fungi persistence [5]. However, other infected tissues rather than fruit, including blossoms, branches, spurs, twigs and fruit peduncles, can entail a source of *Monilinia* spp. infection [6]. Infected blossoms can lead to tree damage together with a reduction on the number of viable fruit and thereby a decrease on fruit production. In fact, *Monilinia* spp. infections can occur throughout all fruit development, although the degree of susceptibility to infection can largely vary among the different phenological stages [7,8].

All plant tissues share, to some extent, response mechanisms triggered by the interaction with pathogens such as *Monilinia*. Such responses are in many cases mediated by a complex and coordinated crosstalk among different hormones. Both ethylene and jasmonate, apart from being involved in response to numerous biotic stresses (drought, salt and heat tolerance; reviewed in [13]), also modulate the response against necrotrophic pathogens [14]. Ethylene, for instance, is perceived within the plant tissues through endoplasmic reticulum receptors and biosynthesized through methionine by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO),

being both gene families comprised of different isogenes [15]. The role of ethylene in determining the disease onset has been studied in multiple pathosystems [16,17]. However, controversy still exists on how this hormone could promote or avoid the pathogen spread.

Ethylene signalling, together with other hormones such as jasmonic acid, leads to the activation of the plant immune responses, through the modulation of the plant transcriptome, and specifically, the regulation of the Ethylene Response Factors (ERF) [18]. ERFs, one of the major groups of plant transcription factors [19], display GCC box–specific binding activity. The interaction of ERF proteins to their target sequences, including the GCC-box containing genes, lead to the induction of responsive genes, such as pathogenesis-related (PR) genes [20,21]. There are five different ERFs characterized in peach, which include *PpERF1a*, *PpERF1b*, *PpERF2a*, *PpERF2b* and *PpERF2c* [22], located in two evolutionary divergent groups. Both ERF member groups are homologous to other host proteins involved in enhancing resistance to pathogens [23,24]. Indeed, evidences suggest that ethylene and jasmonate can differentially modulate ERF**s** in plants, such *Arabidopsis thaliana* [25] but also in peach [22]. To date, no studies are available investigating the role of ethylene on the peach-*Monilinia* pathosystem during the flowering stage. Taking into account that infected blossoms can imply a potential source for future fruit infections, a better understanding on how *Monilinia* spp. can infect peach blossoms and the subsequent response of the host, could represent a crucial step to prevent fruit infection. Hence, the aim of this study was to investigate for the first time i) the role of ethylene and the peach petal transcription reprograming upon the interaction with the necrotrophic pathogen *Monilinia* spp. and ii) the ethylene-dependent ability of both *M. laxa* and *M. fructicola* to infect peach petals.

2. Material and methods

2.1. Plant material

Experiments were conducted with 'Merrill O'Henry' peach blossom petals (*Prunus persica* (L.) Batch) obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain), during the flowering seasons (March) of 2019 and 2020. Branches containing multiple inflorescences, free of physical injuries and rot were picked at full bloom when at least 50 % of flowers were opened. After harvest, peach blossoms were immediately transported to IRTA facilities under acclimatised conditions (20 °C). The total number of samples, including petals and replicates used for each measurement is depicted in Supplementary Table S1.

2.2. Pathogen and inoculum preparation

Two single-spore species of *Monilinia* spp. were used in this study: *M. fructicola* (CPMC6) and *M. laxa* (ML8L). The strains CPMC6 and ML8L are deposited in the Spanish Culture Type Collection (CECT 21105 and CECT 21100, respectively). Fungal cultures were grown 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 days. Conidial suspensions of the different fungal cultures were obtained by rubbing the surface of 7-day-old cultures with sterile water containing 0.01 % Tween-80 (*w/v*) and using 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.

2.3. Ethylene and 1-MCP treatments

Branches containing multiple inflorescences were placed in an airtight plastic chamber (65 L). For 1-Methylcyclopropene (1-MCP) treatment, 5 µL L^{-1} of 1-MCP (or 0.6 µL L^{-}

[1] when indicated) was applied using the product Smartfresh™ (Agrofresh Inc.) and following the company recommendations. In the case of ethylene treatment, ethylene gas was injected into the chamber to a final concentration of 10 µL L^{-1} (or 1 µL L^{-1} when indicated). A control was performed by injecting similar volumes of air. Both treatments and control samples were incubated during 20 h at 3 ºC. For *Monilinia in vitro* treatments, PDA plates were inoculated by applying a drop of 10 µL of the conidial suspension of both *M. laxa* and *M. fructicola* at $1 \cdot 10^5$ conidia mL^{-1}. Plates were incubated in the dark at 25 ºC for 24 h. After this time, 1-MCP and ethylene treatments were applied as explained above for 20 h at 20 ºC and then plates were incubated again in the dark at 25 ºC. Colony growth (diameter) was recorded daily, while a visual inspection of colony features was conducted along time. A total of 9 plates were used for each fungus and treatment condition.

2.4. Determination of ethylene production and respiration rate

Ethylene production (pmol Kg^{-1} s^{-1}) was measured at different times post-inoculation or times post-treatment (depending on the assay) after enclosing 10 individual petals on 14 mL glass vials for 2 h at 20 ºC. Measurements were determined on 4 replicates of 10 petals each. One mL gas samples was collected using a syringe and injected into a gas chromatograph (Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column 80/100 (2 m x 3 mm, Tecknokroma, Barcelona, Spain) as previously described [26]. Fruit respiration (nmol Kg^{-1} s^{-1}) 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).

2.5. Petal inoculation and experimental design

After ethylene and 1-MCP treatments, 'Merrill O'Henry' petals were detached from the branches, pooled and distributed onto plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample). They were separated into different batches depending on whether they were used for: i) assessment of brown rot susceptibility and senescence, ii) determination of ethylene production and respiration rate, and iii) gene expression analysis. Inoculation was performed by applying one droplet (5 μL) of conidial suspension ($5 \cdot 10^4$ conidia mL^{-1}) onto the top part of the host surface. A mock-inoculated control (CK) was also performed by applying one droplet (5 μL) of sterile water with 0.01 % Tween-80 (w/v). All peach petals were incubated in a chamber at 20 °C and 85 % RH. Brown rot incidence was assessed on 4 replicates of 10 petals each. Likewise, ethylene and respiration rate was determined on 4 replicates of 10 petals each, while 3 replicates of 20 petals each were used for gene expression analysis.

2.5.1. Assessment of brown rot susceptibility

Infection capacity was assessed by determining the percentage of infected petals (with necrotic spots) for each fungus at different times post-inoculation. A total of 4 replicates per each fungus were performed, using 10 petals for each replicate.

2.6. Gene expression analysis

RNA was extracted from both control and inoculated petals with *M. laxa* and *M. fructicola* species using the RNeasy kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. Both RNA quantity and quality was determined spectrophotometrically using a Nanodrop 2000 spectrophotometer (Thermo Scientific, DE, USA). Extracted RNA was treated with Turbo DNA-free DNase (Ambion, TX, USA) to remove contaminant DNA, following the manufacturer's recommendation. Both the absence of contaminant genomic DNA and the RNA integrity was assessed

after electrophoresis on an agarose gel. Total RNA was extracted from 3 biological replicates for each treatment and time condition.

First-strand cDNA synthesis was performed on 1 μg of DNase-treated RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qPCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The reaction mix consisted of KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA), 100 nM of each primer and the amount of diluted cDNA, according to standard curves. The ACS and ACO gene members, *PpACS1*, *PpACS2*, *PpACO1*, *PpACO2* and *PpACO3* were analyzed based on their expression profiles in blossom tissues [27], while *PpERF* family members, *PpERF1a*, *PpERF1b*, *PpERF2a*, *PpERF2b* and *PpERF2c* were selected according to previous results on different susceptible peach cultivars [22]. The oligonucleotide primers used for qPCR analysis (Supplementary Table S2) were adopted from the literature [22,27]. Annealing temperature conditions for all primers were optimized in a range of 58-62 ºC in a Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Non-amplification of cDNA derived from *M. laxa* and *M. fructicola* was also verified for all primers. Primer efficiency was determined using 5-fold serial dilutions from a mix of all cDNA samples (Supplementary Table S2). Thermal conditions applied for qPCR analysis 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 cDNA. Genes encoding for translation elongation factor 2 (*TEF2*) and RNA polymerase II (*RPII*) were used as independent reference genes in all the experiments due to its high statistical reliability

191 [28]. Relative quantification of target genes normalized to the geometrical mean of
192 reference gens was determined by the standard Cq method [29]. Three technical
193 replicates were analysed for each biological replicate for both the target and the
194 reference gene.

