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Stone fruit phenolic and triterpenoid compounds more significantly modulate gene expression of *Monilinia* spp. than its characteristics during *in vitro* growth

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Abstract:

Phenolic and triterpenoid compounds are essential components in stone fruit skin and flesh tissues. They are thought to possess general antimicrobial activity. However, regarding brown rot disease, investigations were only confined to a limited number of phenolics, especially chlorogenic acid. The activity of triterpenoids against *Monilinia* spp., as an essential part of the peach cuticular wax, has not been studied before. In this work, the anti-fungal effect of some phenolics, triterpenoids, and fruit surface compound (FSC) extracts of peach fruit were investigated at two developmental stages on *Monilinia fructicola* and *Monilinia laxa* characteristics during *in vitro* growth. A new procedure for assaying anti-fungal activity of triterpenoids, which are notoriously difficult to assess *in vitro* because of their hydrophobicity, has been developed. Measurements of colony diameter, sporulation, and germination of second-generation conidia were recorded. Furthermore, the expression of twelve genes of *M. fructicola* associated with germination and/or appressorium formation and virulence-related genes was studied relative to the presence of the compounds. The study revealed that certain phenolics and triterpenoids showed modest anti-fungal activity while dramatically modulating *M. fructicola* gene expression. *MfRGAE1* gene was overexpressed by chlorogenic and ferulic acids and *MfCUT1* by betulinic acid, at 4- and 7- days of mycelium incubation. The stage II FSC extract, corresponding to the period when the fruit is resistant to *Monilinia* spp., considerably up-regulated the *MfLAE1* gene. These findings effectively contribute to the knowledge of biochemical compounds effects on fungi on *in vitro* conditions.

Keywords:

Anti-fungal activity; gene expression; *Monilinia fructicola*; *Monilinia laxa*; Phenolics; Triterpenoids; Virulence-related genes

1. Introduction

Brown rot (BR), caused mainly by *Monilinia fructicola* (Wint.) Honey and *Monilinia laxa* (Aderh. & Ruhl.) Honey, is one of the most damaging fungal diseases of stone fruit worldwide (Byrde and Willetts, 1977; Landgraf and Zehr, 1982). Because resistant cultivars are not commercially available, fungicide remains the primary tool for reducing the incidence of BR (Rungjindamai et al., 2014). Ascospores and conidia are the primary sources of inoculum for *M. fructicola* and *M. laxa*, respectively, during seasonal crop infection cycles (De Cal et al., 2014; Martini and Mari, 2014). Dispersed conidia clinging to the fruit surface eventually germinate under suitable temperature and humidity conditions, and mycelia break through the fruit cuticle. Despite the fact that *Monilinia* spp. enter through stomata (Garcia-Benitez et al., 2016), wounds and microcracks on the fruit skin (Gibert et al., 2009; Mustafa et al., 2021; Oliveira Lino et al., 2016), they can also employ degrading enzymes (exoenzymes) during fruit surface penetration, invasion, and colonization, like many other necrotrophic pathogens (Byrde and Willetts, 1977).

Cuticle is a hydrophobic coating that covers the surface of all terrestrial plants' aerial parts (leaves, flowers, fruit, and non-woody stems) (Müller and Riederer, 2005). It has two main components, a structural matrix called cutin and cuticular waxes, distinguished by their solubility in organic solvents. The former cannot be dissolved due to its polymer structure, while the latter is dissolved in solvent extraction (Pollard et al., 2008; Walton, 1997). Waxes are embedded in the cutin and form a continuous layer on the surfaces, making it possible to discriminate between intracuticular and epicuticular wax layers (Jetter et al., 2000). The cuticle is an inert mechanical support and barrier against biotic and abiotic stresses; but also a dynamic and metabolically active tissue (Walton, 1997; Ziv et al., 2018). Even though the cuticle provides a source of nourishment for microbes and animals, it also includes toxic peroxidases, phenolics, and activated oxygen compounds (Lattanzio et al., 2006; Oliveira Lino et al., 2016).

Secondary metabolites embedded in fruit skin and flesh may oppose or reduce fungal infection to some extent (Pusztahelyi et al., 2015). The variability of total phenolics between different peach cultivars and the decline pattern along fruit development was proposed to explain the differential behavior of cultivars towards BR resistance and the fruit resistance evolution between stages (Andreotti et al., 2008; Kubota et al., 2000; Lee et al., 1990; Obi et al., 2020). Fruit from specific peach accessions, such as the South American cv. Bolinha shows a high level of resistance to *M. fructicola* and up to three times the level of chlorogenic acid (CGA) of susceptible accessions at equal stages of maturation (Bostock et al., 1999). CGA, neochlorogenic, and caffeic acids decline considerably from stage II to maturity (Bostock et al., 1999; Lee and Bostock, 2007), while fruit susceptibility to BR increases, supporting the hypothesis that they may play an important role in fruit resistance. CGA, neochlorogenic acid, caffeic acid (CA), and epicatechin were among the primary phenolic acids studied *in vitro* for anti-fungal efficacy on the spore germination and mycelial growth in *Monilinia* spp. (Bostock et al., 1999; Lee et al., 1990; Lee and Bostock, 2007; Villarino et al., 2011). Unfortunately, CGA and CA at concentrations up to 5.0 mM did not inhibit spore germination or mycelial growth in culture, while epicatechin appeared to reduce spore germination of *M. fructicola* (Bostock et al.,

1999; Lee and Bostock, 2007). In another study, CGA and pyroquilon did not affect *M. laxa* growth while reducing the level of melanin-like pigment (Villarino et al., 2011). However, CGA and CA markedly inhibited the production of cell wall degrading enzymes such as polygalacturonase and cutinase (Bostock et al., 1999; Lee and Bostock, 2007) and suppressed the production of melanin-like pigments in *M. fructicola*. Villarino et al. (2011) suggested that it could interfere with the early skin penetration process. In addition, Lee & Bostock (2007) have investigated the penetration of *M. fructicola* at different peach fruit developmental stages, observing fewer appressoria production by *M. fructicola* on immature fruit (stage I) and mature fruit (stage III) compared to the stage II (pit hardening). They suggested that the production of numerous appressoria on the stage II could play a role in quiescence, perhaps by serving as resting structures until fruit reach maturity. Besides these extensive works, few studies investigated the effects of other phenolic compounds. Recently, Hernández et al. (2021) reported the conidial germination and mycelium growth inhibitory effect of other phenolic acids, namely ferulic and p-coumaric, and flavonoids (naringin, hesperidin and neohesperidin) on *in vitro* cultures of *M. fructicola*.

Triterpenes are another important class of secondary metabolites included in cuticular waxes. They potentially act as signaling molecules and protect against pathogens and pests (Thimmappa et al., 2014). Cuticular waxes include a high concentration of triterpenoids, some of which are considered phytoanticipins, these anti-fungal metabolites are preformed inhibitors constitutively present in healthy plants (Ribera and Zuñiga, 2012). In a study by Oliveira Lino et al. (2020), several triterpenoids were associated with peach BR disease resistance in immature fruit of Zephir nectarine. When the fruits had the least infection probability in stage II, ursolic acid (UA) and oleanolic acid (OA) were the most prevalent compounds. These two triterpenoids are known for their broad antimicrobial properties (Jesus et al., 2015), and these compounds and their derivatives have been characterized for their anti-fungal activity (Shai et al., 2008). However, the literature relative to the effects on *Monilinia* spp. of triterpenoids is scarce. Indeed, when evaluating compounds with high hydrophilicity, the success of the interaction between the tested compounds and fungi is not always guaranteed. As a result, based on our knowledge, no study on the inhibitory effects of triterpenoids has been conducted in *Monilinia*. The use of an airbrush to apply wax components on agar media or coverslips, as described by Belding et al. (2000), brings up interesting perspectives to assess these kind of compounds.

Besides the exploration of colony traits, such as growth speed, sporulation, and germination rates, and traits related to the infection process, such as formation of appressorium and melanization, it is also interesting to focus studies on the enzymatic and proteomic arsenal of the fungi, especially these involved in virulence and fitness functions. In addition, gene expression perturbations may also be a good indicator of the impact of a compound on fungal growth. In this way, works reported the impact of some compounds on enzyme production by *Monilinia* spp. and on the expression of genes potentially linked to the pathogenicity of *Monilinia* spp. (Baró-Montel et al., 2019; Bostock et al., 1999; De Cal et al., 2013; Lee et al., 2010; Rodríguez-Pires et al., 2020a; Wang et al., 2002). Various essential degrading enzymes are synchronized during fungal infection to overcome the physical and chemical barriers buried in the skin (Garcia-Benitez et

al., 2019; Kubicek et al., 2014; ten Have et al., 2002). The role of these enzymes is well established in the closely related necrotrophic pathogen *Botrytis cinerea* (Li et al., 2020), and they have been used as homologs to study their roles in *Monilinia* (Balsells-Llauradó et al., 2020; De Miccolis Angelini et al., 2018; Garcia-Benitez et al., 2019). In particular, cell wall degrading enzymes (CWDEs), such as glycosyl hydrolases, endopolygalacturonase and oxidoreductases in *B. cinerea* (Blanco-Ulate et al., 2014; Choquer et al., 2007; Li et al., 2020; ten Have et al., 1998; Viefhues et al., 2014), and glycosyl hydrolases, endopolygalacturonase, lyases, and esterases in *Monilinia* (Chou et al., 2015; Lee et al., 2010; Rodríguez-Pires et al., 2020a, 2020b), have been reported to be related to virulence. All potential pathogens encounter cutin during direct host penetration of above-ground plant parts (Walton, 1994). Therefore, cutinase is an enzyme that pathogenic fungi may use to facilitate penetration of the cuticle (Bostock et al., 1999). Cutinases, pectin lyases, α -glucosidases, polygalacturonases, proteases, and xylanases are among the few CWDEs examined in *Monilinia* spp. in terms of pathogenicity (Byrde and Willetts, 1977; Chou et al., 2015; De Cal et al., 2013; Garcia-Benitez et al., 2019; Rodríguez-Pires et al., 2020a; Wang et al., 2002).

