Emission of volatile organic compounds during nectarine-*Monilinia laxa* interaction and its relationship with fruit susceptibility to brown rot

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Abstract

Fruit volatile organic compounds (VOCs) can be emitted by stone fruit in response to biotic stress. One of the main fungal diseases of stone fruit is brown rot, caused by species of *Monilinia* spp. Hence, we aimed to reveal the most relevant VOCs that participate either in resistance or susceptibility to *Monilinia laxa* in nectarines. To this aim, we analyzed the VOCs emitted by two developmental stages (immature, one month before harvest; mature, commercial harvest) of two nectarine cultivars, with different susceptibility to *M. laxa*. Furthermore, we also determined the VOCs profile of *M. laxa* grown in *in vitro* culture media based on peach juice. Results elucidated 34 VOCs whose production pattern was different among samples (control and inoculated of both stages and cultivars), being 13 VOCs also emitted by *M. laxa* culture. A hierarchical analysis and a multivariate analysis exhibited the variations in the VOCs profile of all samples according to their susceptibility to *M. laxa*, and the suitability of the model to predict the *M. laxa* disease (91.94 % of the total variation). In general, results highlighted i) a group of VOCs, positively correlated with *M. laxa* disease, that were emitted by visual *M. laxa* symptomatic tissues (e.g., aldehyde (E,E)-2,6-nonadienal) and also by *M. laxa* itself (e.g., terpenoids alpha-muurolene and (E)-beta-ionone), and ii) a group of VOCs, negatively correlated with brown rot disease, that were emitted by tissues with no visual *M. laxa* symptoms (e.g., ketone butyrolactone and aldehyde (E)-2-decenal) and also by *M. laxa* itself (e.g., aldehyde decanal), suggesting an antifungal role of these compounds. Therefore, this study provides putative potential VOCs that not only will help to improve the knowledge of brown rot development on nectarines, but also provides target volatiles that may serve as potential brown rot control compounds.

Keywords: postharvest, storage, developmental stages, stone fruit, fruit volatiles, fungal volatiles

1. Introduction

Fruit are continuously exposed to a variety of biotic and abiotic stresses. All these stresses cause, among others, an oxidative stress, metabolic imbalances, alteration of hormone responsive pathways and programmed cell death (Vickers et al., 2009; Alkan and Fortes, 2015). The most common biotic stress affecting fruit, in particular stone fruit, is caused by phytopathogenic fungi, being *Monilinia* spp., the causal agent of brown rot, one of the main fungal diseases of this fruit (Mustafa et al., 2021). *Monilinia* spp. can remain latent or quiescent on flowers and fruit surfaces until favorable factors trigger the disease cycle (Luo et al., 2005; Prusky et al., 2013). To protect themselves against these biotic stresses, fruit can produce a plethora of secondary metabolites, including the emission of volatile organic compounds (VOCs) (Baldwin et al., 2006). Volatiles are classified in several chemical categories, among which the most common in peach are alcohols, aldehydes, C6 compounds, C9 compounds, C13 norisoprenoid, esters, lactones, ketones, phenylalanine derived compounds and terpenoids (Wang et al., 2009; Montero-Prado et al., 2013; Xi et al., 2017). Terpenoid-derived VOCs are of special interest since they are considered the largest class of plant VOCs (Abbas et al., 2017). In fact, in a recent study, Balsells-Llauradó, et al. (unpublished) showed the importance of this metabolism in determining the resistance/susceptibility of nectarines against *M. laxa*.

Volatile organic compounds have direct defensive functions by acting on the pathogen, such as inhibiting the germination, the *in vitro* growth and development, or changing the activity of specific enzymes, among others, but in contrast, they can also favor fungal colonization (Mari et al., 2016; Gong et al., 2022). Alternatively, VOCs can act by activating the defensive response of the plant (induced resistance), and thus are also considered a sustainable strategy to control postharvest decay (Romanazzi et al., 2016). For instance, treatments with volatile esters in tomato resulted in stomatal closure, induction of pathogenesis-related genes, and enhanced resistance to *Pseudomonas syringae* pv. *tomato* (López-Gresa et al., 2018). In the specific case of brown rot, several VOCs have shown antimicrobial activity against

Monilinia spp. in *in vitro* and *in vivo* conditions, controlling the postharvest brown rot decay (Mari et al., 2016; Gotor-Vila et al., 2017). For instance, thyme oil vapor (with monoterpene thymol as active ingredient) increases the activity of defense-related enzymes (e.g., chitinase) and total phenolic content, which results in a reduction of *Monilinia laxa* incidence in peaches (Cindi et al., 2016). Previous studies have demonstrated that peaches inoculated with major postharvest fungi of stone fruit (*Botrytis cinerea, Monilinia fructicola* and *Rhizopus stolonifer*) significantly emitted up to eight VOCs different to mock-inoculated fruit (Liu et al., 2018). The cited study was conducted to test the use of volatiles as marker molecules to detect early fungal infections in postharvest chambers. Only recently, Dini (2019) studied the VOCs emitted in immature nectarines compared with wounded immature nectarines in which *M. laxa* progressed slowly compared to ripe fruit, although they did not find a promising relation.

Considering the importance of VOCs in plant-pathogen interactions, we aimed to elucidate the most relevant VOCs emitted by unwounded nectarine tissues of two cultivars which present different susceptibility to *M. laxa* according to their developmental stage. Besides, VOCs detected in fungus-infected fruit can be released from either by the host or by the pathogen (Gong et al., 2022), and since *Monilinia* spp. are also able to emit VOCs in *in vitro* conditions (Mang et al., 2015), we also aimed to analyze the volatile profile of *M. laxa* during *in vitro* growth on media based on peach juice in order to try to discern among volatiles emitted by either the pathosystem or the pathogen itself. Findings from this study would lead to identify volatiles emitted by nectarines in response to brown rot but also, those volatiles that may be helpful to further define nectarine defense mechanisms against *M. laxa*, and thus, useful in brown rot control strategies development.

2. Materials and methods

2.1. Plant material, fruit quality, and fungal culture

Two organically grown cultivars ('Venus' and 'Albared') of nectarine [*Prunus persica* var. *nucipersica* (Borkh.) Schneider] were used for the experiments. Nectarines were obtained

from an orchard located in Lleida (Catalonia, Spain). White paper bags, impregnated with paraffin wax, were used to bag fruit at least 6 weeks before harvest to avoid the presence of natural occurring inoculum. Fruit was harvested at two different fruit developmental stages: "immature" and "mature" fruit. The mature stage corresponded to the commercial harvest date, established according to the grower's recommendations, and the immature stage was harvested 3 and 4 weeks before the mature stage for 'Venus' and 'Albared', respectively. Fruit was homogenized by using a portable DA-Meter (TR-Turoni, Forli, Italy), based on the single index of absorbance difference. Fruit quality between stages was further confirmed by assessing the flesh firmness (FF), total soluble solids content (SSC), and titratable acidity (TA), following previously described protocols (Baró-Montel et al., 2019a) (**Table 1**).

Table 1. Fruit quality parameters of immature and mature 'Venus' and 'Albared' cultivars on harvest day. Harvest date, minimum and maximum values of the single index of absorbance difference (I_{AD}), flesh firmness (FF), soluble solids content (SSC), and titratable acidity (TA) of 'Venus' and 'Albared' nectarine cultivars. Data represents the mean (n = 20 fruit) ± Standard Error.