195 **2.7. Statistical analysis**

196 Data were subjected to analysis of variance (ANOVA) using JMP® software version 13.1.0
197 (SAS Institute Inc., Cary, NC, USA). When the analysis was statistically significant, the
198 Tukey's HSD test at the level $p \leq 0.05$ was performed for comparison of means.
199 Comparisons of brown rot incidence *(M. laxa vs. M. fructicola*) were performed using the
200 Student's T-test ($p \leq 0.05$). Comparisons between treatments and inoculums along time,
201 were determined by using the least significance difference value (LSD; p = 0.05), using
202 critical values of *t* for two-tailed tests. Growth rate was determined as the slope of a linear
203 polynomial regression obtained by plotting growth diameter (cm) *vs*. time (days).

204

205 **3. Results**

206 **3.1. Effect of 1-MCP and ethylene treatment on *Monilinia* spp. incidence**

207 To unravel how ethylene could be involved in the onset of brown rot, peach blossoms were
208 pre-treated with high doses of 1-MCP (5 µL L^{-1}) and ethylene (10 µL L^{-1}), before petal
209 inoculation. At 72 h post-inoculation (hpi) brown rot incidence in control petals (CK) was
210 much higher (3.6-fold) in *M. fructicola*- than in *M. laxa*-inoculated samples (Fig. 1A).
211 However, when treatments were applied, a different tendency was observed for both
212 species. In *M. laxa*-inoculated petals brown rot incidence was significantly higher (1.6-fold
213 and 2.2-fold, respectively) when blossom petals were submitted to both 1-MCP and

ethylene treatment if compared to the control (air treatment). A completely different pattern was observed for *M. fructicola*, since a decrease of 1.3-fold and 1.6-fold in brown rot incidence occurred in both 1-MCP and ethylene treatments respectively, if compared to non-treated condition (Fig. 1A). Although no differences were observed regarding the incidence of both species when exogenous ethylene was applied, brown rot caused by *M. fructicola* was still significantly higher (1.8-fold) than that observed for *M. laxa* in 1-MCP treated petals (Fig. 1A). Brown rot of both *M. fructicola* and *M. laxa* inoculated petals consisted of necrotic lesions around the inoculation point (Fig. 1B). Visual inspection of *Monilinia* infection patterns at 72 hpi led us to confirm that *M. fructicola* species presented a higher severity than *M. laxa* in the control condition when infecting 'Merrill O'Henry' peach petals. However, *M. laxa* aggressiveness was clearly enhanced in petals treated with both 1-MCP and ethylene if compared to the control (Fig. 1B). In any case, sporulation of neither *M. laxa* nor *M. fructicola* was observed on peach petals.

3.2. Effect of 1-MCP and ethylene treatment on petal ethylene production and senescence

The effect of both 1-MCP and exogenous ethylene on ethylene production of detached peach petals was assessed by applying two different doses of 1-MCP (0.6 and 5 μL L^{-1}) and ethylene (1 and 10 μL L^{-1}), using air as a control (CK). Significant differences in the hormone production were recorded among times but also when comparing the different treatments (Fig 2A). At 5 h post-treatment (hpt), ethylene levels peaked in both control and 1-MCP treated petals, while this peak in 10 μL L^{-1} ethylene treated petals remained significantly lower (2-fold) if compared to control. At 16 hpt and up to 24 hpt, ethylene production started to decrease with respect to 5 hpt. At these time points, only 1-MCP treated petals (0.6 μL L^{-1}) sustained its production, being significantly higher than control and ethylene treated petals (1.31- and 2.8-fold, respectively). However, the profile changed

239 at 48 hpt and particularly, at 72 hpt when blossoms treated with high doses of 1-MCP (5 µL
240 L^{-1}) and submitted to both ethylene treatments (1 and 10 µL L^{-1}), experienced a significant
241 increase in their ethylene production levels (2.5-fold, 2.4-fold and 2-fold, respectively) if
242 compared to control (Fig. 2A). For all treatments, respiration rate was greatest at 0 hpi and
243 constantly decreased thereafter (Fig. 2B). Despite some minor yet significant differences
244 were found between treatments at different time points, the respiratory patterns were very
245 similar among treatments. The induction of ethylene levels upon 1-MCP and ethylene
246 applications did not seem to be associated to a senescence process, since no apparent
247 morphological changes typically associated to senescence [30], such as colour bleaching or
248 petal rolling and wilting, were visualized in the control and/or the different treatments (Fig.
249 2C).
250
251 **3.3. *In vitro* treatment of *Monilinia* species with exogenous ethylene and 1-MCP**
252 To evaluate the performance of *Monilinia* upon the 1-MCP and ethylene treatments and to
253 complement the results observed *in vivo*, the same treatments applied to the branches
254 containing multiple inflorescences were also conducted to *in vitro M. laxa and M. fructicola*
255 cultures. Results demonstrated that both doses of 1-MCP (5 and 0.6 µL L^{-1}) and ethylene (1
256 and 10 µL L^{-1}) had no effect on *M. laxa* growth rate (Fig. 3A) or on phenotypical features
257 (Fig. 3B). Only at 0 hpt, significant changes were obtained among treatments, although
258 such differences completely subsided through time. The same lack of treatment effect was
259 also observed on *M. fructicola* cultures, although *M. fructicola* grew more rapidly (1.53 cm
260 day^{-1}) than *M. laxa* (1.04 cm day^{-1}) species (Fig. 3C). Again, although some significant
261 differences seemed to appear at 24 hpt and 48 hpt among treatments, no differences on the
262 final growth of *M. fructicola* were detected. Moreover, as observed for *M. laxa*, the colony
263 phenotype was identical for all tested conditions (Fig. 3D), both at 24 hpt and at the end of

the assay (6 dpt). Hence, such treatments had no effect on either *M. laxa* or *M. fructicola*, as no differences on growth and colony features including colour, morphology and sporulation were observed.

3.4. Effect on peach petal ethylene biosynthetic pathway and host response upon *Monilinia* spp. inoculation

Taking into account that changes in the host (petal) ethylene profile in response to 1-MCP or ethylene differently affected the incidence of both *Monilinia* species, a deeper analysis to decipher ethylene mediated responses in 'Merrill O'Henry' peach petals when inoculated with both *M. laxa* and *M. fructicola* was performed.

Regarding ethylene biosynthetic genes, two *PpACS* and three *PpACO* were analysed (Fig. 4). In general, results demonstrated a completely different pattern among the control and both *Monilinia* infected petals. As refers to *PpACS1* (Fig. 4 and Fig. S1A), expression levels seemed to increase along the time course of the experiment, specially, for both *M. laxa* and *M. fructicola* inoculated petals, in which a significant and important up-regulation of this transcript levels occurred at 72 hpi in comparison to 0 hpi (5.1-fold and 9.4-fold, respectively). When comparing the expression profile between conditions in each time point, *M. laxa* showed no significant changes if compared to control, although a tendency to up-regulation of *PpACS1* levels (5.9-fold) occurred at 72 hpi. In contrast, *M. fructicola* induced an early and significant up-regulation at 5 hpi (3.7-fold), which was even enhanced at 72 hpi (10.8-fold). *PpACS2* transcript levels (Fig. 4 and Fig. S1A) were also up-regulated along the time in all tested conditions, showing a similar pattern to that observed for *PpACS1* yet with higher gene expression. *M. laxa* significantly induced *PpACS2* transcripts levels at 24 hpi (4.4-fold) and an important up-regulation took place at 72 hpi (6.8-fold if compared to control), while response to *M. fructicola* occurred earlier.

PpACO family members recorded a completely different profile than *PpACS* (Fig. 4 and Fig. S1B). Regarding *PpACO1*, and even if it was the most expressed among the other family members, expression levels merely changed over time, and only a significant up-regulation with respect to 0 hpi occurred at 24 hpi (1.5-fold) and 72 hpi (1.3-fold) for *M. laxa* and *M. fructicola*, respectively. The comparison of the expression pattern among the three tested conditions revealed that only *M. laxa* triggered a significant up-regulation (1.4-fold) of *PpACO1* at 24 hpi. On the other hand, even though *PpACO2* reported no significant differences between control and *Monilinia* inoculated petals at any time point analysed, it was significantly up-regulated over time in the three tested conditions. Accordingly, an increase of 7.8-, 8- and 9.3-fold occurred at 72 hpi in control, *M. laxa* and *M. fructicola* inoculated petals, respectively, if compared to 0 hpi. Contrary to that observed for the other paralogs, expression levels of *PpACO3* remained lower than that of *PpACO2* (for instance, 35.5-fold less expressed in the control condition at 0 hpi) and showed no differences on its expression levels between conditions at any time point. Besides, transcript levels were mainly stable over time, observing only a significant reduction at 24 hpi (1.4-fold) and 72 hpi (1.5-fold) with respect to 5 hpi in *M. fructicola* inoculated petals.