Little is known about the inhibitory effects of triterpenoids on the growth and fitness of *Monilinia* spp., and on the impact of phenolics and triterpenoids on the modulation of CWDE genes. Therefore, in this study, two complementary objectives were pursued: (1) investigating the inhibitory effects of phenolic compounds (chlorogenic and ferulic acids), terpenoids (oleanolic, ursolic, betulinic acids), and fruit surface compound (FSC) extracts on the growth *in vitro* of *M. fructicola* and *M. laxa*; (2) surveying the effect on mycelial growth of *M. fructicola* on culture medium to these compounds on the regulation of genes linked to fitness and virulence.

2. Material and methods

2.1. Fungal Isolates and Inoculum preparation

Monospore isolates of *M. fructicola* and *M. laxa* were obtained from an infected apricot branch and mummified apricot, respectively, from an experimental orchard belonging to INRAE, located near Valence (Drôme, France) in March 2011. Species-specific primers were used to confirm both *Monilinia* spp. as reported by Hughes et al. (2000). Both fungi were grown and maintained on V8 agar medium at 24 °C under a 16/8-hour light-dark cycle for short periods. While conidia suspensions were stored in monopotassic phosphate buffer with 20% glycerol at -20 °C for prolonged preservation. Conidial suspensions were made from active cultures that were 7–10 days old whenever needed for running any experiments. Petri cultures were flooded with 3 ml of sterile distilled water and gently scraped with a spatula. The total conidia and mycelia were collected and shaken for 1 min in a tube containing glass beads to release conidial chains. Subsequently, the suspension was passed through a falcon strainer with pore size of 40 μ m. Malassez counting chamber was used to determine conidia enumeration and adjusted to the desired concentration.

2.2. Reagents

Chlorogenic acid (CGA), oleanolic acid (OA) and ferulic acid (FA) were purchased from Sigma-Aldrich (Saint-Quentin-Favallier, France). Other compounds, ursolic acid (UA) and betulinic acid (BA) were purchased from Extrasynthèse (Genay, France).

2.2.1. Preparations of fruit surface compounds (FSC) extract

Two immature fruit stages of Zephir nectarine were selected for extracting fruit surface compounds (FSCs): stage I and stage II (pit hardening), which correlate to high and low susceptibility to brown rot (Mari et al., 2003), respectively. The mass extraction method was carried out according to Oliveira Lino et al. (2020). Around a hundred fruit were harvested at stage I, and about fifty at stage II. A representative part of the lots was measured for the three diameters to determine the average surface area of fruit and calculate the total surface area of the fruit lots. All the fruit were washed in reverse osmosis treated water, air-dried, and then immersed in chloroform (VWR, Normapur) for 30 seconds under frequent agitation. Extracts were then filtered through a paper filter to remove any debris, and the volume were measured as well. Liquid chromatography was used to analyze the amounts of triterpenoids and phenolic acids.

2.2.2. Application of compounds and FSC extracts onto the medium

CGA. CGA stock solutions were prepared by dissolving it in pure ethanol (EtOH) and then diluted in ultra-pure water to reach concentrations of 5, 10 and 20 mM in 10% EtOH. The stocks were first filtered on a 0.22 μm pore size filter (Falcon®). The solutions were amended to an autoclaved V8 juice Agar medium to obtain 0.5, 1.0, and 2.0 mM concentrations at 1% EtOH, and the pH was adjusted to 5.2 to minimize CGA degradation. Altogether, 20 ml of media and the amended compounds were distributed onto plastic Petri dishes. Petri dishes with media at 1% EtOH were employed as a control.

Triterpenoid and FSC extracts. Three triterpenoid compounds OA, UA, and BA at 2.3 mM concentration and one phenolic compound FA (used to test the efficacy of the protocol) at 13 mM were dissolved in chloroform. A 3 ml of each compound was spread on the surface of a glass Petri plate (9 cm diameter) containing V8 juice agar using a glass pipette. When applied on the plates, the proportions of each AO, UA and BA compounds were around 50 $\mu\text{g}/\text{cm}^2$. This amount was determined to represent the actual concentration found on fruit surface (Table 1), while FA concentration was 122 $\mu\text{g}/\text{cm}^2$.

By the same approach, 3 ml of FSC extracts (stage I and stage II) were spread on the surface of glass Petri plates (9 cm diameter) containing V8 juice agar using a glass pipette. This amount was calculated to evenly distribute on Petri dishes a concentration per unit area of oleanolic and ursolic acids comparable to that found on the fruit surface, at each stage.

Initial extracts were concentrated on a vacuum rotary evaporator, and then a calculation was made to determine the concentration required to deposit the extracts on the surface of Petri dishes to form a layer of compound mixture which resemble the spread on fruit skin. In both the triterpenoid and FSC extract tests, 3 ml of pure chloroform was spread on plates containing the same quantity of medium (only V8 juice agar)

and was used as a control. Plates loaded with solutions of triterpenoids, FSC extracts, and their controls were kept uncovered for about 30 minutes under a sterile chemical fume hood to allow the chloroform to evaporate completely, and they were regularly agitated by hand to leave a layer of compounds on the medium surface.

2.3. Inoculation and incubation of culture plates

Plates of all cultures (control, CGA, FA, triterpenoids and FSC extracts) were inoculated the same way by, either *M. fructicola* or *M. laxa*, placing 10 µl conidial suspension in sterilized water at 10^5 conidia/ml⁻¹ concentration to the center of the medium. After the droplet was dried, inoculated Petri dishes were sealed with Parafilm® and incubated in a culture chamber at 24 °C under a 16/8 h light-dark cycle. Each test consisted of 3–4 Petri culture replicates per treatment and control, and each trial was repeated at least twice independently.

2.4. Assessment of fungal response to compounds and FSC extracts

Growth, Sporulation and Germination of second-generation conidia

Colony diameters of 3–4 replicate plates were measured in two perpendicular lines crossing from the sowing center. Values were compared to their corresponding control (untreated culture). Measurements of mycelial growth were started at three days post-inoculation (dpi) and continued up to 10 dpi. Only measures from the 3 and 7 dpi were included in the final analysis.

Each culture plate was flooded with 3 ml of sterile distilled water and gently scraped the entire plate. Total conidia and mycelia were gathered and added to a tube containing glass beads. The mixture was agitated for 1 minute to dismantle conidia chains from mycelia. Then the suspension was separated and passed through a strainer (Falcon®) with pore size of 40 µm. Colony sporulation was measured after 11 dpi. Conidial concentration was determined using a Malassez counting chamber.

Sporulation from the previous step was diluted several times based on the concentration determined and adjusted to a manageable concentration to count. The conidial germination was determined by placing 4 droplets with the size of 50 µl of diluted suspension per replication on Petri dishes containing a thin layer of PDA. Plates were incubated for 20±3 h in a closed plastic box with maximized humidity at room temperature. Under a light microscope with 100x magnification, germinated and ungerminated conidia of more than 100 conidia per plate were recorded. The percentage of germinated conidia for three replicates was compared to the control.

2.5. Modulation of mycelial *Monilinia fructicola* gene expression by compounds and FSC extracts in culture media

2.5.1. Cultural conditions and Mycelia harvest

For evaluating the impact of studied compounds and FSC extracts on the regulation of virulence-related genes, three Petri plates of each treatment and control were harvested at each incubation period. For CGA, the entire culture (approximately 3.0 g of total mycelium and conidia) was harvested from each plate

separately, as three biological replicates, at three incubation periods of 4, 7 and 11 dpi. While other compounds and FSC extracts were collected at 4 and 7 dpi and instantly stored at -80 °C for qPCR.

2.5.2. Primer design of candidate genes

Candidate genes from *B. cinerea* and *M. laxa* were analyzed *in silico* using BLAST Batch Entrez tools (<https://www.ncbi.nlm.nih.gov/sites/batchentrez>) from the NCBI database to produce an expressed sequence tag (EST) ortholog in *M. fructicola*. Primers were designed using software Primer 3.0 (<https://primer3.ut.ee>). The primer sequences and amplicon lengths are listed in Table 2.

2.5.3. Isolation of total RNA and cDNA synthesis from culture media

Total RNA was isolated from frozen fungal samples from each culture media using the ready kit NucleoSpin®RNA Plant and Fungi (Macherey-Nagel) according to the manufacturer's instructions. RNA was quantified using a spectrophotometric at 260 and 280 nm absorbance (Thermo Scientific NanoDrop™) and tested for integrity by agarose gel electrophoresis. According to the manufacturer's instructions, one microgram of total RNA was used as the template for cDNA synthesis using oligo (dT) primer (AffinityScript QPCR cDNA Synthesis Kit; Agilent Technologies) and specific reverse primer of genes. The completed first-strand cDNA synthesis reactions were diluted (1:10), and diluted cDNA was used as the template for qPCR analysis.

2.5.4. Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) was used to quantify the mycelial gene expression in the presence of compounds or extracts on culture media, compared to their corresponding control. qRT-PCR was performed on a Stratagene MX Pro 3005 amplification system. For amplification, 7 µl of SYBR Green PCR master mix (Brilliant III with High ROX, Agilent Technologies), 0.5 µl of each primer (10 µM), 2 µl of diluted cDNA (1:10), and 5 µl RNase free water were used in a 15 µl reaction mixture. The thermocycling program consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, and 60 °C for 1 min. Each reaction was carried out in triplicate. All qPCR reactions were normalized by the threshold cycle value (Ct) compared to three internal reference genes *α-Tubulin*, *RPL13* and *TEF2* in *M. fructicola* following the $2^{-\Delta\Delta CT}$ method for relative quantification as according to Livak & Schmittgen (2001). The reference genes were considerably expressed and did not demonstrate any significant changes between modalities. Statistical analyses were performed for the normalized gene expressions by adapting scripts from the R package (RqPCRBase) developed by Hilliou and Tran (2013). Thereby, for each gene normalized expression (GRE) averaged over 3 biological replicates, a fold change relative to control was computed for all modality tested (modality) and incubation time (t): $(\text{mean GRE}_{\text{modality, t}} - \text{mean GRE}_{\text{control, t}}) / (\text{mean GRE}_{\text{control, t}})$. Positive and negative values correspond respectively to the genes over and under expression due to adding the compound in the culture compared to the control (no compound).