Cultivar	Harvest date	I_{AD}	FF (N)	SSC (%)	TA (g malic acid L^{-1})
'Venus' Immature	07-July-20 (189)[1]	1.6 - 2.1	83.3 ± 3.4	9.0 ± 0.2	5.2 ± 0.1
'Venus' Mature	24-July-20 (206)	0.4 - 1.3	63.6 ± 2.9	10.4 ± 0.3	9.6 ± 0.1
'Albared' Immature	27-July-20 (209)	1.9 - 2.2	94.5 ± 1.8	12.9 ± 0.3	5.3 ± 0.1
'Albared' Mature	28-Aug-20 (241)	0.2 - 1.4	84.4 ± 2.4	16.0 ± 0.3	11.1 ± 0.28

[1] Date expressed as Julian days (e.g., January 1st is considered as day 1).

Monilinia laxa single-spore strain 8L (ML8L, Spanish Culture Type Collection number CECT 21100) was used and conidial suspensions were maintained and prepared as previously

111 described by Baró-Montel et al. (2019b). Potato dextrose agar (PDA; Biokar Diagnostics, 39
112 g L^{-1}) supplemented with 25 % tomato pulp was used for culture media, and incubation was
113 conducted under photoperiod conditions (12 h light at 25 ºC/12 h dark at 18 ºC). Conidial
114 suspensions were obtained by rubbing the surface of a 7-day-old culture with sterile water
115 containing 0.01 % (w/v) Tween-80 and filtered conidia suspensions were diluted to the desired
116 concentration using an hemocytometer.

117 2.2. Fruit inoculations and sampling

118 Inoculations, incubation, and sampling for volatile organic compounds (VOCs) profile
119 analyses were conducted as previously described by Balsells-Llauradó et al. (2020). For
120 disease evaluation, one drop of 30 µL of conidial suspension (10^6 conidia mL^{-1}) was applied
121 on the fruit surface. Fruit was incubated in containers with a relative humidity of 97 % ± 3 and
122 20 ºC ± 1 of temperature under darkness. Disease development was examined daily as
123 disease incidence (% of brown rot) and severity (lesion diameter, cm), and were calculated
124 for each stage and cultivar (n = 20) at 3 days post-inoculation (dpi). Immature 'Venus'
125 nectarines were incubated until 7 dpi to confirm the absence of disease symptoms.

126 For tissue sampling, six drops of 30 µL of *M. laxa* conidial suspension (10^6 conidia mL^{-1}) or
127 sterile water containing 0.01 % (w/v) Tween-80 (control) were applied on each unwounded
128 fruit. Fruit was incubated under the same conditions as described before. The assay was
129 conducted with three replicates consisting of seven fruit each per treatment. Sampling was
130 carried out at 3 dpi by freezing in liquid nitrogen six cylinders of peel and pulp tissue (1 cm)
131 encompassing the inoculation sites. Frozen samples were ground into powder and stored at
132 -80 ºC until further analysis.

133

2.3. *In vitro* growth of *M. laxa* and sampling

To assess the VOCs emitted by *M. laxa*, 50 mL-flasks containing 30 mL of peach juice based-medium (100 % of organic peach juice, pH = 4.0) were inoculated with conidial suspensions to a final concentration of 2 x 10^4 conidia mL^{-1}. Flasks were incubated at 20 ºC ± 1 under complete darkness. Sampling was conducted at 3 and 7 dpi by extracting the mycelium from the top of the liquid media and rinsing it with sterile water to remove the medium residues. Mycelia were immediately flash-frozen in liquid nitrogen, ground into powder, and stored at −80 °C until further analysis. Three biological replicates were conducted.

2.4. Analyses of VOCs

2.4.1. Sample preparation and headspace solid-phase microextraction (HS-SPME)

Headspace solid-phase microextraction (HS-SPME) was performed for extracting and determining the VOCs emitted both by the nectarine-*M. laxa* study and *M. laxa in vitro* study. SPME fiber coated with a 50/30 µm layer of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco Co., Bellefonte, PA, USA) was used after being activated, according to the manufacturer's instructions. For each extraction, 5 or 1.5 g of frozen homogenized plant tissue or *M. laxa* mycelium were mixed with 5 mL or 1.5 mL, respectively, of 20 % (w/v) NaCl into a 28 mL screw-capped glass vial (previously cooled) to facilitate the release of VOCs. A volume (2 µL) of 3-nonanone (0.82 g L^{-1}) was added as an internal standard, whose absence was previously checked in all samples. Vials were immediately sealed with a magnetic screw cap provided with a PTFE/silicone septum. To undergo the same temperature treatment, once prepared, samples were stored at -20 ºC until use. Slowly thawing was performed one hour before the incubation at room temperature. For volatile extraction and determination, each sample was incubated for 20 min at 40 ºC with stirring (600 rpm), and then, the SPME fiber was exposed to the headspace of the sample for 30 min under the same conditions for volatiles absorption.

2.4.2. Gas chromatography/mass spectrometry (GC-MS)

The compounds were separated, identified, and quantified with a 7890A gas chromatograph in conjunction with 5977A MSD mass spectrometry (GC-MS) (Agilent Technologies, Inc.). The volatile compounds were desorbed from the fiber for 5 min at 220 °C into the injection port (spitless mode) of the chromatograph, which had a cross-linked polyethylene glycol-TPA (FFAP) (50 m × 200 µm × 0.33 µm) as the capillary column. Helium at 1.0 mL min^{-1} was used as the carrier gas. The solvent delay was 5 min. Temperatures of source and quadrupole were 230 and 150 ºC, respectively. The oven program was 60 ºC for 1 min, then the temperature rose at 3 °C min^{-1} to 135 °C, followed by another constant ramp of 4 °C min^{-1} to 225 °C, and held at that temperature for 15 min. The total run time was 63.25 min. Mass spectra for each compound were obtained by electron impact ionization at 70 eV. The scan mode was used to detect all the compounds from 30 to 300 m/z. Compounds were identified by comparing the mass spectral data obtained with those from standards from the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST11.L). Data of VOCs for each sample were relativized using the concentration of the internal standard (3-nonanone).

2.5. Statistical analysis

Data were analyzed with JMP® software version 16.0.0 (SAS Institute Inc., Cary, NC, USA). Brown rot incidence was analyzed using the generalized linear model (GLM) based on a binomial distribution and logit-link function. When the analysis was statistically significant, orthogonal contrasts ($P \leq 0.05$) were performed for means separation among stages and cultivars. Severity were subjected to analysis of variance (ANOVA). For means comparison between stages and cultivars, Tukey's HSD test ($P \leq 0.05$) was conducted. Regarding data of VOCs, as a pre-treatment, data were adjusted for the relation between dry fresh weight/fresh weight aiming to obviate the changes in water content occurring during fruit development. A hierarchical cluster analysis (HCA) dendrogram was conducted based on

185 Ward's method. The dendrogram graph of the HCA and heat maps were conducted to
186 establish a relationship between all analyzed VOCs (n=34) among cultivars, developmental
187 stages, and treatments (8 samples). As a pre-treatment, data were centered and weighted by
188 the inverse of the standard deviation for each variable. A partial least square (PLS) analysis
189 was conducted to correlate all 34 VOCs (X variables or explanatory variables) with brown rot
190 incidence and severity (Y variable or response). The non-linear iterative partial least squares
191 (NIPALS) algorithm with two factors was used for estimating the model parameters. Data for
192 selected VOCs were subjected to analysis of variance (ANOVA). Tukey's HSD test ($P \leq 0.05$)
193 was performed for means separation among all 8 fruit samples. Student's T-test ($P \leq 0.05$)
194 was conducted between 3 and 7 dpi *M. laxa* samples.