Changes on *PpACS* and *PpACO* gene families could ultimately lead to distinct hormone production levels. In fact, different ethylene profiles were found depending on the inoculated species (Fig. 4). Control petals displayed an increase of ethylene levels at 5 hpi, likely associated to a stress-induced ethylene peak, which decrease thereafter and remain low and stable during all the time course of the experiment. On the other hand, in *M. laxa* inoculated petals, the ethylene production remained quite constant over time up to 16 hpi but increased later, observing 7-fold significantly higher levels at 72 hpi if compared to control. Contrary to what observed for *M. laxa*, *M. fructicola* induced a transient ethylene stress peak at 5 hpi, as also observed in the control, but thereafter, ethylene levels decreased

314 and remained low until 48 hpi to later (72 hpi) increase again (15-fold higher if compared to
315 the control). Thus, while both control and *M. fructicola* inoculated petals enhanced its
316 production at 5 hpi, *M. laxa* inoculated petals exhibited a completely different profile at this
317 time point, with a strong inhibition (41.3-fold if compared to control) of the ethylene
318 production levels.
319 Such ethylene levels could ultimately lead to different ethylene-dependent responses which
320 are mediated by ERFs. Concerning such transcription factors, we examined changes in the
321 transcript profile of the two evolutionary divergent groups (Fig. 4). *PpERF1a* exhibited a
322 similar profile with no significant differences among all the analysed conditions (Fig. 4 and
323 Fig. S2A). Only at 72 hpi, a repression (1.7-fold) induced by *M. fructicola* took place if
324 compared to 24 hpi, although no differences were reported among the different tested
325 conditions at any time point. As regards to *PpERF1b*, some differences on its expression
326 profile were obtained along the time course of the experiment (Fig. 4 and Fig. S2A). Hence,
327 the three conditions recorded significant changes at any time if compared to 0 hpi. At 24
328 hpi, both *M. laxa* and *M. fructicola* triggered a 2.2-fold and a 2.3-fold up-regulation of these
329 transcripts levels, respectively, if compared to control. Notwithstanding, only *M. fructicola*
330 was able to hold this induction until 72 hpi. On the other hand, *PpERF2a* in control petals
331 remained stable at 5 hpi, but experienced an up-regulation (1.3-fold) at 24 hpi followed by a
332 significant down-regulation (1.9-fold) at 72 hpi (Fig. 4 and Fig. S2B). Similarly, petals
333 inoculated with *M. laxa* also showed a 1.9-fold down-regulation of *PpERF2a* levels from
334 24 to 72 hpi, contrary to *M. fructicola* inoculated petals, for which *PpERF2a* was stable
335 among all the analysed times. When comparing the different conditions in each time point,
336 a significant reduction (1.6-fold) in its expression levels if compared to control occurred at
337 24 hpi when peach petals were inoculated with *M. fructicola*. Such tendency changed
338 abruptly at 72 hpi when *PpERF2a* levels were significantly higher (1.7-fold) in *M.*

fructicola-inoculated petals in comparison to the control (Fig. 4 and Fig. S2B). *PpERF2b* did not show significant changes neither along the time nor among the different tested conditions at both 5 hpi and 72 hpi. However, a significant up-regulation of this transcript (1.1-fold) took place at 24 hpi in *M. laxa* inoculated tissue, while a repression of 1.2-fold and 1.4-fold was promoted by *M. fructicola* if compared to the control and *M. laxa* inoculated petals, respectively. Finally, although no differences on *PpERF2c* expression profile were detected at any time point among the three tested conditions, an over-expression of the transcript occurred through time. A 6.1-fold induction of its expression levels from 0 hpi to 24 hpi took place in the control condition and, to a lesser extent, in *M. laxa* (4.4-fold) and *M. fructicola* (3.6-fold) inoculated petals. However, while control petals clearly down-regulated *PpERF2c* levels at 72 hpi with respect to 24 hpi (2.3-fold), petals inoculated with both *M. laxa* and *M. fructicola* showed a sustained induction (Fig. 4 and Fig. S2B).

Differences on ERFs transcription factor expression give rise to different ethylene responsive genes that ultimately can impair host ability to cope with the presence of pathogens. In this way, different susceptibility patterns to both *Monilinia* species were observed in infected peach petals (Fig. 4). Although no differences were recorded at 24 hpi between both fungi, significant differences in brown rot incidence were obtained after 72 hpi in *M. laxa* (33 %) and *M. fructicola* (57.8 %) inoculated peach petals. Besides, the incubation period of *M. fructicola* was shorter than for *M. laxa*, as *M. fructicola* infection symptoms were visible before 24 hpi, opposite to *M. laxa*, which symptoms were detected only at 48 hpi and thereafter.

4. Discussion

Both ethylene biosynthetic pathway and ethylene response factors are known to be involved in the response of plants and fruit to abiotic and biotic stress conditions [31]. In this study,

we performed an in-depth analysis to determine the role of ethylene in the interaction peach petals – *Monilinia* spp. For the first time, we characterized the ethylene effects on both host and pathogen, as well as the peach petal responses, both at the physiological and molecular level, including ethylene biosynthesis and ethylene-mediated responses upon *M. fructicola* and *M. laxa* interaction.

1-MCP is a synthetic cyclic olefin capable of inhibiting ethylene perception and has been widely used to control fruit ripening and senescence and hence, prolonging fruit shelf life and lengthening the life of cut flowers [32]. Seglie *et al*. [33] already demonstrated the benefits of 1-MCP on inhibiting the detrimental effects of *Botrytis cinerea* on petals of cut flowers. In this work, significant differences were found between the response of two fungal species to 1-MCP or ethylene treatment. Hence, while *M. fructicola* infection capacity was reduced in response to these treatments, *M. laxa* took advantage of it and showed enhanced infection capacity.

1-MCP applied to branches containing multiple inflorescences did not inhibit the ethylene production of subsequently detached petals but rather increase it, especially when high doses of 1–MCP were applied. A similar effect was described in grapefruit [34] and flowers [35] where 1-MCP application significantly enhanced ethylene production. Such induction could be explained by the fact that 1-MCP shuts down the system of feedback regulation of ethylene biosynthesis pathway by binding to physiological ethylene receptors [36], inhibiting the ethylene binding protein [37] and hence disabling the host to perceived any ethylene quantities already synthetized. Moreover, exogenous ethylene treatment also enhanced ethylene production, in agreement with previous results in both climacteric and non-climacteric systems [35,38], probably through the induction of *ACS* expression [39] and/or ethylene receptors [40].

Results presented herein demonstrate that *M. fructicola* infection capacity was reduced in response to treatments accompanying the increase in ethylene host production, while *M. laxa* took advantage of it and showed an enhanced infection capacity. Hence, results point out a differential role of ethylene on the pathogen infection strategy and a different ability of both species to overcome the induction of ethylene-dependent responses displayed by the host [41]. A previous study determined that continuous application of ethylene on *M. fructicola* inoculated stone fruit do not exhibit any changes neither on brown rot incidence nor severity [42]. However, the discrepancy with the study presented herein could be related to the fact that such measurements were performed on fruit and during cold storage conditions. To further investigate the role of both 1-MCP and ethylene, not on the host itself but on *Monilinia* spp. performance, *M. laxa* and *M. fructicola* cultures were also submitted to these treatments. Results revealed that phenotype of both fungi, including its growth and colony morphology, were not affected by the treatments. Overall, our data indicates that differences on brown rot incidence in treated petals were not due to the treatment effects on growth and phenotype of the fungi, but uniquely dependent on the response of the host to the treatment itself.

In this sense, an in-depth analysis of the host responses when inoculated with the two *Monilinia* species was carried out. In such analysis, the different steps of the host ethylene biosynthetic pathway at the molecular level, including ethylene production and the subsequent ethylene-dependent response triggered by the host to brown rot were considered. Incidence of brown rot in peach petals caused by *M. fructicola* was significantly higher if compared to *M. laxa* at 72 hpi as previously described by Bernat *et al*. [2] in stone fruit. Besides, the shorter incubation period of *M. fructicola* if compared to *M. laxa* (less than 24 hpi and more than 24 hpi, respectively) agreed with previous results on peach fruit [9] and could be related to specific aggressiveness components of this fungus [43].