2.6. Statistical analysis

Graphical representations and statistical analyses were performed using R (version 4.0.5) (r Core Team, 2014). Results of colony diameter underwent a two-way analysis of variance in which the days post-inoculation was considered a second parameter. A one-way ANOVA was used with a *t*-test using an R-based tool and ggplot2 to generate graphs to evaluate the mean differences for sporulation and germination on each culture media. Treatments were compared to controls, and a *P* value of 0.05 was used to indicate significance. All studies were carried out at least twice independently, with each treatment receiving at least three replicates. A one-way ANOVA was performed on normalized gene expression to test the effect of compounds and FSC extracts compared to control on culture media, at each incubation time. Three biological replicates (3 independent plates) were available for each modality (control, compounds and FSC extracts).

3. Results

3.1. Oleanolic and ursolic contents in the fruit extracts and applied on the Petri dishes

The extracts from three years of collection were used for the tests. The amount of oleanolic acid deposited was around 5 $\mu\text{g}/\text{cm}^2$ at stage I and 36 $\mu\text{g}/\text{cm}^2$ at stage II, and the amount of ursolic acid was around 19 $\mu\text{g}/\text{cm}^2$ at stage I and 99 $\mu\text{g}/\text{cm}^2$ at stage II (Table 1).

Table 1. Oleanolic and ursolic contents in the fruit extracts at the two stages and on the Petri dishes.

Fruit content			Content in the extracts applied to the Petri plates			
Year (season)	Fruit stage	Number of fruits	oleanolic acid ($\mu\text{g}/\text{cm}^2$)	ursolic acid ($\mu\text{g}/\text{cm}^2$)	oleanolic acid ($\mu\text{g}/\text{cm}^2$)	ursolic acid ($\mu\text{g}/\text{cm}^2$)
2013	stage I	292	3.7	13.2	4.0	13.0
2017	stage I	159	4.8	20.7	6.0	27.0
2018	stage I	60	9.0	33.6	5.0	18.0
Average	stage I	170.3	5.8	22.5	5.0	19.3
2013	stage II	50	33.5	101.0	34.0	103.0
2017	stage II	50	34.1	99.0	39.0	113.0
2018	stage II	40	37.7	84.6	36.0	80.0
Average	stage II	46.7	35.1	94.9	36.3	98.7

3.2. Anti-fungal Effect of Phenolic and terpenoid compounds and FSC on *Monilinia laxa* and *M. fructicola*

3.2.1. Chlorogenic acid

The amendment of CGA in media proved to have no inhibitory effect on growth of both *Monilinia* spp., whatever the concentrations. On the contrary, a significant speed increase of colony growth was observed for *M. laxa* at both 3- and 7-days post-inoculation (dpi) compared to their respective control (untreated

culture). As for *M. fructicola*, colony growth was less enhanced at 3 dpi (only 1.0 mM was significant), whereas the increase in growth was also obvious at 7 dpi. Contrarywise, the sporulation of both species was reduced significantly, with less effect on *M. fructicola* compared to *M. laxa* at 11 dpi after full colony growth. Furthermore, germination of second-generation conidia of *M. laxa* gathered in the assays with 0.5 and 1.0 mM CGA showed significant reduction; however, no statistical reduction of germination was observed for *M. fructicola* compared to control (Fig. 1).

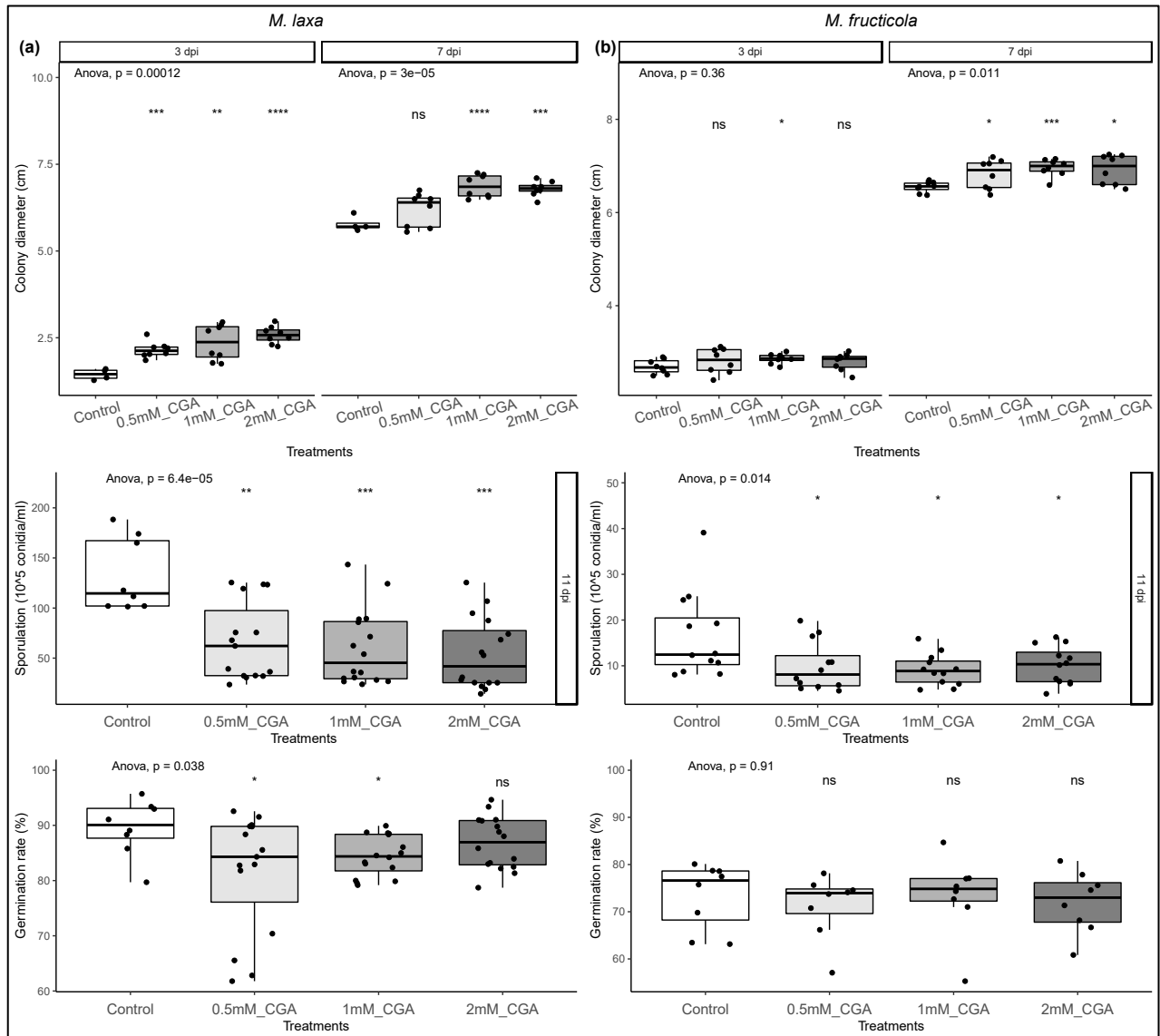


Fig. 1. Anti-fungal effect of chlorogenic acid (CGA) on *Monilinia laxa* (a) and *M. fructicola* (b), by *in vitro* assessments, at three concentrations amended to V8 juice agar. Colony growth diameter (cm) was measured at 3 and 7 dpi, sporulation (10^5 conidia/ml) at 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. A final concentration of 1% EtOH in the medium was employed as a control. The differences are assessed using a *t*-test with a 0.05 *P* value.

3.2.2. Triterpenoid compounds and Ferulic acid

The suitability of the protocol to test hydrophobic compounds was confirmed by the obtention of significant effects of ferulic acid on the three characteristics of fungi growth in Petri dishes. Indeed, with both *Monilinia* spp., a significant inhibitory effect was observed on the fungal growth (especially at 7dpi) and sporulation,

while the effect on germination of second generation of conidia was limited to *M. laxa* on culture media (Fig 2). The anti-fungal effect of oleanolic acid against *M. laxa*, and ursolic and betulinic acids against *M. fructicola* were then observed. Applying a thin layer on the Petri dishes with 50 $\mu\text{g}/\text{cm}^2$ of each compound did not induce any inhibitory effect on growth of the two *Monilinia* species.