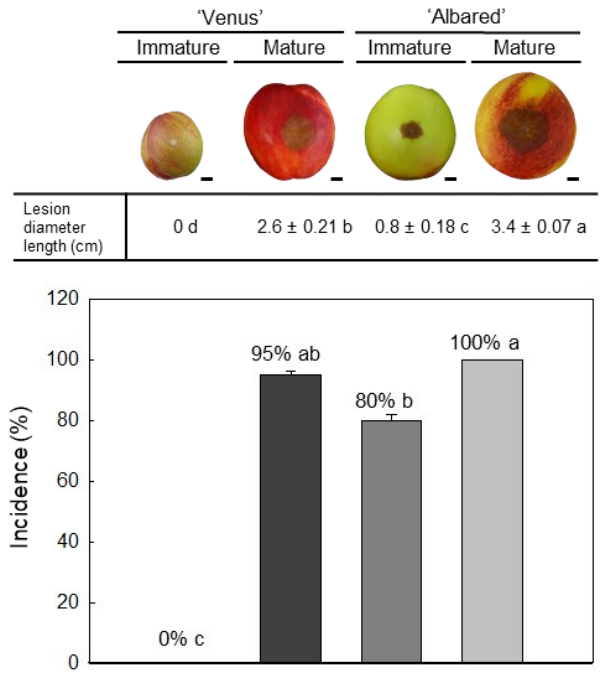
195 **3. Results**

196 3.1. The distribution of VOCs is associated with the degree of brown rot symptoms

197 Results for brown rot disease evaluation showed that *M. laxa*-inoculated 'Albared' mature
198 nectarines exhibited the highest incidence (100 %) and severity (3.4 cm ± 0.07), followed by
199 'Venus' mature (95 % and 2.6 cm ± 0.21) and 'Albared' immature (80 % and 0.8 cm ± 0.18)
200 and that no visual disease symptoms were observed in *M. laxa*-inoculated 'Venus' immature
201 nectarines (**Figure 1**). To evaluate the volatile profile of tissues with different susceptibility to
202 *M. laxa*, the two developmental stages of two different cultivars were artificially inoculated with
203 *M. laxa* (**Supplementary Figure S1**). In the VOCs analysis of the nectarine-*M. laxa* study, a
204 total of 34 VOCs were finally identified and quantified among all groups of samples. These
205 VOCs included 10 aldehydes, 7 ketones, 5 acids, 4 alcohols, 3 benzenoids, 3 terpenoids**,** 1
206 ester, and 1 furan (**Figure 2, Supp. Table S1**). To explore the variations in the VOCs profile
207 of both cultivars harvested at two different developmental stages and tissue (control or
208 inoculated), a HCA was performed integrating all VOCs data (**Figure 3**). The hierarchical
209 graph showed that samples can be grouped in two main clusters, *i*) *M. laxa*-inoculated mature

fruit (P1), and *ii*) the rest of samples subdivided into control mature 'Venus' samples (P2), and control mature 'Albared' samples and all immature tissues (P3).

Figure 1. Brown rot evaluation in 'Venus' and 'Albared' nectarines at immature and mature stages. Severity (cm of rotted fruit) and incidence (% of brown rot) and of *M. laxa* at 3 dpi. The black line indicates the scale (1 cm). Different letters indicate significant differences ($P \leq 0.05$) of incidence and severity, among tissues. Values represent the mean and error bars represent the standard error of the means (n = 20).



220 **Figure 2. Content of each VOC chemical category detected in the nectarine-*M. laxa* study at 3 dpi, for all tissues and cultivars.** For the list of VOCs corresponding to each

221 chemical category the reader is referred to Supplementary Table S1.