413 Characterization studies on stone fruit has determined that, compare to other species, *M.*
414 *fructicola* is the most aggressive and has the fastest growth rate [9]. Notwithstanding, while
415 *M. fructicola* is more common on fruit, *M. laxa* was found to be equally present both in
416 blossoms and fruit [10], probably due to the better performance of *M. laxa* at lower
417 temperatures [11,12], such as those occurring during the flowering period.
418 When analysing the host ethylene profile during the interaction, a massive production of
419 ethylene at 5 hpi was observed for both the control and *M. fructicola* inoculated petals.
420 However, *M. laxa* when interacting with its host induced a completely different profile, as
421 an inhibition of ethylene production occurred. Such increased in ethylene levels at 5 hpi
422 was likely due to a stress response caused by the petal detachment from the blossom, but
423 the different modulation of this ethylene stress associated peak by *Monilinia* spp. warrants
424 further investigation. We cannot discard that the fungus may be modulating the hormone
425 production to prevent the activation of defence responses. In fact, although no effectors are
426 yet characterized for *Monilinia* spp., in other pathogens such as *Pseudomonas syringae*, it
427 was described its ability to produce coronatine, a jasmonic acid like compound that
428 suppresses salicylic-acid-mediated defence response [44,45]. In line with these results, a
429 recently published study also demonstrated that the interaction of nectarine fruit with the *M.*
430 *laxa* ML8L strain also exhibit a decrease on ethylene production levels at early time points
431 [8, Balsells-Llauradó *et al*., unpublished results]. Hence, we demonstrated that this altered
432 ethylene response is conserved among the *Prunus persica* host players regardless of their
433 climacteric or non-climacteric nature and that not the fungus but the host itself was altering
434 its ethylene production pattern in an attempt to avoid or slow down the *M. laxa* progression.
435 To determine a possible molecular regulation of the ethylene production, gene expression
436 analysis of different ACS and ACO paralogs involved in the hormone biosynthesis were
437 further analysed. As reported in earlier studies, either on apples [46] or in peach cultivars

[47] *ACS1* is also the rate-limiting enzyme for the observed petals ethylene production. A significant up-regulation of both *PpACS1* and *PpACS2* occurred in *M. laxa* and *M. fructicola* infected peach petals if compared to the control, coinciding with those time points when an ethylene burst occurred. A similar up-regulation of these genes was previously reported in peach fruit infected with *Monilinia* spp. [8]. The *ACO* gene family also completes ethylene biosynthesis. Similar expression level patterns were found between the different conditions analysed. An induction of *PpACO1* levels took place in petals inoculated with *M. laxa*, only at 24 hpi coinciding with the start of the increase of ethylene production. However, such induction did not occur in *M. fructicola* inoculated peach petals neither at 24 hpi nor at early time points, although the ethylene production pattern was similar in both species from 16 hpi to 48 hpi. Besides, expression levels of *PpACO2*, which seems not to be strictly involved in the transition from system-I to system-II [27], were clearly lower compared to *PpACO1*, and were induced over time, irrespective of the inoculated condition. Contrary to this pattern, *PpACO3*, also involved in the transition from system-I to system-II remained stable along the time course of the experiment. Vilanova *et al*., [17] demonstrated the ability of *Penicillium expansum* to alter *MdACO3* in the apple-*Penicillium* pathosystem in an attempt of the fungi to infect the host. The fact that *PpACO3* levels remained stable upon the infection with *Monilinia* spp. demonstrates that both the ability to modify ethylene production and the mechanism involved are not ubiquitous for all fungal species. Although molecular changes described were generally in line with the subsequent ethylene production profile, there were no significant changes regarding ethylene biosynthesis genes on *M. laxa* inoculated petals that could evidence the observed inhibition at 5 hpi. A possible explanation could be that changes at molecular level occurred earlier than 5 hpi, as it is known that many responses including reactive oxygen species (ROS) accumulation occurred already at 4 hpi on rose petals infected with *M. fructicola*

463 [48]. In this sense, the role of the redox environment and its effects on the fungi
464 performance and the fungi ability to counteract the host response should be also considered
465 [49,50]. The same authors also reported that other pathogen responses such as expression of
466 *M. fructicola* polygalacturonases took also place as early as 4 hpi. A recently published
467 proteomic study revealed that 1-aminocyclopropane-1-carboxylate deaminase is increased
468 in peach fruits inoculated with *M. laxa* [51]. Thus, the increased abundance of this enzyme,
469 involved in the conversion of ACC to α-ketobutylate, could, in part, explain the reduced
470 ethylene levels observed in *M. laxa* inoculated blossoms. It is therefore evident that further
471 studies are needed to decipher the origin of the inhibition observed herein.

472 Plant-pathogen interactions can trigger a signalling cascade in which plant hormones are
473 involved, and that ultimately, will lead to the activation of the plant defence responses
474 [49,52]. The activation of ethylene and jasmonic acid-mediated signalling commonly
475 occurs during necrotrophic interactions [14,53]. Indeed a synergistic interaction between
476 both ethylene and jasmonate has been described in the *B. cinerea*-tomato pathosystem [54].
477 Such crosstalk converge in the activation of *ERF*s, and concretely, *ERF1* [25] as a way to
478 regulate plant defence response genes. Accordingly, we also examined the regulation of
479 *PpERF* expression upon the infection of peach petals with *M. laxa* and *M. fructicola*. All
480 tested conditions showed differences on *PpERF1b* but not in *PpERF1a* expression levels.
481 Any changes in *PpERF1b* occurred at 5 hpi when infection symptoms were not already
482 visible. However, an induction took place at 24 hpi in both *M. laxa* and *M. fructicola*
483 inoculated petals, concomitantly with the onset of petal necrosis, and hence likely indicating
484 the activation of the cascade response, in a failed attempt of the host to overcome the fungal
485 disease. A previous study [22] demonstrated that *PpERF1a* is highly induced in a resistant
486 peach cultivar when inoculated with *Xanthomonas campestris* (*Xcp*) pointing out the
487 importance of *PpERF1a* on the outbreak of the disease. Although the differences on the

pathosystem should be considered and hence, the associated responses, the fact that our results showed no changes on *PpERF1a* could in part explain the susceptibility of peach petals to *Monilinia* spp. and the ability of the pathogens to overcome the *PpERF1b*-mediated activation of the ethylene response cascade. The same authors [22], also demonstrated that members of this group are mainly induced by methyl jasmonat (MeJA) and ethephon treatments, being the treatment with the ethylene analogue the one showing more rapid and stronger effects. However, in our results, the expression profile of *PpERF1b* seemed to be not related to the host ethylene production. Only at 72 hpi, the induced ethylene levels mediated by *M. fructicola* lead to an overexpression of *PpERF1b*, the same time point when brown rot incidence was significantly different between the two *Monilinia* species.

As refers to both *PpERF2a* and *PpERF2b*, *M. fructicola* triggered a down-regulation of these transcripts levels at 24 hpi, which could in part explain, the greater incidence and severity observed for this species. Thus, although *M. fructicola* inoculated peach petals overexpressed *PpERF2a* levels at 72 hpi, the infection was already established as the fungus already colonized completely the tissue. Contrary to the other family members, *PpERF2c* remained stable between conditions at all times analysed. Overall, these results led us to conclude that peach petals modulate the expression of *PpERF1b*, *PpERF2a* and *PpERF2b* in response to *Monilinia* spp. infection in a species-specific dependent manner. Sherif *et al*. [22] described that while a resistant peach cultivar showed higher *PpERF2a* and *PpERF2c* gene expression, *PpERF2b* exhibited a contrary profile. Such discrepancies between our and previous results could be, in part, related to differences on the pathosystem itself. *Monilinia* is a necrotrophic fungus, while *Xcp* is considered a hemibiotrophic bacterium. Differences on pathogen-associated molecular patterns (PAMPs) specific of each type of pathogen may lead to a differentially activation of the host signalling cascades

513 [55]. Hence, the strength, the intensity and ultimately, the effectiveness of effector-triggered
514 immunity (ETI) and PAMP-triggered immunity (PTI) responses, and therefore the defence
515 activation, could be different and dependent on the interaction [56]. Consequently, the
516 differentially altered cascade described in the petals-*Monilinia* spp. interaction potentially
517 leads to different ability of peach petal to cope with both species infection.

518 **5. Conclusions**

519 To the best of our knowledge, this is the first study providing a detailed description of the
520 mechanisms underlying the interaction between *Monilinia* spp. and peach petals as well as
521 demonstrating: (1) the importance of ethylene on such interaction and its species-specific
522 effect and (2) the different ability of both *Monilinia* species to overcome the host ethylene
523 response. Importantly, we described the different ability of either *M. laxa* or the host to
524 inhibit the ethylene production, a mechanism conserved among the different phenological
525 stages of peach, regardless of their climacteric or non-climacteric nature. Besides,
526 *PpERF1b*, *PpERF2a* and *PpERF2b* were differently modulated in response to *Monilinia*
527 spp. in a species-specific dependent manner, although ethylene inhibition in *M. laxa*
528 inoculated petals did not altered the ERF gene expression. Further studies to decipher the
529 cause of such modulation and specifically of the observed inhibition are encouraged. A
530 better understanding of the interaction between *Monilinia* spp. and peach petals occurring at
531 the peach flowering stage can lead to reduced fruit losses on the field, together with more
532 specific and rational brown rot management strategies.