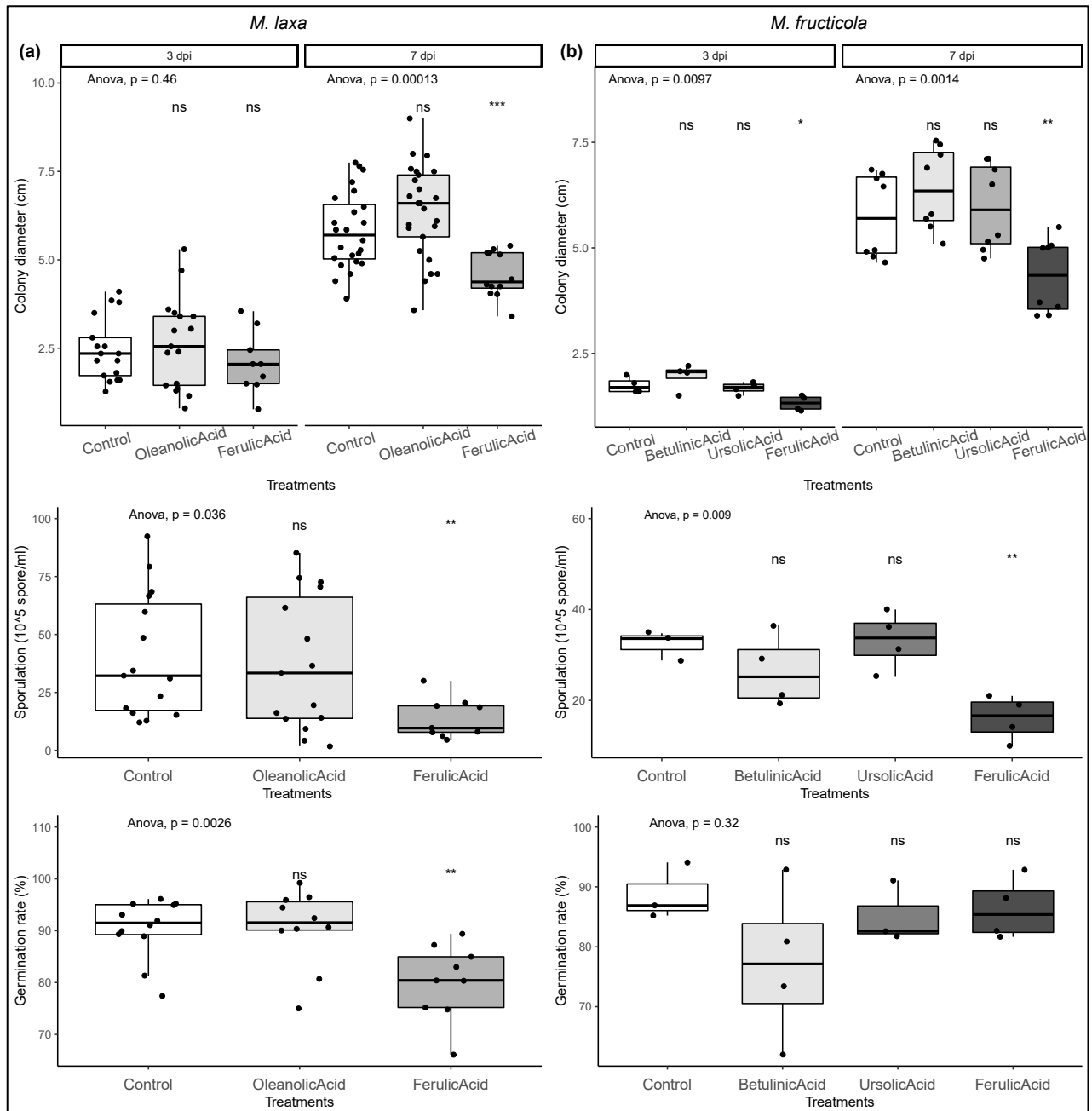


Fig. 2. Anti-fungal effect of OA and FA on *Monilinia laxa* (a) and BA, UA and FA on *M. fructicola* (b), by *in vitro* assessments. The concentration of compounds applied was 2.3 mM (or 50 $\mu\text{g}/\text{cm}^2$ on medium surface) for each, while ferulic acid was 13.4 mM (or 122 $\mu\text{g}/\text{cm}^2$), dissolved in chloroform. Three ml of each compound was spread on the surface of V8 juice agar medium. Colony growth diameter (cm) was measured at 3 and 7 dpi, and sporulation (10^5 conidia/ml) after 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. Control was only 3 ml chloroform (pure) spread on the medium. The differences are assessed using a *t*-test with a 0.05 *P* value.

3.2.3. Effect of Fruit Surface compound (FSC) extracts

The two FSC extracts of Zephir nectarine stages I and II were assessed on *M. laxa* and *M. fructicola* cultures. No inhibitory effects were observed; instead, in the case of *M. laxa*, the colony growth diameter was increased at 7 dpi compared to control, and a significant increase was observed at 3 dpi for *M. fructicola*. At full colony growth, after 11 dpi, the sporulation and germination of second generation were not affected compared to their respective control for both FSC extracts and with both *Monilinia* spp. (Fig. 3). In Zephir nectarine at stage II, the average of three seasons quantity of UA and OA were estimated at very high levels of 95 and 35 $\mu\text{g}\cdot\text{cm}^{-2}$, respectively (Table 1), 4 and 7 times more than at stage I. The concentrations of OA and UA present in the layers made with stage II FSC extracts (around 36 and 99 $\mu\text{g}/\text{cm}^2$ respectively) were very similar to the concentrations applied for separately testing OA and UA products (50 $\mu\text{g}/\text{cm}^2$), except they were applied together and mixed with the other minor constituents of the waxes. Comparing FSC extracts, OA and UA tests, same results were obtained (no effect) apart from the promotion of colony growth of *M. laxa*, which can be attributed to the minor compounds in FSC extracts.

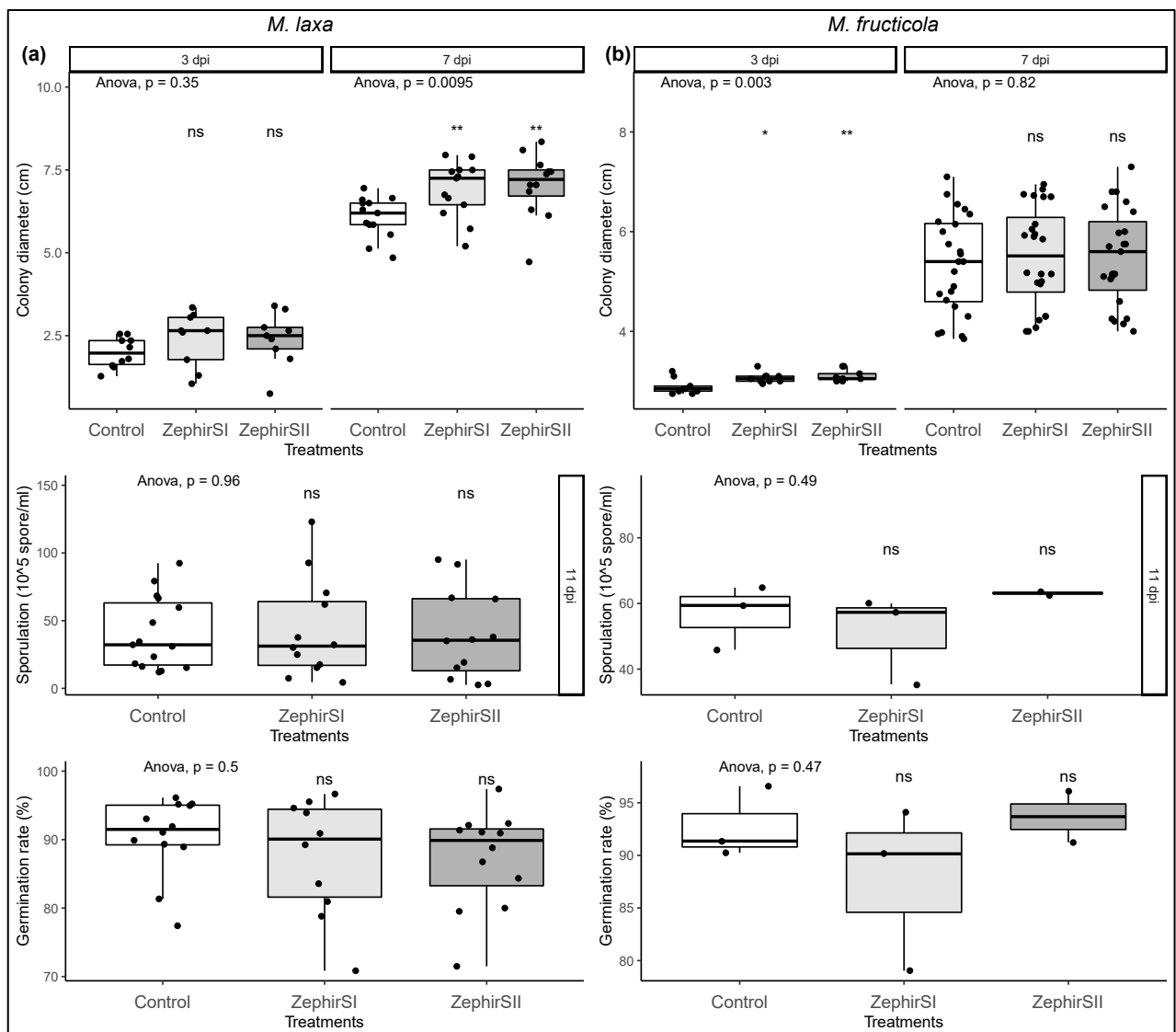


Fig. 3. Anti-fungal effect of two fruit surface compound extracts at stage I and II of fruit development of cultivar “Zephir” on *Monilinia laxa* (a) and *M. fructicola* (b), by *in vitro* assessments. Three ml of each

compound was spread on the surface of V8 juice agar medium. Colony growth diameter (cm) was measured at 3 and 7 dpi and sporulation (10^5 conidia/ml) after 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. Control was only 3 ml chloroform (pure) spread on the medium. The differences are assessed using a *t*-test with a 0.05 *P* value.

3.3. Effect of phenolic and terpenoid compounds and FSC extracts on Gene expression

A total of 12 genes were selected from the literature according to their putative functions described either on *Monilinia* spp. or *B. cinerea* (Table 2).

Table 2. Details of the candidate genes with their putative function, primer sequence and amplicon size, were considered in this study for *Monilinia fructicola*.