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223

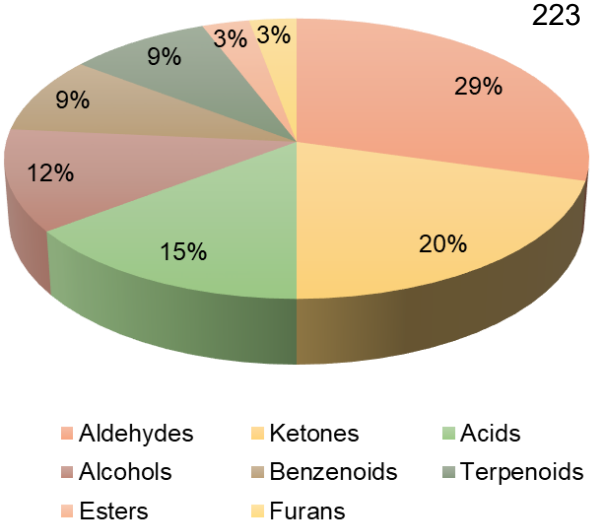

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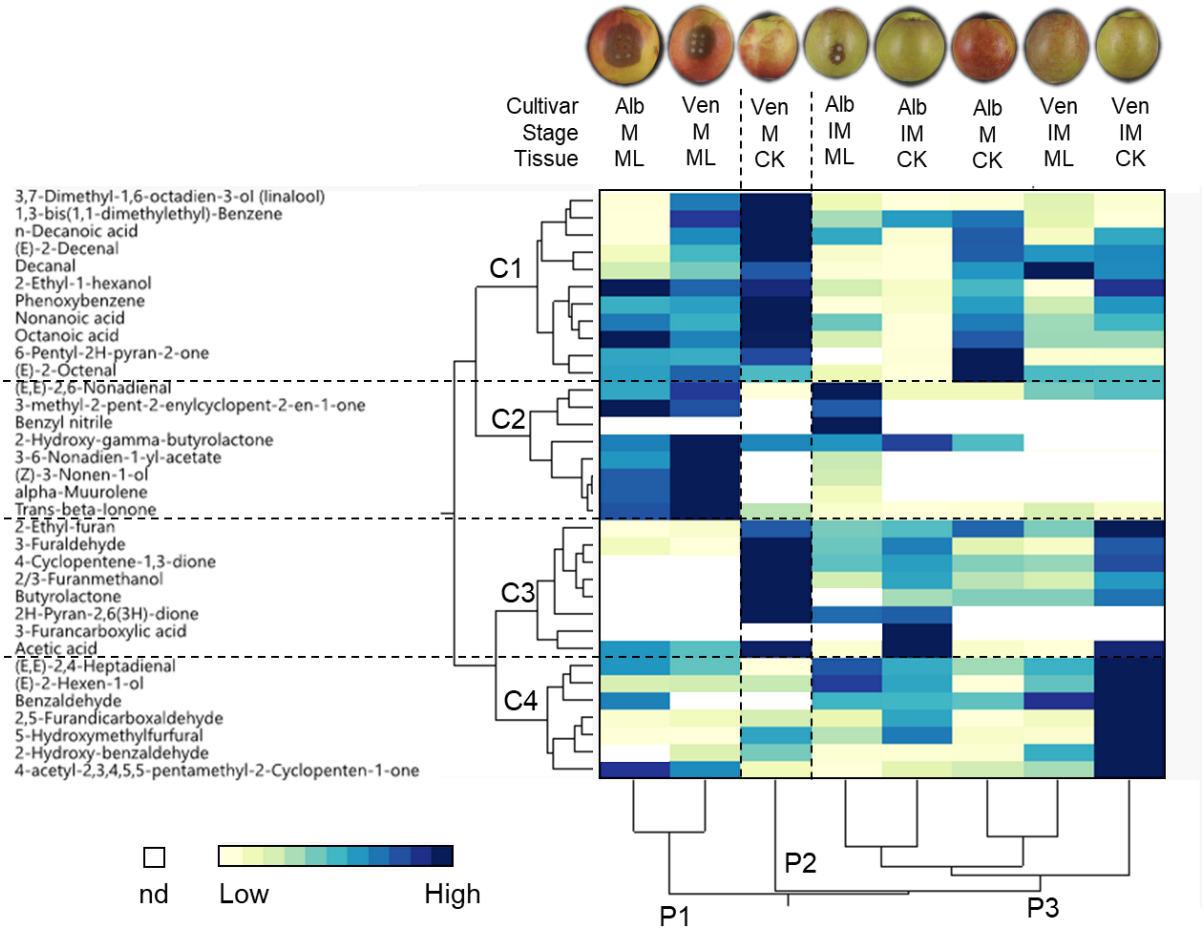
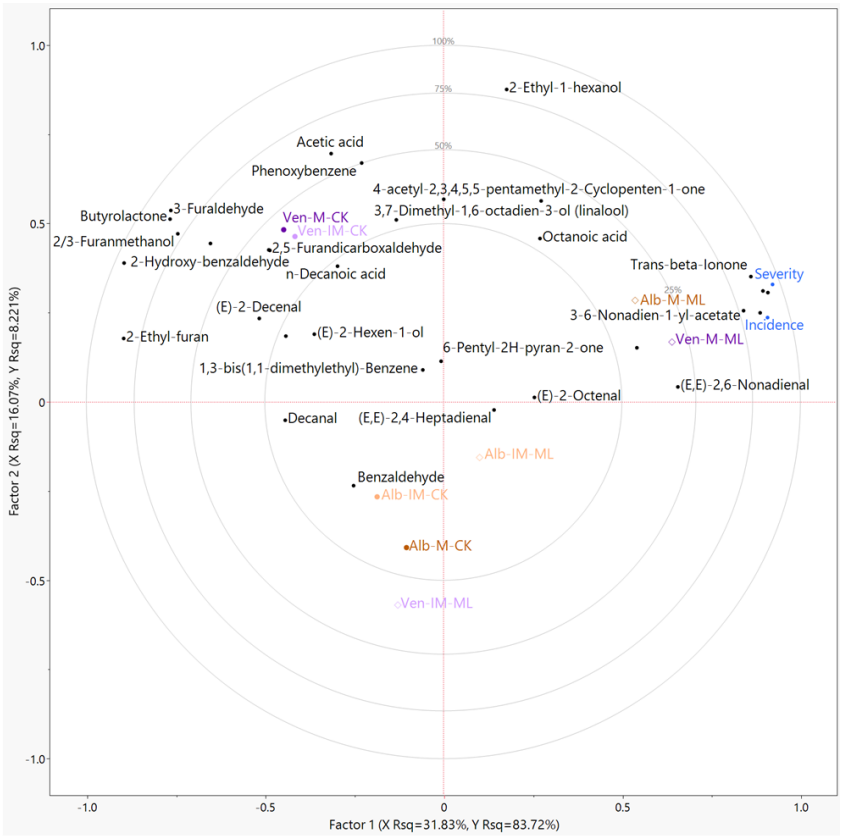
Figure 3. A two-way hierarchical cluster analysis (HCA) and heat map of the VOCs identified in each cultivar, stage, and tissue. For each group of replicates (n=3), the cultivar ('Venus', Ven; 'Albared', Alb), the developmental stage (immature, IM; mature, M), and tissue (control, CK; *M. laxa*-inoculated, ML) are specified. Fruit images correspond to each sample at 3 dpi. (Suppl. Figure S1). Clusters for compounds (Lines; C1 to C4) and for samples (Columns; P1 to P3) are indicated. Colors indicate the relative quantity to the internal standard (3-nonanone) for each VOC, where yellow represents low concentration and blue depicts high concentration. Empty cells (white) indicate a non-detected compound ("nd"). Detailed data is available in Suppl. Table S2.



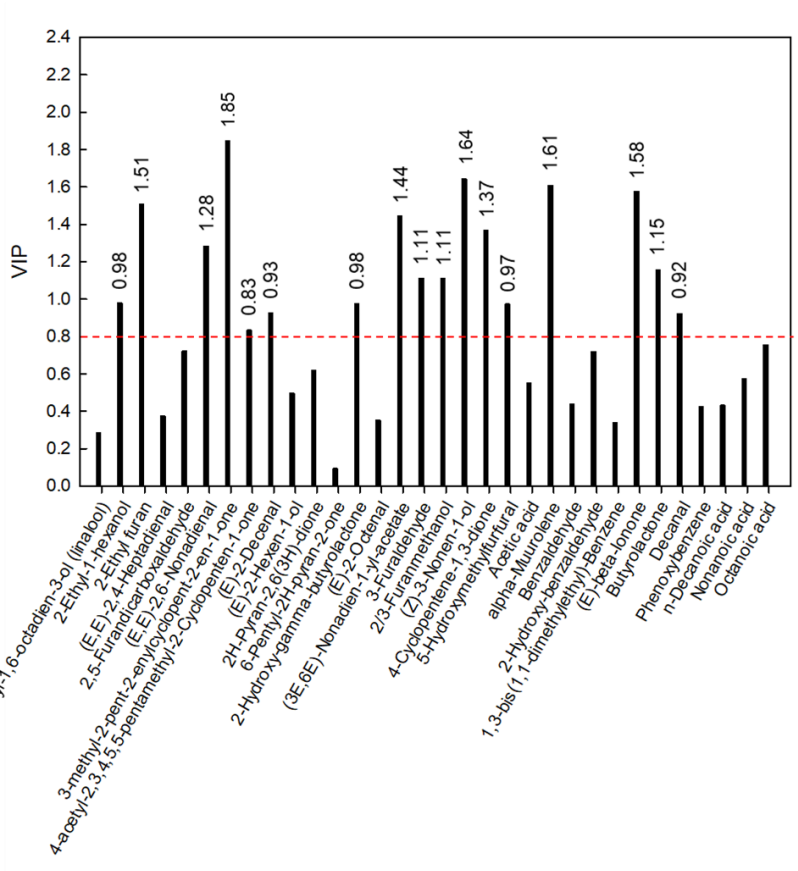
To further explore the relationship between the VOCs emitted during the *M. laxa*-nectarine interaction and the susceptibility to the pathogen, data were integrated on a multivariate analysis, correlating the VOCs produced during the interaction (X explanatory variables) with the incidence and severity of *M. laxa* (Y variables) (**Figure 4**). The PLS model showed that the two PLS factors accounted for 91.94 % of the variation observed in the total *M. laxa* incidence and severity (**Figure 4A**). In particular, the first factor of the PLS correlation loading plot explained 83.72 % of the incidence and severity of *M. laxa* and clearly separated the tissues with *M. laxa* symptoms from the visual asymptomatic or control samples. Besides, the correlation between measured and predicted incidence and severity demonstrated the effectiveness of the model (R^2 = 0.8803 and R^2 = 0.9585, respectively) for predicting brown rot incidence and severity, respectively. The variable importance plot (VIP) of the PLS model revealed 17 VOCs whose values were equal to or higher than 0.8 (**Figure 4B**), and hence considered the most influential volatiles determining the PLS projection model and explaining the variable susceptibility to *M. laxa* among the different samples analyzed.

Figure 4. A) Partial Least Squares (PLS) correlation loading plot showing the contribution of each volatile to *M. laxa* incidence and severity. Black labels indicate VOCs (explanatory variables). Blue labels indicate the incidence and severity of *M. laxa* (Y variables). Color dots (●) and diamonds (◊) indicate control and *M. laxa*-inoculated samples, respectively, of 'Venus' (purple) and 'Albared' (orange) cultivars. Their labels indicate the cultivar ('Venus', Ven; 'Albared', Alb), the developmental stage (mature, M; immature, IM), and tissue (control, CK; *M. laxa*-inoculated, ML). **B) Variable importance plot (VIP) of the PLS model.** The number of VIP ≥ 0.8 (red dashed line) indicates which predictors are important in explaining the Y variables (*M. laxa* incidence and severity) used in the PLS model. VIP values of the VOCs that have VIP ≥ 0.8, are indicated.