533

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Conflict of interests

All authors declare no conflict of interest.

Author contributions

RT, NV and JGB conceived and designed the experiments. NV carried out the experiments and wrote the manuscript. JGB and RT assisted in reviewing and editing the manuscript. JU and NT assisted the statistical analysis. JGB, RT and CL supervised all experimental procedures. All authors contributed in improving the final version of the manuscript.

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List of Figures:

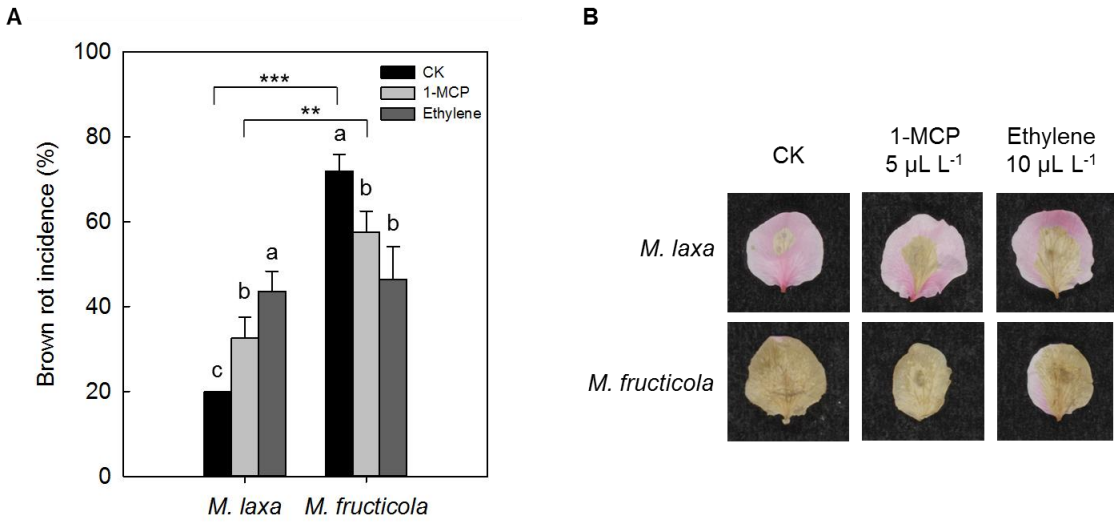
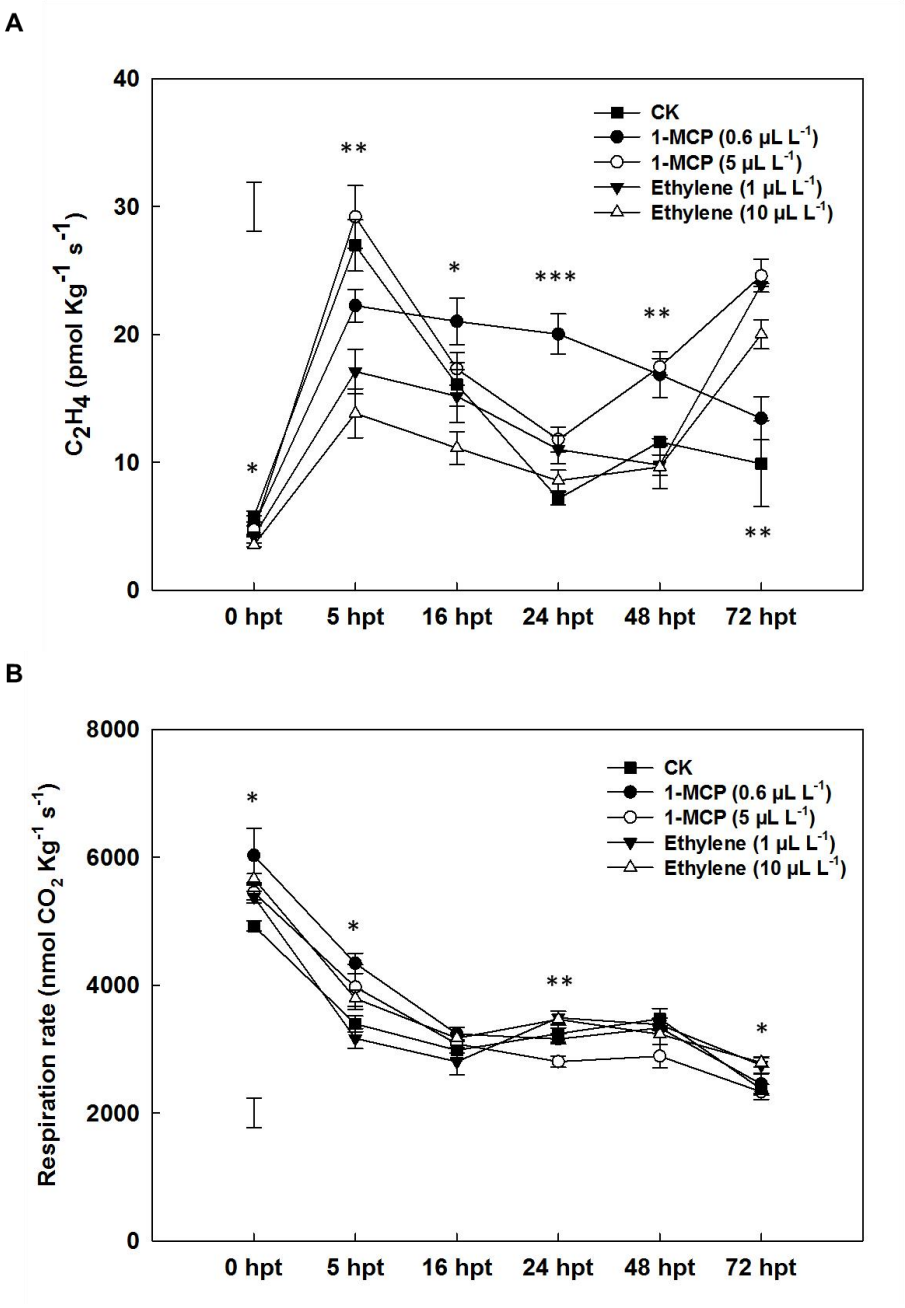


Figure 1. Brown rot susceptibility in 1-MCP and ethylene treated petals. Changes in brown rot susceptibility (A) and phenotypic differences (B) of 'Merrill O'Henry' peach petals inoculated with *M. laxa* and *M. fructicola* after the treatment with air (CK) (■), 5 µL L^{-1} of 1-MCP (■) and 10 µL L^{-1} of ethylene (■) for 20 h at 3 ºC. Petals were detached from the branch and infected with a $5 \cdot 10^4$ conidia mL^{-1} suspension of *M. laxa* and *M. fructicola*, and incubated for 72 h at 20ºC and 85 % relative humidity. Each point represents the mean of four biological replicates and vertical bars indicate the standard error of the mean. Different letters indicate significant differences between treatments ($p < 0.05$). For each treatment, asterisks denote significant differences between *M. laxa* and *M. fructicola* (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$).


A
40
30
20
10
0
C_2H_4 (pmol Kg^{-1} s^{-1})
CK
1-MCP (0.6 μL L^{-1})
1-MCP (5 μL L^{-1})
Ethylene (1 μL L^{-1})
Ethylene (10 μL L^{-1})
*
**
*
***
**
**
0 hpt
5 hpt
16 hpt
24 hpt
48 hpt
72 hpt
B
8000
6000
4000
2000
0
Respiration rate (nmol CO_2 Kg^{-1} s^{-1})
CK
1-MCP (0.6 μL L^{-1})
1-MCP (5 μL L^{-1})
Ethylene (1 μL L^{-1})
Ethylene (10 μL L^{-1})
*
*
**
*
0 hpt
5 hpt
16 hpt
24 hpt
48 hpt
72 hpt
C
5 hpt
24 hpt
48 hpt
72 hpt
6 dpt
CK
1-MCP
5 μL L^{-1}
Ethylene
10 μL L^{-1}

773

774 **Figure 2. Ethylene and respiration profile of 1-MCP and ethylene treated petals.**
775 Ethylene production (A), respiration (B) and phenotypic differences (C) of 'Merrill
776 O'Henry' peach petals treated with 1-MCP or ethylene at different h post-treatment
777 (hpt). Branches containing multiple inflorescences were treated with air (CK), 0.6 and 5
778 µL L^{-1} of 1-MCP, and with 1 and 10 µL L^{-1} of ethylene for 20 h at 3 ºC. Each
779 point represents the mean of four biological replicates and vertical bars indicate the
780 standard error of the mean. For each time post-treatment, asterisks denote significant
781 differences between treatments (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$). Individual error
782 bars depict the LSD for the interaction treatment*time.
783