No.	Gene name	Putative function	5' to 3' sequence [oligonucleotides]	Amplicon (bp)	Described in	Reference	In <i>M. fructicola</i>
<u>Germination/appressorium formation-related genes</u>							
1	<i>MfBMP1</i>	Germination induction and pathogenicity	F: AAAGACGGAGTTGCTGCTTC R: CGGATCCGAATCCTTTTCGAG	116	<i>B. cinerea</i>	(Zheng et al., 2000) (Doehlemann et al., 2006)	This study
2	<i>MfPLS1</i>	Appressorium mediated penetration into intact host plant leaves (virulence).	F: GTCTCGGCGAATCTCTTGT R: CATGTAGCCGTGCACCTTGA	143	<i>B. cinerea</i>	(Gourgues et al., 2004)	This study
<u>Cell wall degrading enzyme (CWDE) genes</u>							
3	<i>MfCUT1</i>	Production of cutinase which breaks down the cutin on the fruit surface	F: GGATCCCCAGTTCAAGGTG R: GTCTCCGGCGTGACAGAT	60	<i>M. fructicola</i>	(Wang et al., 2002)	
4	<i>MfPNL3</i>	Pectin lyase 3.	F: GCGTCTCCAACGTCATCATC R: TGAGCCTTCCAGCAGCATT	176	<i>M. laxa</i>	(Rodríguez-Pires et al., 2020a)	This study
5	<i>MfRGAE1</i>	Pectin dependent; The assistance of rhamnogalacturonan acetyl esterase.	F: CGGAGAGACCGTCTACACAT R: GGGAGGTGTAGGACCAAGTG	135	<i>M. laxa</i>	(Rodríguez-Pires et al., 2020a)	This study
6	<i>MfPG1</i>	Endopolygalacturonase, overexpression of <i>Mfpg1</i> reduces virulence in <i>M. fructicola</i> .	F: TGTCAACGTTACCGGTGGT R: TTGACACCAGATGGGAGACC	60	<i>M. fructicola</i>	(De Cal et al., 2013)	
7	<i>MfPG3</i>	Endopolygalacturonase: Not clear function, expressed with high pH (in <i>M. fructicola</i> during fruit infection)	F: ATGGTAGAGGTATCACGATCACTG R: CGGCACCATTTCCTTCAAT	60	<i>M. fructicola</i>	(De Cal et al., 2013)	
8	<i>MfPG6</i>	Endopolygalacturonase: Not clear function, expressed with high pH (in <i>M. fructicola</i> during fruit infection) acidify	F: GGCCCGTTCATCTTTTTCAC R: CCCTGCTGTGTGTTGAGAG	77	<i>M. fructicola</i>	(Chou et al., 2015)	
9	<i>Mfcdal</i>	Putative chitin deacetylase gene (<i>cdal</i>) and conidial germination.	F: AAGGCCCTTGCTTCAACAAC R: AAAGTGCAGTGGGAAGATCGC	168	<i>B. cinerea</i>	(Leroch et al., 2013)	This study
10	<i>MfLAE1</i>	[ROS] Involved in secondary metabolism and virulence	F: GGTGAAGTTCTTGGGCTTGA R: TCCAGGACTCCACTTCTAGT	118	<i>B. cinerea</i>	(Schumacher et al., 2015)	This study
<u>Reactive oxygen system (ROS)-related genes</u>							

11	<i>MfNoxR</i>	ROS, actin cytoskeleton regulation, decrease in sporulation	F: AGGCGCTGAAGGAATTTGAC R: TCGAAACGCCTTGTGGAAA	168	<i>B. cinerea</i>	(Siegmund et al., 2013)	This study
12	<i>MfOfd1</i>	Stress responses and virulence, increased ROS.	F: GGATGGGGCGATGATGGTAATG R: GGCTCTCCTTGGGCTCTGGT	149	<i>M. fructicola</i>	(Zhang et al., 2020a)	

3.3.1. Chlorogenic acid (CGA)

Among the 12 genes tested for mycelial gene expression in response to the presence of 2.0 mM CGA amended, *MfRGAE1*, *MfPG1*, *MfLAE1* and *Mfcdal* showed significant overexpression at 4 dpi, and *MfRGAE1* also overexpressed at 7 dpi. In addition, *MfOfd1* and *MfPG3* were slightly overexpressed at 4 dpi, though significantly. On the contrary, *MfCUT1* was the only gene down-regulated throughout the period compared to control. Besides, *MfNoxR*, *MfOfd1*, *MfPG1* and *MfPLS1* displayed significant under-expression at 11 dpi only. Some genes such as *MfPG1*, *MfOfd1* and *MfPLS1*, shifted from up-regulation at 4 and 7 dpi to significant down-regulation at 11 dpi compared to control (Fig. 4).

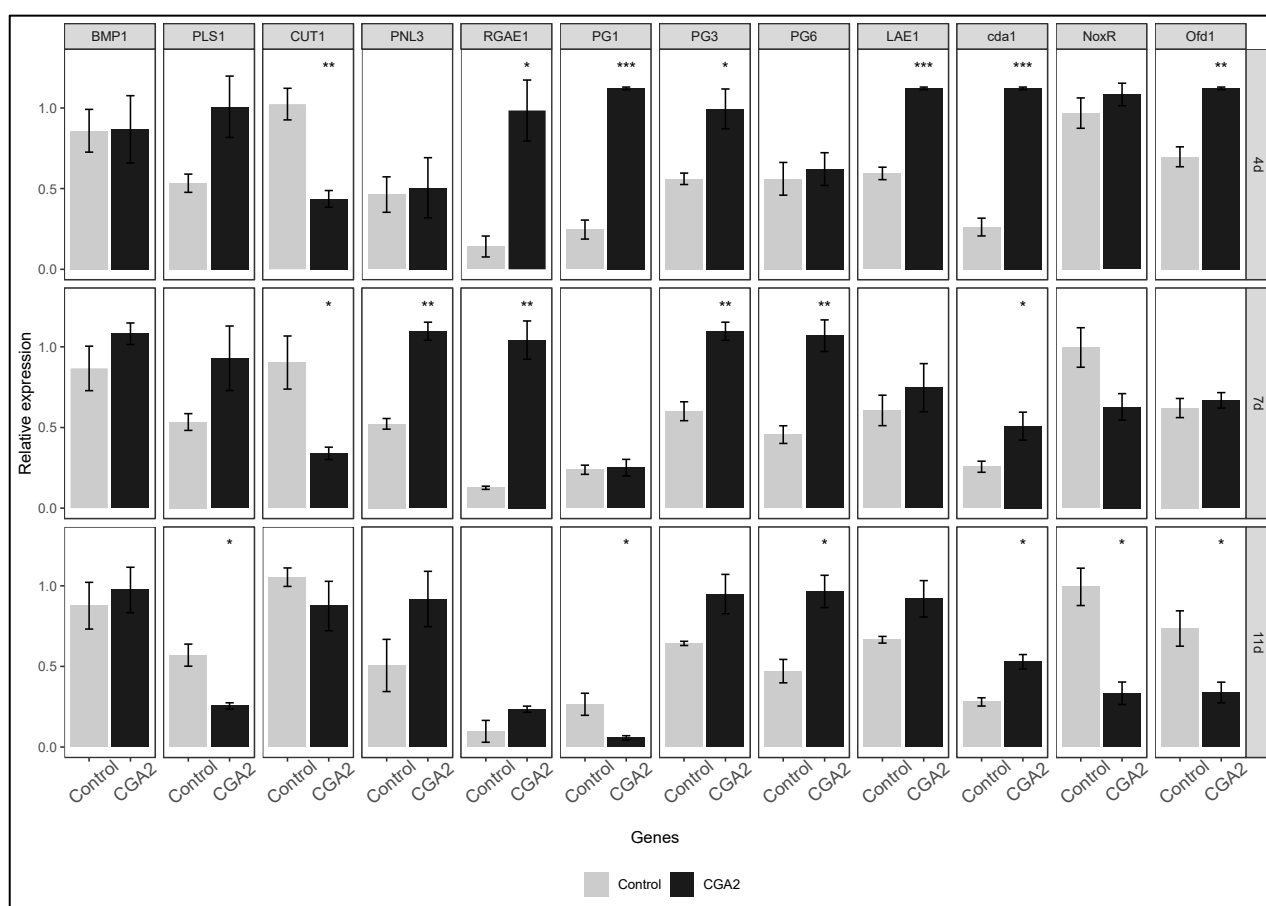


Fig. 4. Chlorogenic acid (CGA) effect on the expression of 12 virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.0 mM CGA concentration at different incubation periods (4, 7, and 11 dpi).

3.3.2. Ursolic and Betulinic acids

In general, ursolic and betulinic acids displayed similar profiles of modulation of mycelial gene expression over the 12 genes tested (Fig. 5). However, ursolic acid had a more negligible effect than betulinic acid. Indeed, many genes were significantly affected by adding 2.3 mM ursolic acid to the medium but only

moderately and this effect remained at 4 and 7 dpi. *Mfcdal*, *MfCUT1*, *MfOfd1*, *MfPG1*, and *MfPNL3* were significantly overexpressed at 4 and 7 dpi and *MfRGAE1* at 4 dpi only. On the contrary, *MfNoxR* was the only gene significantly down-regulated by ursolic acid relative to control. Surprisingly, betulinic acid distinguishingly up-regulated *MfCUT1* mainly at 7 dpi. On the other hand, *MfPG1* and *MfPG3* were significantly down-regulated at 7 dpi. In addition, *MfPG1*, again (as with CGA), shifted from significant up-regulation at 4 dpi to significant down-regulation at 7 dpi.