A


100%
75%
50%
25%
2-Ethyl-1-hexanol
Acetic acid
Phenoxybenzene
4-acetyl-2,3,4,5,5-pentamethyl-2-Cyclopenten-1-one
3,7-Dimethyl-1,6-octadien-3-ol (linalool)
Butyrolactone
3-Furaldehyde
Ven-M-CK
Ven-IM-CK
2/3-Furanmethanol
2,5-Furandicarboxaldehyde
Octanoic acid
2-Hydroxy-benzaldehyde
n-Decanoic acid
Trans-beta-Ionone
Severity
Alb-M-ML
(E)-2-Decenal
3-6-Nonadien-1-yl-acetate
Incidence
2-Ethyl-furan
(E)-2-Hexen-1-ol
Ven-M-ML
6-Pentyl-2H-pyran-2-one
1,3-bis(1,1-dimethylethyl)-Benzene
(E,E)-2,6-Nonadienal
(E)-2-Octenal
Decanal
(E,E)-2,4-Heptadienal
Alb-IM-ML
Benzaldehyde
Alb-IM-CK
Alb-M-CK
Ven-IM-ML
Factor 2 (X Rsq=16.07%, Y Rsq=8.221%)
Factor 1 (X Rsq=31.83%, Y Rsq=83.72%)

B


VIP
0.98
1.51
1.28
1.85
0.83
0.93
0.98
1.44
1.11
1.11
1.64
1.37
0.97
1.61
1.58
1.15
0.92
3,7-Dimethyl-1,6-octadien-3-ol (linalool)
2-Ethyl-1-hexanol
2-Ethyl furan
(E,E)-2,4-Heptadienal
2,5-Furandicarboxaldehyde
(E,E)-2,6-Nonadienal
3-methyl-2-pent-2-enylcyclopent-2-en-1-one
4-acetyl-2,3,4,5,5-pentamethyl-2-Cyclopenten-1-one
(E)-2-Decenal
(E)-2-Hexen-1-ol
2H-Pyran-2,6(3H)-dione
6-Pentyl-2H-pyran-2-one
2-Hydroxy-gamma-butyrolactone
(E)-2-Octenal
(3E,6E)-Nonadien-1-yl-acetate
3-Furaldehyde
2/3-Furanmethanol
(Z)-3-Nonen-1-ol
4-Cyclopentene-1,3-dione
5-Hydroxymethylfurfural
Acetic acid
alpha-Muurolene
Benzaldehyde
2-Hydroxy-benzaldehyde
1,3-bis(1,1-dimethylethyl)-Benzene
(E)-beta-Ionone
Butyrolactone
Decanal
Phenoxybenzene
n-Decanoic acid
Nonanoic acid
Octanoic acid

263

264 3.2. VOCs can be grouped by clusters and by their correlation with brown rot disease

265 Analyzing in detail **Figure 3**, 4 main clusters were deployed according to the relationship
266 between VOCs. Cluster 1 (C1) groups VOCs that, in general, were abundantly emitted by
267 mature tissues (for both control and inoculated fruit). Among them, (E)-2-decenal and decanal
268 had a significant VIP value (VIP ≥ 0.8) and were negatively correlated with the incidence and
269 severity of *M. laxa*, whereas 2-ethyl-1-hexanol (VIP ≥ 0.8) was positively correlated with *M.*
270 *laxa* disease (**Figure 4**). However, their relative quantification to the internal standard (3-
271 nonanone) was almost similar across all samples (**Tables 2 and 3**).
272

Table 2. Relative quantification of VOCs (VIP ≥ 0.8) negatively correlated with *M. laxa* incidence and severity. Data is presented relative to the internal standard (3-nonanone). Each value represents the mean (n = 3) ± Standard Error. Different letters indicate significant differences ($P \leq 0.05$) among all samples for each VOC. Non-detected compound is indicated as "nd".

		Immature				Mature			
		'Venus'		'Albared'		'Venus'		'Albared'	
Cluster	Compound	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.
C1	(E)-2-Decenal	1.26 ± 0.34 AB	1.18 ± 0.21 AB	0.37 ± 0.09 B	0.43 ± 0.03 B	1.86 ± 0.72 A	0.99 ± 0.04 AB	1.45 ± 0.21 AB	0.48 ± 0.06 AB
C1	Decanal	1.09 ± 0.23 A	1.55 ± 0.01 A	0.43 ± 0 A	0.44 ± 0 A	1.26 ± 0.06 A	0.80 ± 0.05 A	1.04 ± 0.27 A	0.64 ± 0.2 A
C3	2-Ethyl furan	2.33 ± 0.52 A	1.01 ± 0.07 AB	1.16 ± 0.29 AB	1.03 ± 0.11 AB	1.83 ± 0.63 AB	0.40 ± 0.06 B	1.76 ± 0.41 AB	0.32 ± 0.09 B
C3	3-Furaldehyde	3.80 ± 1.12 A	0.87 ± 0.06 B	3.23 ± 1.01 AB	1.97 ± 0.73 AB	5.10 ± 0 A	0.70 ± 0.12 B	1.19 ± 0.11 AB	0.97 ± 0.14 AB
C3	2/3-Furanmethanol	1.27 ± 0 B	0.33 ± 0.02 B	1.09 ± 0.38 B	0.35 ± 0.07 B	3.10 ± 0.46 A	nd	0.45 ± 0.02 B	nd
C3	4-Cyclopentene-1,3-dione	0.90 ± 0 A	0.31 ± 0.01 A	0.58 ± 0.18 A	0.41 ± 0.01 A	1.18 ± 0.49 A	nd	0.34 ± 0.02 A	nd
C3	Butyrolactone	0.52 ± 0.12 AB	0.18 ± 0 AB	0.15 ± 0.03 B	nd	0.92 ± 0 A	nd	0.18 ± 0 AB	nd
C4	5-Hydroxymethylfurfural	20.69 ± 2.38 A	1.11 ± 0.1 C	11.41 ± 3.46 B	3.67 ± 0.58 C	7.71 ± 0 BC	0.83 ± 0.19 C	1.37 ± 0.2 C	0.85 ± 0.13 C

Table 3. Relative quantification of VOCs (VIP ≥ 0.8) positively correlated with *M. laxa* incidence and severity. Data is presented relative to the internal standard (3-nonanone). Each value represents the mean (n = 3) ± Standard Error. Different letters indicate significant differences ($P \leq 0.05$) among all samples for each VOC. Non-detected compound is indicated as "nd".