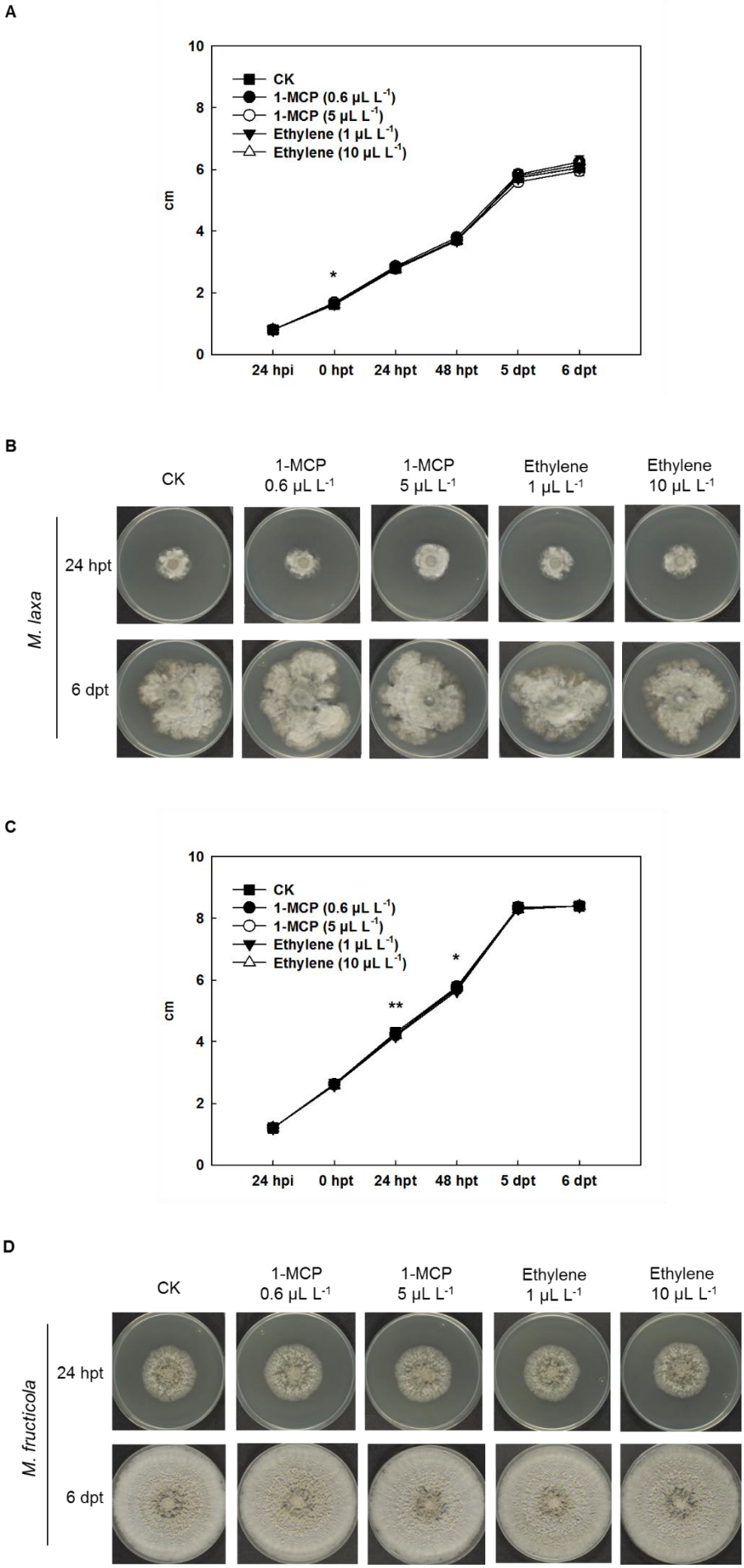

A
CK
1-MCP (0.6 μL L-1)
1-MCP (5 μL L-1)
Ethylene (1 μL L-1)
Ethylene (10 μL L-1)
cm
24 hpi
0 hpt
24 hpt
48 hpt
5 dpt
6 dpt
B
CK
1-MCP 0.6 μL L-1
1-MCP 5 μL L-1
Ethylene 1 μL L-1
Ethylene 10 μL L-1
M. laxa
24 hpt
6 dpt
C
D
M. fructicola

Figure 3. Effect of 1-MCP and ethylene application on *M. laxa* and *M. fructicola* growth. Plates of potato dextrose agar (PDA) were inoculated by applying a drop of 10 µL at 10^5 conidia·mL^{-1} and incubated in the dark at 25 ºC for 24 h. After this time, inoculated plates were treated with air (CK), 0.6 and 5 µL L^{-1} of 1-MCP, and with 1 and 10 µL L^{-1} of ethylene for 20 h at 20 ºC. Growth (cm) for *M. laxa* (A) and *M. fructicola* (B) was recorded at different h/days post treatment (hpt/dpt). Asterisks denote significant changes among treatments (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$). Phenotypic differences among treatments for *M. laxa* (B) and *M. fructicola* (D) were visually inspected.

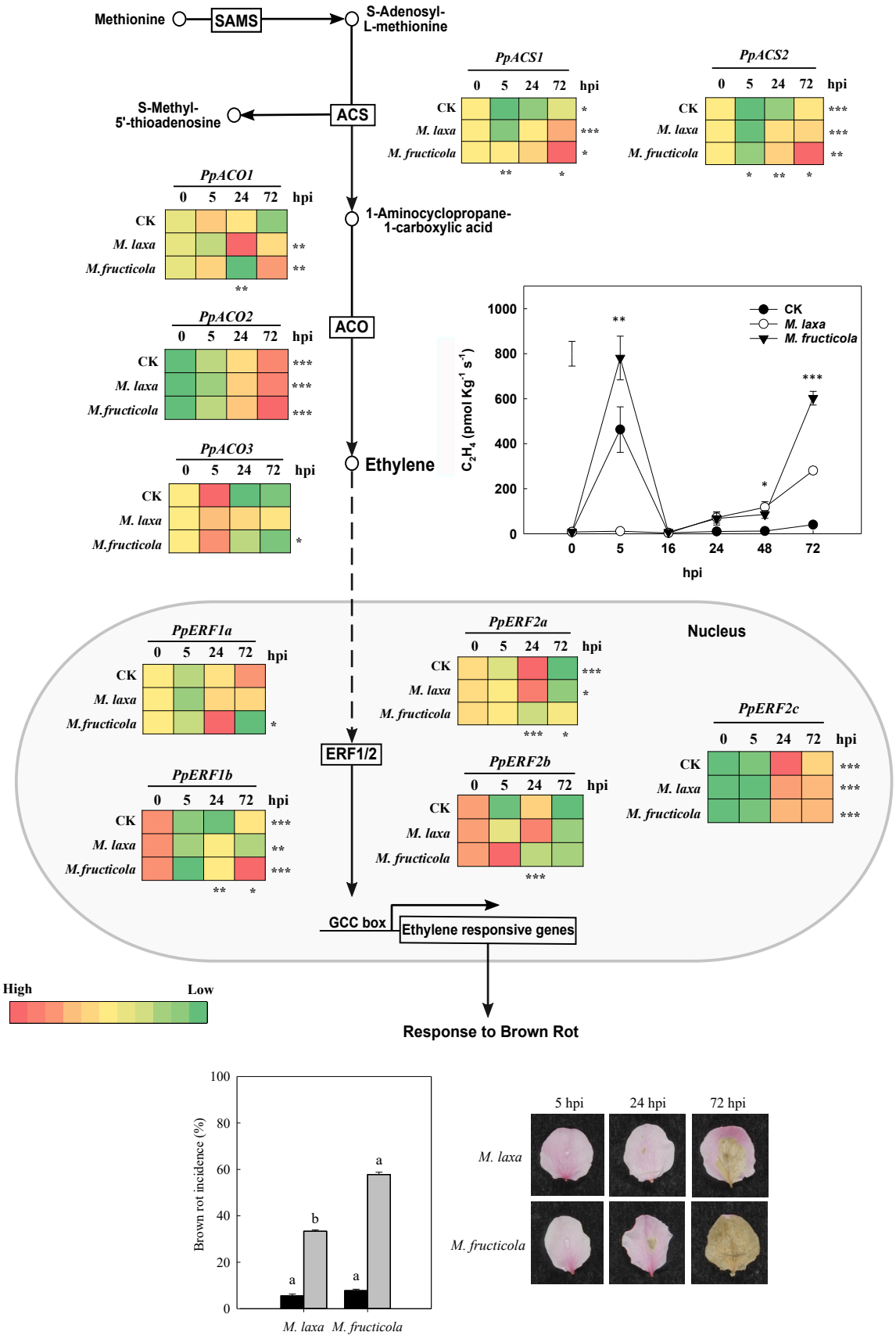
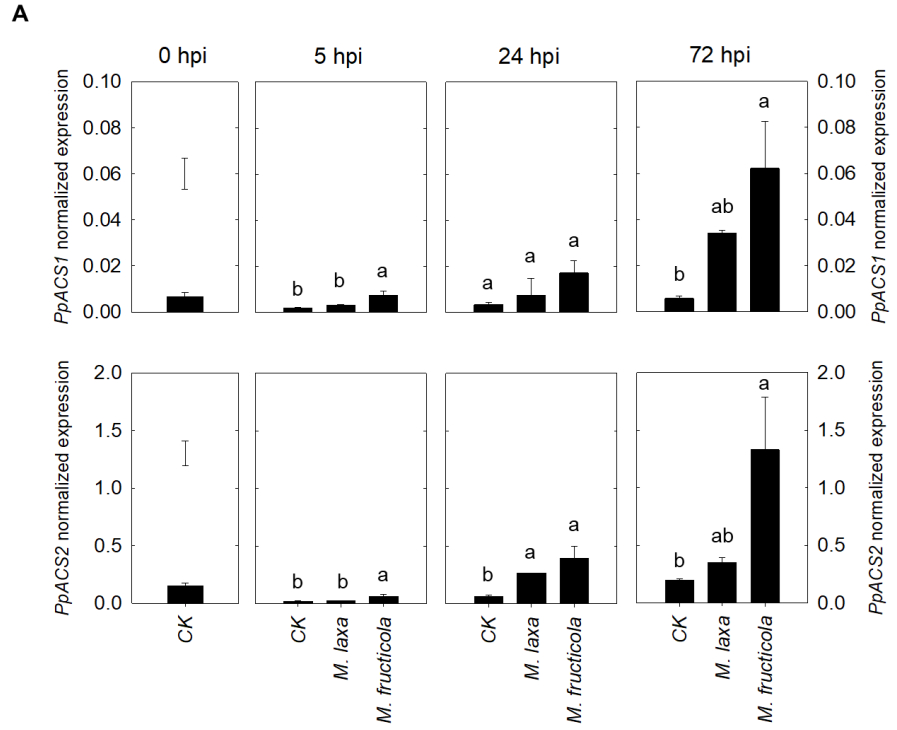