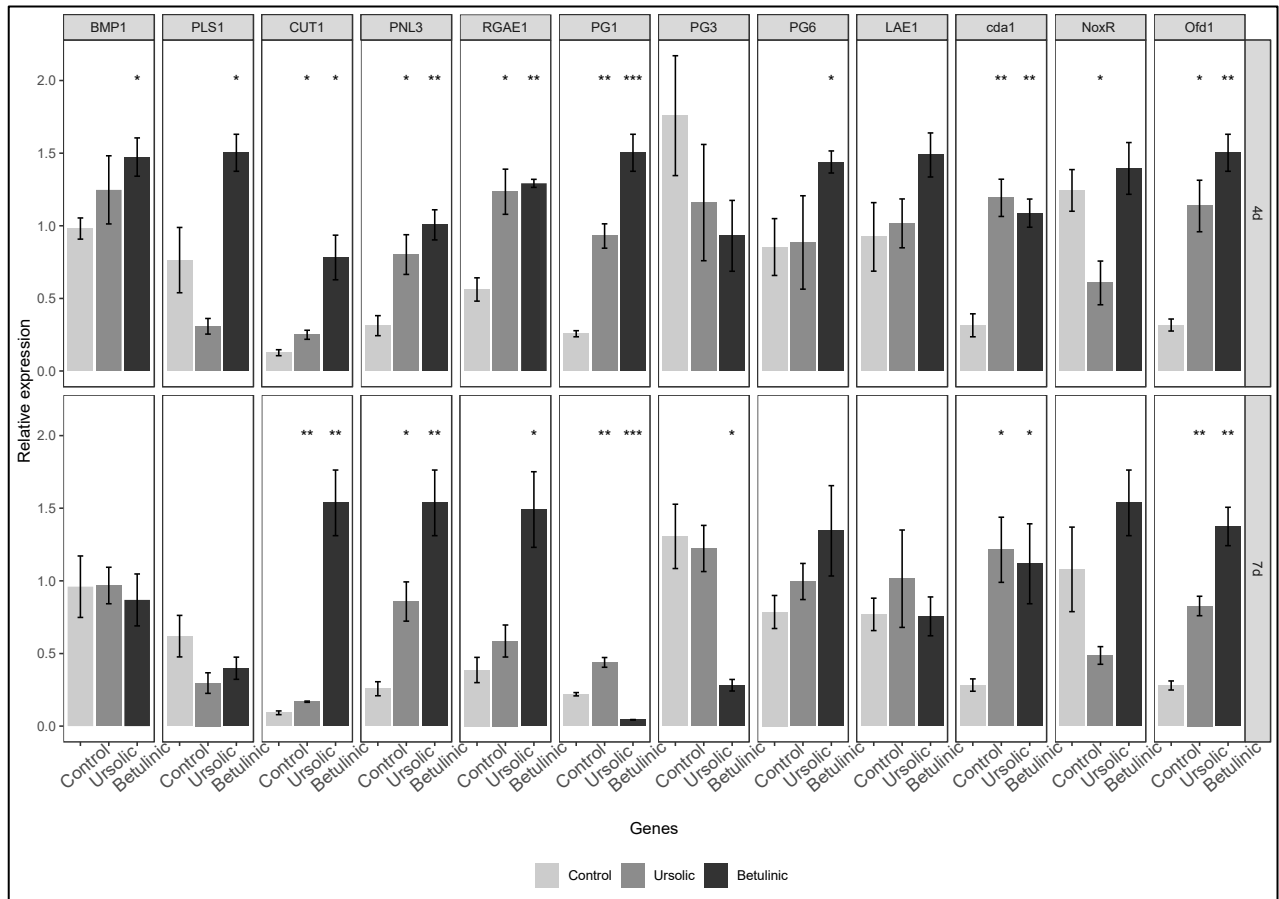


Fig. 5. Ursolic and betulinic acids effect on the expression of 12 virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.3 mM (or 50 $\mu\text{g}/\text{cm}^2$ on medium surface) concentration of each compound at different incubation periods (4 and 7 dpi).

3.3.3. Ferulic and Oleanolic acids

Neither of the two acids, ferulic nor oleanolic, caused the under-expression of any studied genes (Fig. 6). On the contrary, they triggered a strong overexpression of some specific genes on culture media. *MfRGAE1* was largely up-regulated by ferulic acid especially at 4 dpi. Regarding oleanolic acid, *MfPG6* showed significant up-regulation at both 4 and 7 dpi compared to control. *MfRGAE1* and *MfCUT1* were remarkably up-regulated by oleanolic acid, respectively, at 4 and 7 dpi.

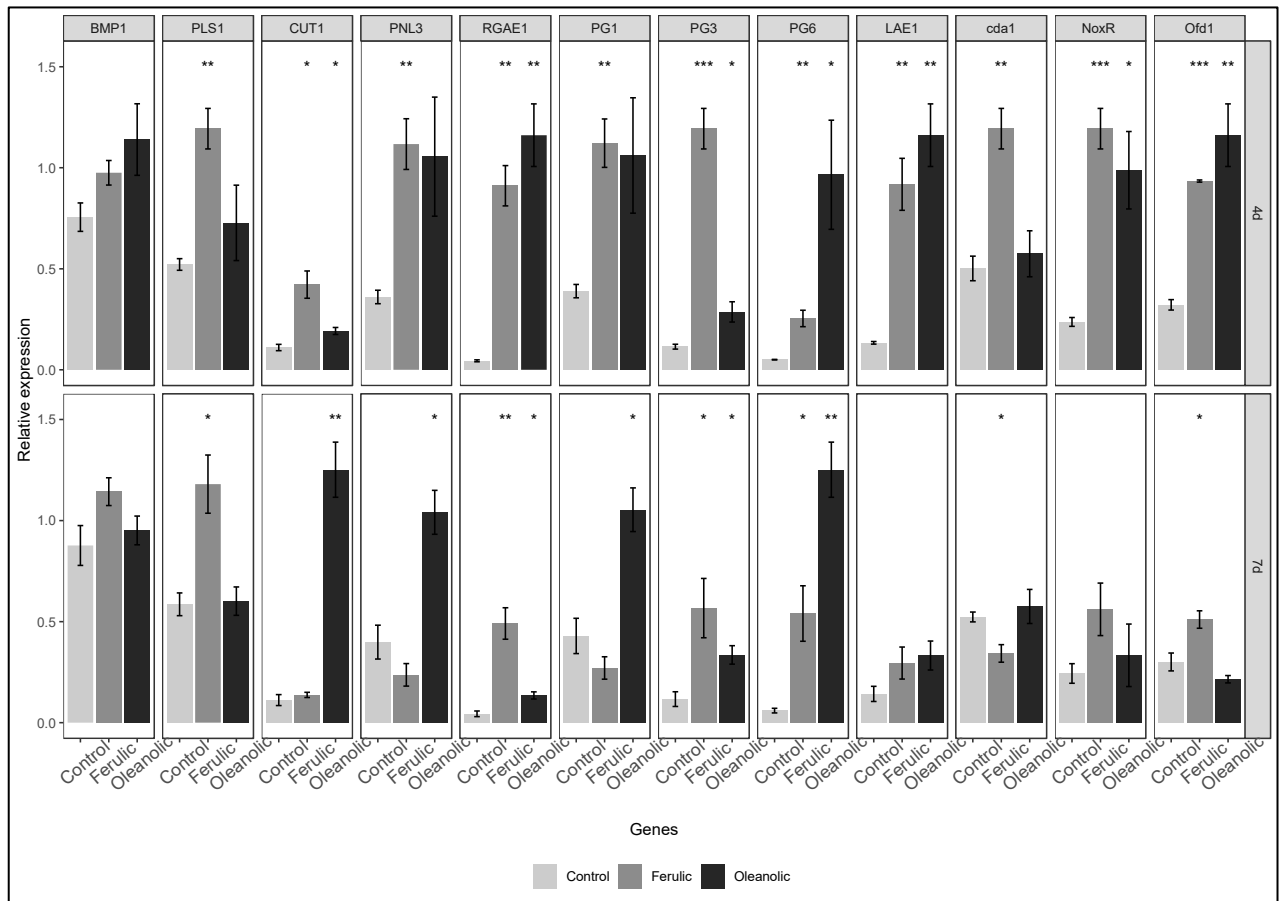


Fig. 6. Ferulic and oleanolic acids effect on the expression of 12 virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.3 mM (or 50 $\mu\text{g}/\text{cm}^2$ on medium surface) for oleanolic acid while for ferulic acid was 13.4 mM (or 122 $\mu\text{g}/\text{cm}^2$) at different incubation periods (4 and 7 dpi).

3.3.4. Fruit surface compound (FSC) extracts

Regardless of the genes, both FSC extracts had a minimal effect at 4 dpi on Petri dishes (Fig. 7). On the contrary, at 7 dpi most of the genes had their expression significantly modulated, with different effects depending on the FSC extracts. It is worth noting that the two genes related to formation, *MfBMP1* and *MfPLS1*, were more over-expressed in the case of stage I FSC extracts (ZeSI) compared to stage II FSC extracts (ZeSII). In addition, *MfOfd1* showed a significant and high overexpression due to ZeSI at 7 dpi. *MfPG3* and *MfPG6* were both up-regulated by the two FC extracts, but *MfPG3* was much more impacted by ZeSII. Also, ZeSII largely impacted *MfLAE1* at 7dpi inducing a large overexpression compared to the control.