		Immature				Mature			
		'Venus'		'Albared'		'Venus'		'Albared'	
Cluster	Compound	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.	Control	*M. laxa*-inoc.
C1	2-Ethyl-1-hexanol	0.89 ± 0.03 A	0.46 ± 0.10 A	0.49 ± 0.08 A	0.57 ± 0.06 A	0.91 ± 0 A	0.83 ± 0.20 A	0.72 ± 0.07 A	0.94 ± 0.17 A
C2	(E,E)-2,6-Nonadienal	0.22 ± 0.04 AB	0.20 ± 0.04 AB	0.11 ± 0.04 B	0.48 ± 0.09 A	0.08 ± 0 AB	0.41 ± 0.13 AB	0.11 ± 0.01 B	0.26 ± 0.07 AB
C2	3-methyl-2-pent-2-enylcyclopent-2-en-1-one	nd	nd	nd	1.23 ± 0.17 A	nd	1.29 ± 0.36 A	nd	1.82 ± 0.33 A
C2	2-Hydroxy-gamma-butyrolactone	nd	nd	1.48 ± 0.26 A	1.13 ± 0.33 A	1.19 ± 0 A	1.73 ± 0 A	0.90 ± 0.32 A	1.22 ± 0.30 A
C2	(3E,6E)-Nonadien-1-yl-acetate	nd	nd	nd	0.32 ± 0.08 B	nd	3.63 ± 0.61 A	nd	1.29 ± 0.42 B
C2	(Z)-3-Nonen-1-ol	nd	nd	nd	0.74 ± 0.12 B	nd	8.05 ± 2.35 A	nd	5.09 ± 1.06 AB
C2	alpha-Muurolene	nd	nd	nd	0.74 ± 0.27 B	nd	21.83 ± 5.12 A	nd	13.64 ± 0.14 A
C2	(E)-beta-Ionone	0.93 ± 0.4 B	1.20 ± 0.21 B	0.84 ± 0.19 B	0.91 ± 0.06 B	1.35 ± 0.10 B	4.67 ± 0.71 A	0.87 ± 0.15 B	3.46 ± 0.57 A
C4	4-acetyl-2,3,4,5,5-pentamethyl-2-Cyclopenten-1-one	0.80 ± 0.33 A	0.46 ± 0.10 A	0.41 ± 0.14 A	0.36 ± 0.01 A	0.39 ± 0 A	0.60 ± 0.18 A	0.43 ± 0.06 A	0.73 ± 0.17 A

Cluster 2 (C2) shows those VOCs that, overall, were produced by *M. laxa* symptomatic tissues (**Figure 3**), indicating that they could be produced either by the host, the pathogen, or both. The alpha-muurolene and (E)-beta-Ionone (both with VIP ≥ 0.8) were positively correlated with *M. laxa* disease (**Figure 4**). Their emission in tissues with high *M. laxa* incidence (mature fruit of both cultivars) were significantly higher, being 24.0- and 4.5-fold higher (in average) than the other tissue with less disease symptoms (*M. laxa*-inoculated immature 'Albared' nectarines) (**Table 3**). Besides, alpha-muurolene was not detected either in the asymptomatic tissue or control samples. Other compounds (3-methyl-2-pent-2-enylcyclopent-2-en-1-one, (3E,6E)-nonadien-1-yl-acetate and (Z)-3-nonen-1-ol) had also significant VIP values (VIP ≥ 0.8), were positively correlated with *M. laxa* disease, and exclusive emitted by tissues with visible *M. laxa* symptoms (**Figure 3, 4, Table 3**). Remarkably, benzyl nitrile was only detected in *M. laxa*-inoculated immature 'Albared' nectarines (**Figure 3**).

VOCs from the cluster 3 (C3) of the HCA were, in general, more produced in control mature and immature tissues with low or no visual *M. laxa* symptoms than in tissues with advanced disease symptoms (**Figure 3**). Among them, 5 VOCs had significant VIP values (VIP ≥ 0.8) and were negatively correlated with *M. laxa* disease (**Figure 4**). The quantity of these compounds tends to be higher in control than in *M. laxa*-inoculated fruit, and only few significant differences were detected among all samples. For instance, the emission values of 3-furaldehyde for control immature and mature 'Venus' nectarines were 4.4- and 7.3-fold significantly higher, respectively, than those for *M. laxa*-inoculated 'Venus' fruit (**Table 2**). In turn, in the 'Albared' cultivar, this compound showed a similar pattern among samples, although not statistically significant. For other VOCs such as 2/3-furanmethanol, 4-cyclopentene-1,3-dione, and butyrolactone, their production was lower in inoculated tissues with low or no *M. laxa* disease symptoms compared to control samples, and even not detected in tissues with high disease incidence. These results showed a clear pattern caused by the emission of these VOCs mainly due to the presence of pathogen on nectarine tissue rather than developmental stages and/or cultivar.

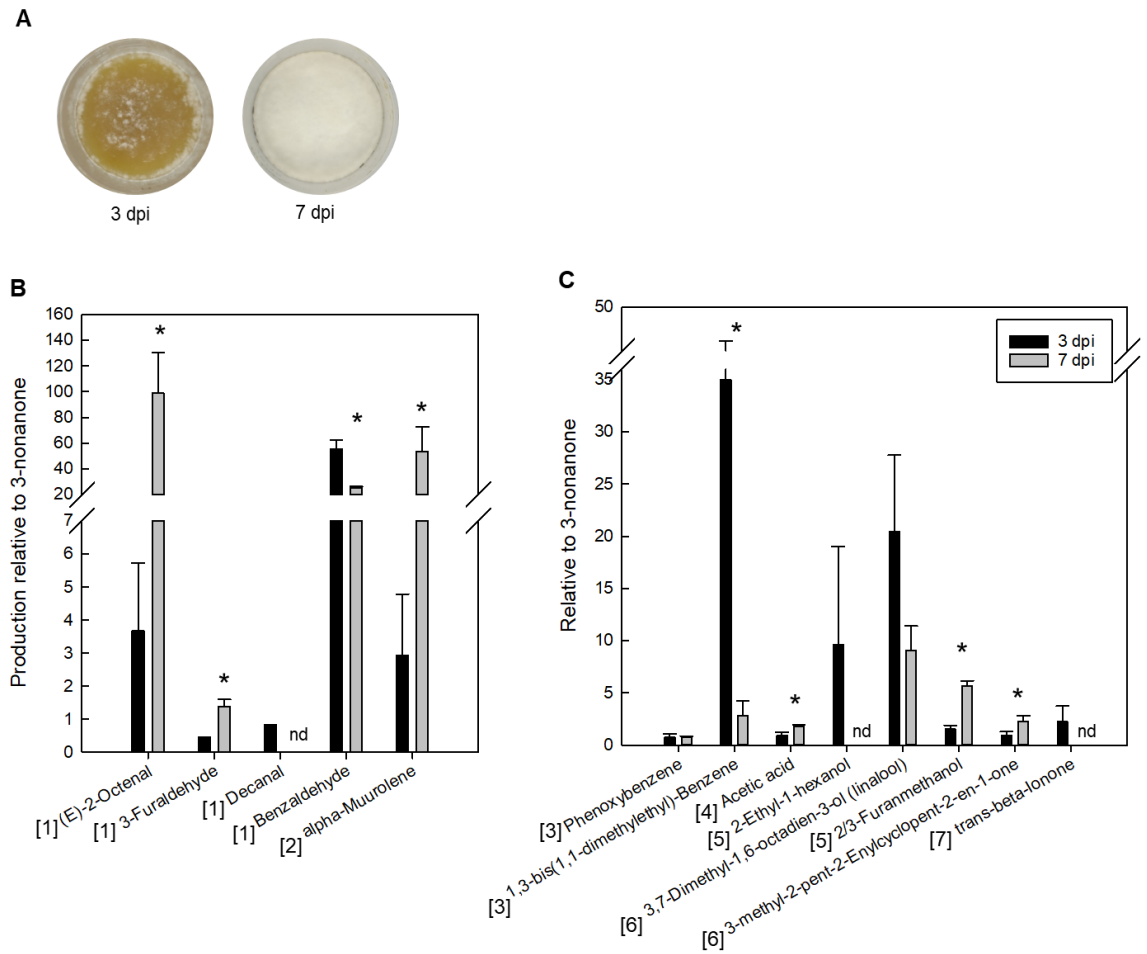
Regarding cluster 4 (C4), VOCs such as (E,E)-2,4-heptadienal, (E)-2-hexen-1-ol, 2,5-furandicarboxaldehyde, 5-hydroxymethylfurfural, and 4-acetyl-2,3,4,5,5-pentamethyl-2-cyclopenten-1-one were widely distributed throughout all samples, although emitted at low quantities in some samples (like mature ones) (**Figure 3**). Among them, 5-hydroxymethylfurfural and 4-acetyl-2,3,4,5,5-pentamethyl-2-cyclopenten-1-one had significant VIP values and were negatively and positively correlated with *M. laxa* disease, respectively (**Figure 4**). Specifically, 5-hydroxymethylfurfural was overall significantly higher in the control immature fruit of both cultivars than the other samples (**Table 2**).