Methionine
SAMS
S-Adenosyl-
L-methionine
S-Methyl-
5'-thioadenosine
ACS
1-Aminocyclopropane-
1-carboxylic acid
ACO
Ethylene
PpACS1
PpACS2
PpACO1
PpACO2
PpACO3
0 5 24 72 hpi
CK
M. laxa
M.fructicola
M. fructicola
C2H4 (pmol Kg-1 s-1)
hpi
Nucleus
PpERF1a
PpERF1b
PpERF2a
PpERF2b
PpERF2c
ERF1/2
GCC box
Ethylene responsive genes
High
Low
Response to Brown Rot
Brown rot incidence (%)
M. laxa M. fructicola
5 hpi
24 hpi
72 hpi

Figure 4. Ethylene biosynthetic pathway and ethylene-dependent ERF response. The heat maps represent changes in the relative gene expression of ACS (1-Aminocyclopropane-1-carboxylic acid synthase), ACO (1-Aminocyclopropane-1-carboxylic acid oxidase) and ERF (Ethylene response factor) family members after 5, 24 and 72 h post-inoculation (hpi) with CK (mock-inoculated), *M. laxa* and *M. fructicola*. The scale colour of the heat maps represents the intensity of the relative gene expression. Dashed lines indicate that some steps within the pathway have been omitted. For each gene, asterisks indicate significant differences among inoculums (bottom) and h post-inoculation (left) (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$). Ethylene production at different times post-inoculation, brown rot susceptibility after 24 h (■) and 72 h post-inoculation (■) and phenotypic differences of peach petals to both *M. laxa* and *M. fructicola* are also shown. Different letters indicate significant differences between both fungi at each incubation time ($p \leq 0.05$). Individual error bar depicts the LSD for the interaction inoculum*time.

Supplementary data:



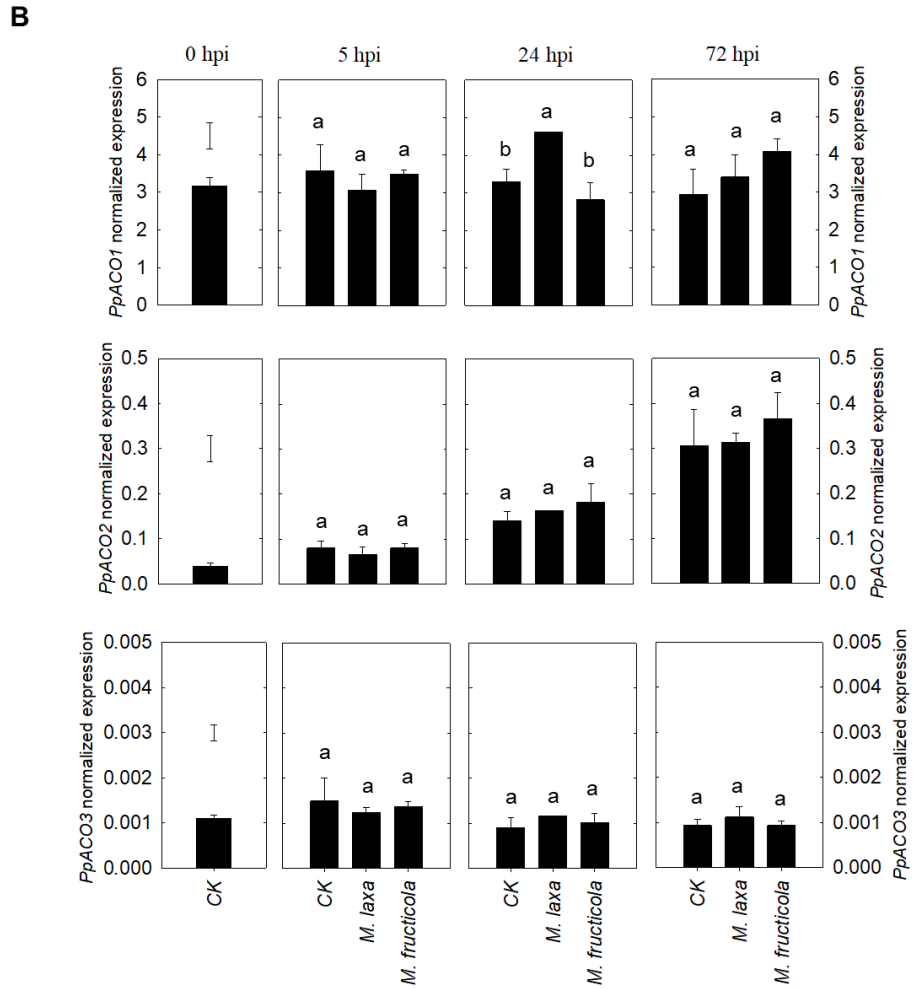
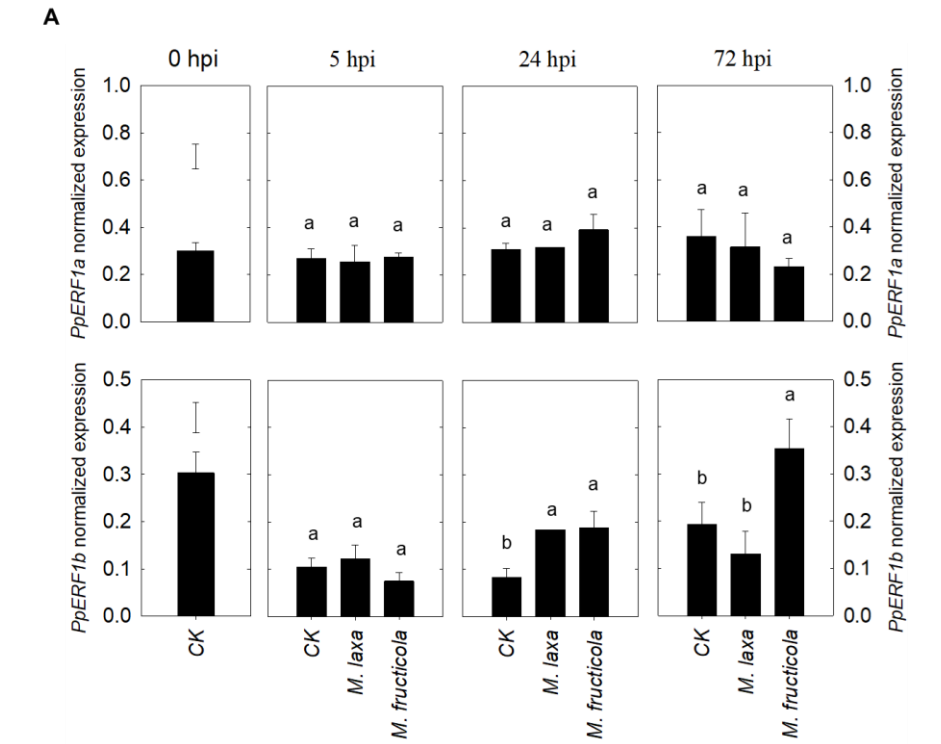