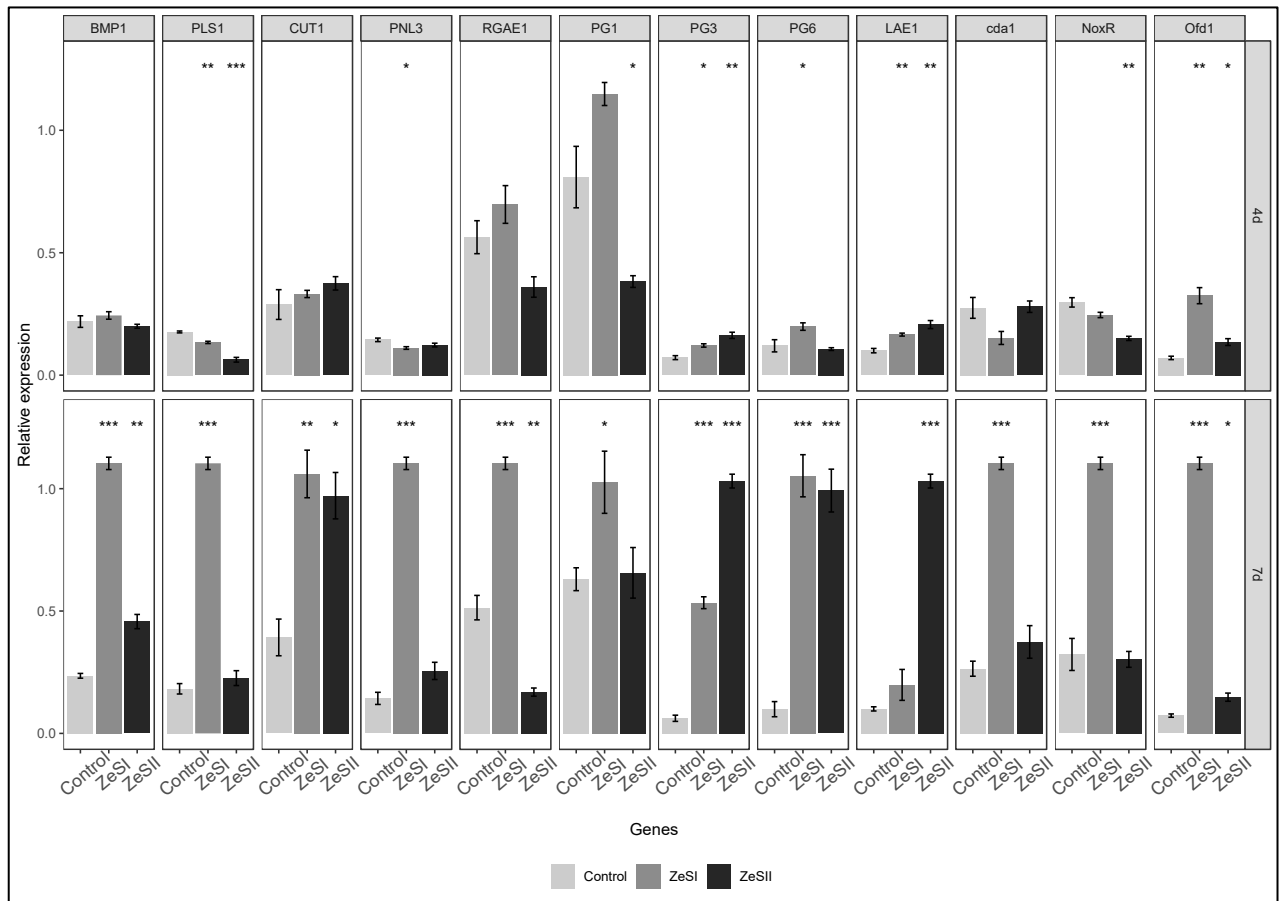


Fig. 7. The effect of Fruit surface compound (FSC) extracts of fruit developmental stage I (ZeSI) and II (ZeSII) of Zephir nectarine on the expression of 12 virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on each extract at different incubation periods (4 and 7 dpi).

4. Discussion

Monilinia fructicola and *M. laxa* are the two most important causal agents of brown rot (BR) in stone fruits in Europe. Because phenolic and terpenoid compounds are found in almost all stone fruits (Lara et al., 2020), and some of them have been correlated to BR resistance in different cultivars and at some fruit developmental stages (Oliveira Lino et al., 2020), their anti-fungal effect was investigated either individually or collectively.

In this study, the anti-fungal activity of two phenolics (chlorogenic and ferulic acids), three triterpenoids (oleanolic, ursolic, betulinic acids), and two fruit surface compound (FSC) extracts from two Zephir nectarine fruit developmental stages have been assessed *in vitro*.

4.1. Anti-fungal effect of the compounds and extracts

4.1.1. Chlorogenic acid (CGA)

At three concentrations, chlorogenic acid (CGA) did not inhibit colony growth diameter and germination of second-generation conidia generated from Petri dishes. On the contrary, it increased the colony growth slightly. However, the sporulation was reduced for both fungi compared to untreated control. These findings align with earlier research that found that even a higher concentration of 5.0 mM CGA did not limit colony growth and spore germination (Bostock et al., 1999). In another study, Lee and Bostock (2007) found that at

a dose of 0.5 mM, conidia germination was not inhibited on flower petals or fruit, but it did reduce appressorium formation from germinated conidia. Furthermore, they reported that applying CGA to peach petals and cherry fruits significantly reduced lesion development caused by *M. fructicola*. The anti-fungal action of CGA against *Monilinia* spp. is ambiguous in literature, as it is mainly dependent on both the observed fungal features and the test settings (methodologies) of *in vitro* culture and/or on plant tissue.

4.1.2. Ferulic acid

The validation of anti-fungal properties of triterpenes via *in vitro* tests requires setting up specific protocols since they are difficult to dissolve and amend in the medium due to their waxy and very hydrophobic nature. So, it is challenging to study triterpenoids in liquid form or mixed with other nutritious media. As ferulic acid (FA) is broadly recognized as an effective anti-fungal against many pathogenic fungi (Barral et al., 2017; Boutigny et al., 2009; Hernández et al., 2021; Liu et al., 2019; Sarma and Singh, 2003), it was taken as control to test the protocol to be further used for the triterpenoids. Since we obtained the expected results for FA anti-fungal effects and the deposit was homogeneous without voids and clumps, we concluded that the protocol developed in this study was adequate to study the inhibitory effect of other complex compounds to be dissolved such as triterpenoids.

For both *M. fructicola* and *M. laxa*, FA appeared to significantly inhibit colony development, sporulation, and germination of second-generation conidia compared to control. Our findings are in accordance with a recent study that brought out an anti-fungal activity of FA against *M. fructicola* (Hernández et al., 2021). However, their results should be taken with caution. Indeed, using a measure of medium turbidity as an indicator of fungal development seems risky since phenolic compounds such as FA and CGA interfere with the color of the mycelium. Although the study by Fernández et al. (2011) reports the presence of FA in peach fruit, Oliveira Lino and colleagues (2020) did not detect FA in its free form; instead, it was found in the form of feruloyl quinic acid in leaves and feruloyl-2,3- dihydroxy-urs-12-en-28-oic acid in peach fruit. In conclusion, FA is a promising anti-fungal compound against BR, although its absence in peach fruit seems to prevent the increase of its content to create resistant varieties against *Monilinia* spp.

4.1.3. Triterpenoid compounds

Triterpenoids are major components of fruit cuticular waxes, among them ursolic acid (UA) and oleanolic acid (OA) being the most prevalent in peach (Ludeña-Huaman and Ramos-Inquiltupa, 2019) and nectarine (Oliveira Lino et al., 2020). The concentrations of UA and OA reported in this study are comparable to the results reported by Oliveira Lino et al. (2020), with variations depending on the fruit stage, the cultivar and the season. The literature available on the evolution of individual triterpenoid compounds along fruit development, and especially nectarine, is very limited (Belge et al., 2019, 2014; Oliveira Lino et al., 2020; Szakiel et al., 2012). However, the study by Oliveira Lino et al. (2020) showed that UA and OA are found in very low amounts in cuticle of very young nectarines, then they increase sharply at stage I and reach a peak at stage II, and start to decline in later stages of fruit development. Particularly, UA is considered an active anti-fungal compound (Mahlo et al., 2013; Shai et al., 2008; Shaik et al., 2016; Shu et al., 2019), especially

against *Alternaria alternata*, since it induces disturbance of membrane permeability and integrity, and intracellular ROS accumulation, resulting in the lysis of the pathogen (Shu et al., 2019). Regarding OA, fewer anti-fungal activities have been reported, for example, against *B. cinerea* and *S. sclerotiorum* (Zhao et al., 2013). In addition, betulinic acid (BA) also has antimicrobial and/or anti-fungal properties (Shai et al., 2008). BA is not broadly detected in peach fruit waxes except in small amounts in fruit peel of *P. davidiana* and some *P. persica* × *P. davidiana* hybrids (Oliveira Lino, 2016). In other plants, it has been found in limited amounts in grape berry and olive cuticular waxes; however, it is mainly found in the bark of the white birch *Betula alba* L. (Yogeeswari and Sriram, 2010) and the cuticular wax of *Eucalyptus globulus* fruit (Pereira et al., 2005). Through a kinetic study, Oliveira Lino et al. (2020) revealed that oleanolic and ursolic acids contents were correlated with BR disease resistance in nectarine ("Zephir"). To our knowledge, oleanolic, ursolic, and betulinic acids had not been studied before concerning brown rot and this is the first assay against *Monilinia* spp. *in vitro*. Our results suggest no significant inhibitory effects against *M. laxa* and *M. fructicola* mycelial growth, sporulation, and germination of second-generation conidia, for any of the triterpenoids tested. This could be due to fungi being unable to digest and utilize waxy triterpenoids, as previously reported in other fungi with apple cuticular waxes (Belding et al., 2000). Compared to the phenolics tested in this study, triterpenoid compounds showed least effect, while FA remains an effective antifungal compound as it is known to induce laccase activity in fungi (Swatek and Staszczak, 2020). Cytological studies are required to reveal the effect of those compounds on the integrity of cell membrane of such fungal cells.