3.3. *Monilinia laxa* VOCs profile reveals shared compounds with control and *M. laxa*-inoculated nectarines

To investigate whether the VOCs detected in the *M. laxa*-nectarine pathosystem were exclusively produced by nectarines as a host response or could be produced by *M. laxa* itself, a VOCs analysis of *M. laxa* grown in an *in vitro* peach-based medium was conducted. Sampling of fungal samples was performed at 3 dpi (the same sampling time point of the *M. laxa*-nectarine interaction study) and at 7 dpi (time in which the pathogen reached its maximum *in vitro* growth) (**Figure 5A**). A total of 72 VOCs were finally identified and quantified, being 13 of them, also detected in the nectarine-*M. laxa* interaction study (**Figure 5B and 5C**). Three VOCs were only detected at 3 dpi (decanal, 2-ethyl-1-hexanol, and (E)-beta-Ionone) and two were similarly emitted at both 3 and 7 dpi (e.g., phenoxybenzene and linalool). Remarkably, 6 VOCs detected at 7dpi were significantly higher than at 3 dpi, including (E)-2-octenal, acetic acid, 3-furaldehyde, 2/3-furanmethanol, alpha-muurolene, and 3-methyl-2-pent-2-enylcyclopent-2-en-1-one, ranging from 1.8 to 26.9-fold higher. Finally, the emission of benzaldehyde and 1,3-bis(1,1-dimethylethyl)-benzene at 3 dpi was 2.2- and 12.3-fold higher at 3 dpi than at 7 dpi.

335 **Figure 5. VOCs detected in *M. laxa in vitro* culture that were commonly detected in the**
336 **nectarine-*M. laxa* study.** (**A**) Images of *M. laxa* growth on the top of peach-based medium
337 corresponding to each sampling point (3 and 7 dpi). VOCs are grouped into (**B**) aldehydes [1]
338 and sesquiterpene [2] and (**C**) benzenoids [3], acids [4], alcohols [5], monoterpenes [6], and
339 ketones [6]. Data is presented relative to the internal standard (3-nonanone). Each value
340 represents the mean (n = 3) ± Standard Error. Symbol (*) indicates significant differences ($P \leq$
341 0.05) between 3- and 7-dpi for each VOC. Non-detected compound are indicated as "nd".

342



343

344 **4. Discussion**

345 By analyzing the VOCs profile of nectarine tissues with different susceptibility to *M. laxa,*
346 results allowed to group VOCs according to their relationship with fruit susceptibility to the

pathogen. Results also suggested the most influential volatiles explaining (i.e., positively correlated) the progression of *M. laxa*. Besides, VOCs can be emitted either by fruit in response to *M. laxa*, by the pathogen as development or virulence mechanisms, or by both organisms. Herein, out of the total VOCs detected in the nectarine-*M. laxa* study, 20 compounds were emitted by all samples. Cluster 1 groups, in general, VOCs that were more abundant in mature than in immature tissues, but also, some VOCs that were more emitted in control than in *M. laxa*-inoculated tissues. For instance, acid compounds such as nonanoic and octanoic, produced by all the analyzed samples, were also detected in slices of mature nectarines (Giné-Bordonaba et al., 2014) as well as the ketone 6-pentyl-2H-pyran-2-one, detected in skin and pulp of mature peaches (Aubert and Milhet, 2007). Regarding the production of VOCs by *M. laxa* grown in an *in vitro* peach-based medium, these results are the closest approach to the nectarine tissue for discerning among which VOCs can be also emitted by the pathogen, but in any case, we cannot assume that the rest of VOCs are exclusively emitted by the host. Hence, out of VOCs in C1, five of them were not detected in *M. laxa in vitro* culture, suggesting that they were produced by the host at the mature stage, and probably, involved in susceptibility factors. In this sense, El-Sayed et al. (2014), who also detected 6-pentyl-2H-pyran-2-one in ripe but not in unripe peach (with attached leaves), found that this compound is attractive to the New Zealand Flower Thrips (causing pest in mature stone fruit), and hence, contributing to the onset of the pest. Altogether points out that compounds such as 6-pentyl-2H-pyran-2-one could act as susceptibility factors promoting brown rot.

Among the VOCs emitted by all samples and, in general, more abundant in immature fruit (especially immature control ‘Venus’ nectarine, grouped in C4), almost all were emitted by the host (i.e., not detected in *M. laxa in vitro* culture) except for aldehyde benzaldehyde. Among the compounds exclusively emitted by the host, 2,5-furandicarboxaldehyde (aldehyde) and (E)-2-hexen-1-ol (alcohol) were more emitted in immature than in mature samples, and the presence of *M. laxa* only impaired the VOCs profile in immature but not in mature tissues.

374 Other authors also reported the production of 2,5-furandicarboxaldehyde in immature
375 peaches (Bacvonkralj et al., 2014) and (E)-2-hexen-1-ol in unripe but also in commercially
376 ripe nectarines (Aubert et al., 2003). Besides, (E)-2-hexen-1-ol was also detected in *B.*
377 *cinerea*-inoculated peaches, in which the production was significantly lower than that in
378 healthy fruit after 48 h of storage (Liu et al., 2018), which is a similar pattern to inoculated and
379 control immature 'Venus' nectarines of our study. Hence, all suggest that these VOCs are
380 overall typical of immature tissues and that can be altered by fungi during infection processes
381 as a strategy to infect fruit. Furthermore, in this study, the production of (E,E)-2,4-heptadienal
382 (aldehyde) by the resistant 'Venus' immature tissue was slightly lower than that from its
383 control, and higher in *M. laxa*-inoculated susceptible tissues than their controls. In line with
384 these results, other authors also detected (E,E)-2,4-heptadienal in peaches, which also
385 showed a high positive correlation with flesh firmness, suggesting that it accumulates in
386 immature peach fruit (Sánchez et al., 2012). Besides, this compound is produced from
387 linolenic acid via the lipoxygenase (LOX) pathway in leek (Nielsen et al., 2004), and Balsells-
388 Llauradó et al. (2020) found that the *PpLOX3* gene is upregulated in response to *M. laxa* in
389 resistant immature nectarines if compared to control fruit. Taken all together reveals that these
390 compounds (e.g., (E)-2-hexen-1-ol and (E,E)-2,4-heptadienal) are produced by the host at
391 the immature stage and seem to be involved in resistance to *M. laxa*.