Figure S1. Relative gene expression of *ACS* and *ACO* gene families. Changes on relative gene expression of (A) *ACS* (1-Aminocyclopropane-1-carboxylic acid synthase) and (B) *ACO* (1-Aminocyclopropane-1-carboxylic acid oxidase) family members after 5, 24 and 72 h post-inoculation (hpi) with CK (mock-inoculated), *M. laxa* and *M. fructicola*. Each point represents the mean of three biological replicates and vertical bars indicate the standard error of the mean. Different letters indicate significant differences between conditions at each time point ($p \leq 0.05$). Individual error bars depict the LSD for the interaction inoculum*time.

822


A
0 hpi
5 hpi
24 hpi
72 hpi
PpERF1a normalized expression
PpERF1b normalized expression
CK
M. laxa
M. fructicola

823

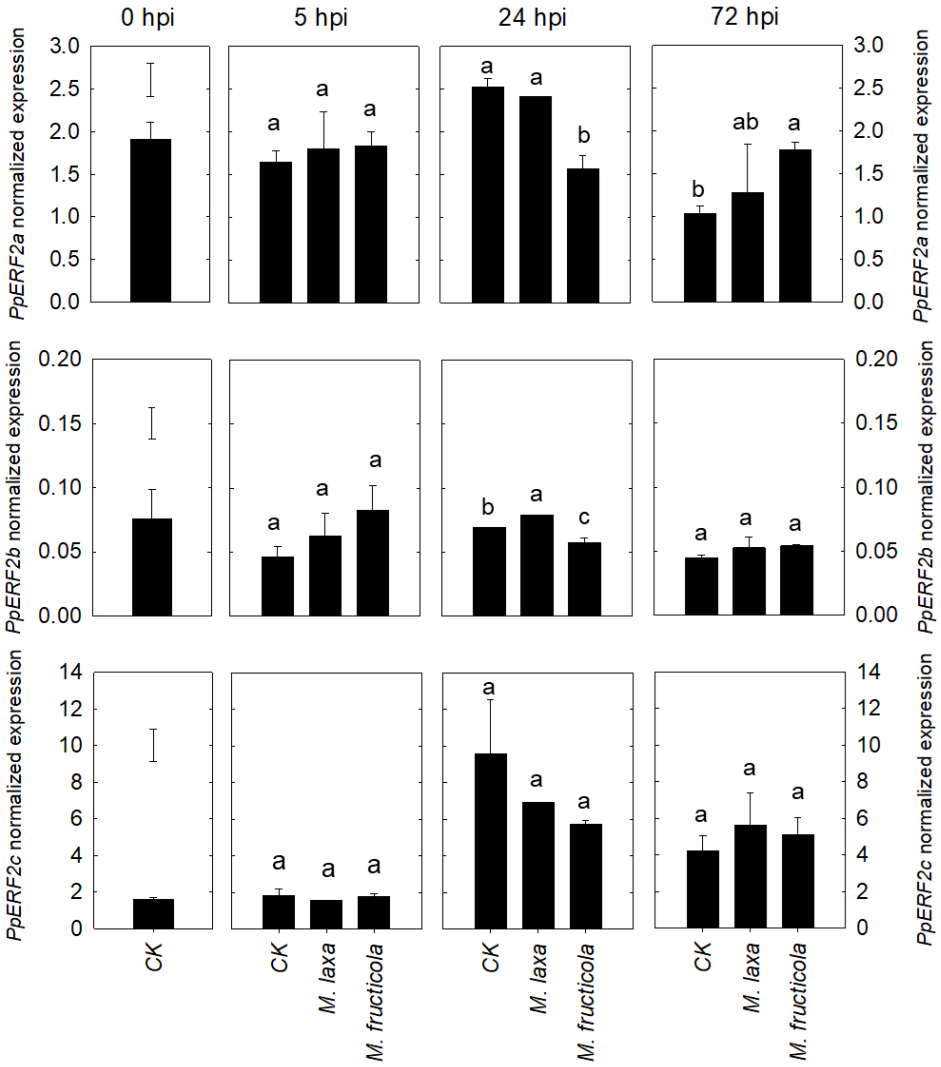

0 hpi
5 hpi
24 hpi
72 hpi
PpERF2a normalized expression
PpERF2b normalized expression
PpERF2c normalized expression
CK
M. laxa
M. fructicola

Figure S2. Relative gene expression of *ERF* families. Changes on relative gene expression of (A) *ERF1* (Ethylene response factor) and (B) *ERF2* family members after 5, 24 and 72 h post-inoculation (hpi) with CK (mock-inoculated), *M. laxa* and *M. fructicola*. Each point represents the mean of three biological replicates and vertical bars indicate the standard error of the mean. Different letters indicate significant differences between conditions at each time point ($p \leq 0.05$). Individual error bars depict the LSD for the interaction inoculum*time.

Measurement	Figure	Flowering season	Number of biological replicates	Number of petals/plates for replicate	Total samples for each condition
Incidence of 1-MCP and ethylene treated blossoms	Fig. 1	2019	4 replicates	10 petals	40 petals
Ethylene production and respiration of 1-MCP and ethylene treated blossoms	Fig. 2	2019 and 2020	4 replicates	10 petals	40 petals
***Monilinia in vitro* treatments with 1-MCP an ethylene**	Fig. 3	2019	3 replicates	3 plates	9 plates
Gene expression analysis	Fig. 4	2020	3 replicates (+ 3 technical replicates)	20 petals	60 petals
Ethylene production of inoculated petals	Fig. 4	2020	4 replicates	10 petals	40 petals
Incidence of inoculated petals	Fig.4	2020	4 replicates	10 petals	40 petals

Table S1. Scheme of the experimental design. The targeted measurement and its corresponding figure, the flowering season when de measurements were performed, the number of biological replicates (reps) for each condition, the number of petals and/or plates used for each replicate and the total of samples analysed for each measurement are indicated.

Gene	Primer	Sequence (5’ – 3’)	Efficiency	Source
PpACS1	Fw Rv	TGTTCAGCTCCCCGACTTTCAC TCTTGCGGCCGATGTTCACC	2.060	[27]
PpACS2	Fw Rv	TTTGAAGAACCCAGAAGCCTCCAT ATAACAATCCGGTCGGGGTCAAA	1.912	[27]
PpACO1	Fw Rv	CCCCCATGCGCCACTCCA CATCACTGCCAGGGTTGTAAAAG	2.089	[27]
PpACO2	Fw Rv	CAGCCGGATGGTACCAGAATGTC ACACAAATTTGGGGTAGGCTGAGA	1.975	[27]
PpACO3	Fw Rv	GAAGTCGACTGTCCATTGCTACCT TGCGGCCCTTGTCAGAAAA	2.090	[27]
PpERF1a	Fw Rv	CTCCTCGGTGGCTGAACAT AGTGGCAGCAGACCCATA	1.926	[22]
PpERF1b	Fw Rv	CGATTTCGGGTCCCATTT GCAAATCGCCCCAGTTTT	1.945	[22]
PpERF2a	Fw Rv	GCTGTCCCGCTGTATTTCA CTTGTTCTCCCCAGCCAAA	1.992	[22]
PpERF2b	Fw Rv	GGGGTTGAGCTCCATGAAT GAACCAGACCCGGAGTTTT	1.966	[22]
PpERF2c	Fw Rv	GGTGGCGAGATTTATGGAAA CCAGACCCAGGAACGATT	1.983	[22]
PpTEF2	Fw Rv	GGTGTGACGATGAAGAGTGATG TGAAGGAGAGGGAAGGTGAAAG	2.006	[28]
PpRPII	Fw Rv	TGAAGCATACACCTATGATGATGAAG CTTTGACAGCACCAGTAGATTCC	1.891	[28]

Table S2. List of primers used for RT-qPCR. From left to right: Gene name, Primer type (Forward/Reverse), Primer sequence (5’ – 3’), Primer efficiency (%) and Primer source.