4.1.4. Fruit surface compound extracts

Many compounds can be found in peach and nectarine exocarp extracts, such as phenolics, flavonoids, hydroxycinnamates and flavonols, which appeared to be more present in the peel than in the pulp (Lara et al., 2015; Yeats and Rose, 2013). In particular, peach cuticle was found to be composed of 53% cutan, 27% waxes, 23% cutin, and 1% hydroxycinnamic acid derivatives (mainly ferulic and p-coumaric acids) (Fernández et al., 2011). The marked evolution of the composition of cuticular waxes in parallel with the evolution of resistance to *Monilinia* spp. during stages I and II of peach fruit growth (Oliveira Lino et al., 2020), invited to validate a cause-and-effect link *in vitro*. Indeed, stages I and II correlated to high and low susceptibility to brown rot (Mari et al., 2003), respectively. The FSC extracts used in this study were primarily comprised of triterpenoid derivatives along with other minor compounds (Oliveira Lino et al., 2020), and the results showed no inhibitory effect of stages I nor II of FSC extracts on *M. laxa* and *M. fructicola* growth and fitness, but a slight colony increase, especially for stage II.

As source of anti-fungal compounds, pomegranate skin extracts were shown to limit growth and conidia germination of *M. laxa* and *M. fructigena* (El Khetabi et al., 2020). Identical results were observed with orange peel extracts and combinations of polyphenolics on *M. fructicola* (Hernández et al., 2021). Such studies seem to suggest synergies between compounds that may be fundamental to yield significant effects against the pathogen and reproduce results obtained directly on fruit. Unfortunately, testing stage II FSC extracts did not show the results that the *in vivo* experiments suggested. Probably the resistance of the fruit at stage II not only come from FSC extracts and may involve other mechanisms that require fruit integrity.

4.2. Gene expression

Our follow-up objective was to investigate the effect of some compounds on the modulation of expression of genes of *M. fructicola*. Many reports highlighted the significance of *M. fructicola*, which appears to be more severe and displacing than *M. laxa* (Villarino et al., 2016). In addition, recently published genome drafts (Angelini et al., 2019; Ma et al., 2019; Rivera et al., 2018; Vilanova et al., 2021) are available for this species, as well as more genetic information regarding its pathogenicity. This prompted us to approach gene expression by focusing on *M. fructicola*. The 12 genes were chosen on the basis of their putative function in the fruit infection process. Considering the lack of literature on the topic, we started from the hypothesis that the evidence of an effect of a compound *in vitro* was a valuable step to launch in-depth studies on fruit, even if it would be inappropriate to directly extrapolate results obtained *in vitro* to the processes taking place during the infection of a fruit.

4.2.1. Appressorium formation and/or Penetration-related genes

MfBMP1, *MfPLS1* homologs to *BMP1* and *PLS1*, respectively, have been reported to play a critical role in the host penetration and appressorium formation in *B. cinerea* (Doehlemann et al., 2006; Gourgues et al., 2004; Zheng et al., 2000). The *MfPLS1* gene, which encodes a tetraspanin-like protein, has been identified in many plant pathogens associated directly with host plant penetration (Clergeot et al., 2001; Gourgues et al., 2004; Siegmund et al., 2013; Veneault-Fourrey et al., 2005). Another study reported a close association between *MfPLS1* and NADPH oxidase complexes (NoxR) in *B. cinerea* and their role in regulating penetration peg emergence (Siegmund et al., 2013). The upregulation of *MfPLS1* observed in presence of FA at both 4 and 7 dpi may suggest a signaling effect of this compound linked to the fruit cuticle which would be perceived by the fungi and would trigger the arsenal of penetration into the fruit.

Regarding the *BMP1* gene, the mutant of *B. cinerea* was unable to infect (penetrate) intact or wounded host tissue, although its growth on the medium was not affected (Gourgues et al., 2004; Zheng et al., 2000). *BMP1* gene was upregulated with both FSC extracts, but twice as much with stage I FSC extracts at 7 dpi in culture media. The makeup of the compounds found in the extracts might explain this differential gene expression. We can thus infer that stage I FSC extracts contained promoting compounds inducing *BMP1* expression that were more present than in stage II FSC extracts. Extra studies are required to fully unravel the effect of the compounds studied. Probably developing tests on fruit could overcome the lack of recognition by the pathogen and resolve the lock linked to the low expression of genes under control conditions.

4.2.2. Cell wall degrading enzymes (CWDEs)

Cutin has been shown to up-regulate genes involved in penetration of host tissues, whether from leaves or fruit. *Monilinia* needs to induce cutinase to break down the fruit's cutin (Bostock et al., 1999; Chiu et al., 2013; Wang et al., 2002). Indeed, cutinase is involved in both the penetration of the host cuticle at the early infection stage and in the degradation of a large amount of cuticle during lesion expansion (Yu et al., 2017). When *MfCUT1* is overexpressed, the pathogenicity of *M. fructicola* rises (Lee et al., 2010). Early studies on CGA and caffeic acid reported their ability to either down-regulate the expression of the *MfCUT1* (Wang et

al., 2002), or inhibit the cutinase activity (Bostock et al., 1999). Therefore, *MfCUTI* was given special attention in our study. In coherence with the literature, we also detected an attenuation of *MfCUTI* with 2.0 mM CGA concentration. On the contrary, we observed overexpression of *MfCUTI* with betulinic and oleanolic acids and may speculate that these compounds may promote pathogenicity on fruit.

MfRGAEI codes for a protein member of the rhamnogalacturonan acetyltransferase (RGAE) protein family (Rodríguez-Pires et al., 2020a). Among the pectin-dependent related genes, *MfRGAEI* has been previously studied and reported to be pectin dependent in *M. laxa* (Rodríguez-Pires et al., 2020a). *MfRGAEI* was considerably up-regulated in the presence of chlorogenic, ferulic, and oleanolic acids in our investigation, even though no pectin was supplemented to the medium. In accordance with previous findings by Rodríguez-Pires et al., (2020a), in our *in vitro* experiment the overexpression of *MfRGAEI* was higher at shorter incubation time (4 dpi) than at longer. Contrary to these three compounds, ursolic and betulinic acids had limited effect on upregulation of this gene. In the presence of FSC extracts, *MfRGAEI* expression was not significantly different from the control at 4 dpi. On the contrary at 7 dpi, the regulation of *MfRGAEI* was opposite between the two FSC extracts since it was significantly overregulated with stage I FSC and down-regulated with stage II FSC extracts. This *in vitro* downregulation was in adequation with the fact fruit is less susceptible at stage II. Compounds differentially present in these extracts probably play a role in the recognition of the cuticle by the pathogen and the activation of cutinase and other genes on fruit.

Three genes from the endoPG family were studied, namely *MfPG1*, *MfPG3*, and *MfPG6*. It was reported that the overexpression of *MfPG1* in *M. fructicola* reduced the lesion infection (Chou et al., 2015). In our study, *MfPG1* was significantly overregulated at 4 dpi in the presence of 2.0 mM CGA and then attenuated to be downregulated at 11 dpi. We could speculate that one reason why CGA correlates well with disease resistance could be its promoting effect on *MfPG1* expression. However, mild or not clear effect was observed in the presence of FSC extracts. Regarding ursolic acid effect, *MfPG1* expression was upregulated at 4 and 7 dpi. Upregulation was also observed with betulinic and ferulic acids at 4 dpi. These effects may further support the idea of a functional link between the overexpression of *MfPG1* and BR resistance.

The function of the other two genes, *MfPG3* and *MfPG6*, is less known apart from the fact that they have been associated with high pHs in the medium (De Cal et al., 2013). Throughout our experiment, these two genes appeared to be upregulated in the presence of both individual compounds or FSC extracts especially at 7 dpi in culture media. This may be due to the modulations of pH medium by the fungus secreting acids to colonize the host cells (De Cal et al., 2013). *MfLAEI* gene was significantly up-regulated by the stage II FSC extract corresponding to the period when the fruit is resistant to *Monilinia* spp. This up-regulation might be due to the higher concentration of OA in FSC extract at stage II than at stage I since comparable overexpression was observed in the presence of OA alone. In *B. cinerea*, the deletion of *LAEI* gene resulted in mutants with reduced pathogenicity, light-independent conidiation and sclerotia formation loss (Schumacher et al., 2015; Zhang et al., 2020b). In another fungus, i.e., *Trichoderma reesei*, it was shown that the putative protein methyltransferase *LAEI* controls cellulase gene expression (Seiboth et al., 2012).

5. Conclusion

In conclusion, our data confirm the limited anti-fungal effect of some phenolic and triterpenoid compounds, and FSC extracts from two fruit development stages on the colony growth of *M. fructicola* and *M. laxa*. Among the studied compounds, ferulic acid showed effective inhibitory effects on *M. fructicola* and *M. laxa* growth, while chlorogenic acid effect was limited to sporulation reduction. The tested compounds might modulate some genes essential for *Monilinia* spp. infection in a two-way manner, with some acting as infection inhibitors and others acting as promoters. The concept of generalized infection inhibitor needs to be reassessed. It could be split into distinctly specialized features such as germination, penetration, growth, sporulation and more based on the infection steps. Two consequences flow from this: firstly, aside from enlarging the list of genes to study in connection with candidate compounds, other features such as appressorium or peg formation, fungal melanization, and sclerotia production are important fungal characteristics that may be modulated by the compounds to investigate in the future; secondly, the solution to reduce fruit infection by *Monilinia* spp. may lie in breeding varieties capable of synthesizing a pool of compounds, each acting on a specific function of the fungus. Finally, the involvement of such natural compounds in fruit disease resistance might instigate the synthesis of eco-friendly fungicides.

Declaration of competing interest

None.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

Conceptualization, B.Q.-T.; investigation, M.H.M., M.-N.C., and L.H.; writing—original draft preparation, M.H.M.; methodology and formal analysis, M.H.M., M.-N.C., and L.H.; resources, B.Q.-T.; writing—review and editing, B.Q.-T., D.B., and M.C.; methodology and formal analysis, M.H.M., M.-N.C., and L.H.; data analysis, M.H.M., and B.Q.-T.; funding acquisition, B.Q.-T., and D.B. All authors have read and agreed to the published version of the manuscript.

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