392 Among the VOCs emitted by visual symptomatic *M. laxa* tissues (rotted fruit), and positively
393 correlated with brown rot (overall located in the C2), 4 VOCs were also found in *M. laxa in*
394 *vitro* culture, indicating that they could be produced by *M. laxa* itself or by the fruit in response
395 to the pathogen. Since the terpene alpha-muurolene and the ketone 3-methyl-2-pent-2-
396 enylcyclopent-2-en-1-one were exclusively produced by rotted tissue, this suggests that they
397 were probably emitted by the pathogen rather than by the host. A BLAST search in either
398 *Rosaceae* or *Prunus* organisms revealed no matches with the codifying gene for the alpha-
399 muurolene synthase (*COP3*) of the fungus *Marasmius oreades* (Hiltunen et al., 2021).
400 Although it cannot be discarded of being a fruit VOC, all evidence points towards that alpha-

401 muurolene is emitted by the pathogen. In this sense, Thelen et al. (2005) detected alpha-
402 muurolene in tomato leaves infected by *B. cinerea* and Mang et al. (2015) found that this VOC
403 is emitted by *M. fructicola* and *M. fructigena* in *in vitro* cultures. The function of this compound
404 is poorly understood, but some authors relate the emissions of this compound with the
405 toxigenicity of the phytopathogenic fungus *Aspergillus flavus* (Josselin et al., 2021) or emitted
406 by the saprotrophic fungi *Hypholoma fasciculare* against ectomycorrhizal fungus *Pisolithus*
407 *tinctorius* (Baptista et al., 2021). Hence, in our study, alpha-muurolene could be emitted by
408 *M. laxa* as a virulence factor.

409 In addition to the VOCs emitted by rotted tissues and shared with the *M. laxa in vitro* culture,
410 the alcohol 2-ethyl-1-hexanol and the terpene (E)-beta-ionone were emitted by all samples of
411 the nectarine-*M. laxa* study. The detection of 2-ethyl-1-hexanol, as previously described in
412 other peach and nectarine cultivars (Giné-Bordonaba et al., 2014; Xin et al., 2018), was
413 similarly emitted in all samples of our study, suggesting that it is a fruit VOC, or seems not to
414 be involved in *M. laxa* response. On the other hand, (E)-beta-Ionone was significantly highly
415 produced in the very rotted fruit of both cultivars if compared to control and immature samples.
416 Hence, although (E)-beta-ionone is produced by several peach cultivars (Montero-Prado et
417 al., 2013; Xin et al., 2018), our results suggest that in the presence of *M. laxa*, the emission
418 of this compound is enhanced, either by the action of the fungus or just because the pathogen
419 itself is able to produce it, leading to an increased susceptibility of the tissue. Some of these
420 VOCs, such as (E)-beta-ionone, are derived from the terpenoid metabolism, and specifically
421 from (9Z)-beta-carotene, which are also highly induced in *M. laxa* infected nectarines
422 (Balsells-Llauradó, unpublished). Recently, Brambilla et al. (2021) found that infected plants
423 of barley emit (E)-beta-ionone, which in turn, induces resistance in neighbor plants. Hence,
424 susceptible tissue of our study could be using such compounds, which can be emitted by the
425 pathogen or by the host, as a fruit-fruit signaling to induce resistance on the neighbor fruit.

426 Out of the VOCs that were emitted by rotted fruit and positively correlated with brown rot, 6
427 were not detected in the *M. laxa in vitro* culture, indicating that they may be exclusively

produced by fruit. Some of them, like the ketone 2-hydroxy-gamma-butyrolactone, have already been described in peach leaf extract (Ozpinar et al., 2017). A closely related compound to the aldehyde (E,E)-2,6-nonadienal (i.e., (E,Z)-2,6-nonadienal) is also emitted by peaches (Wang et al., 2009; Xi et al., 2017), and besides, applied as a fumigant, it reduced *Botrytis* growth on strawberries (Archbold et al., 1997). However, on the contrary to that described in *Botrytis,* this compound seemed to favor, or at least, it did not prevent *M. laxa* infection since the disease developed in the tissues in which it was detected. On the other side, since (3E,6E)-nonadien-1-yl-acetate, (Z)-3-nonen-1-ol and benzyl nitrile (although not considered VIP compound) were exclusively produced by rotted tissues, results presented herein point out that these compounds are produced by the host as a response to the *M. laxa* disease. To our knowledge, (3E,6E)-nonadien-1-yl-acetate and (Z)-3-nonen-1-ol have been detected in several melon cultivars (Shi et al., 2020), but no studies have revealed their implication in fruit diseases nor their emission by fungi. Further studies should be conducted to explore whether these VOCs are produced by the fruit and could somehow favor the disease susceptibility.

Volatile organic compounds that were negatively correlated with brown rot (overall located in C3), were, in general, lower emitted in *M. laxa*-inoculated fruit than in their respective control tissues. However, among them, three compounds (3-furaldehyde, 2/3-furanmethanol, and decanal), were also emitted by *M. laxa in vitro* culture. Although our methodology was not able to discern between the alcohol 2- or 3-furanmethanol, Liu et al. (2018) reported that 2-furanmethanol is emitted by *B. cinerea*-inoculated peaches but neither in *M. fructicola*-inoculated nor control fruit. These results are in line with our study in which 2/3-furanmethanol was not detected in *M. laxa*-inoculated fruit. Besides, 2-furanmethanol is one of the main bioactive compounds produced by a *Bacillus* strain (DM6120) that suppresses the mycelial growth of *Colletotrichum nymphaeae* (Alijani et al., 2022). Regarding the aldehyde decanal, which is commonly emitted by peaches and nectarines (Montero-Prado et al., 2013; Giné-Bordonaba et al., 2014), it is also emitted by active molds on aged model materials (e.g.,

Alternaria alternata on silk and *Cladosporium herbarum* on paper) (Sawoszczuk et al., 2015). Besides, its exogenous application significantly inhibits the germination and development of *Penicillium expansum in vitro*, by decreasing the oxidative phosphorylation as one of the main inhibitory actions (Zhou et al., 2020). Hence, based on our results, two main hypotheses can arise: (1) the fact that they were overall lower or even not detected in rotted tissues, suggests that these VOCs were probably generated by the host rather than emitted by the pathogen itself. In this sense, the host could be reducing its emission since the fruit tissue was already invaded by the pathogen, and thus could drive the energy towards other metabolisms. Alternatively, another hypothesis could be that (2) although *M. laxa* can produce these VOCs for its development (i.e., observed during *in vitro* culture on the peach juice based-medium), the results presented herein suggest that the pathogen was not producing them, since they were low or almost not detected in rotted samples. In turn, *M. laxa* could be repressing the emission of these VOCs in attempt to inhibit the negative effect that these compounds may have on the pathogen. Therefore, further studies are necessary to confirm these hypotheses and validate the negative effects of these compounds on *M. laxa*.

Furthermore, among the VOCs that were lower in *M. laxa*-inoculated fruit than in their respective control tissue, and negatively correlated with *M. laxa* disease, 5 VOCs were not detected in *M. laxa in vitro* culture, indicating its implication exclusively as a fruit response to *M. laxa*. Herein, the compounds 2-ethyl furan and the aldehyde (E)-2-decenal were detected in all samples at different levels of emission (mostly lower in *M. laxa*-inoculated than in their controls), whereas 4-cyclopentene-1,3-dione, butyrolactone, and 5-hydroxymethylfurfural, were almost not or lower emitted by rotted tissues compared to the other tissues. For instance, (E)-2-decenal is also detected in bean infected with *Colletotrichum lindemuthianum* and besides, it completely inhibits the mycelia growth of *C. lindemuthianum* and *B. cinerea* when the compound is exposed to the atmosphere of each pathogen (Quintana-Rodriguez et al., 2018). Furthermore, derivates of the ketone butyrolactone, also detected in peach cultivars (Xin et al., 2018), showed antifungal effects towards *B. cinerea* (Cazar et al., 2005). In this

line, the aldehyde 5-hydroxymethylfurfural, a product of the degradation of furfural, which is also emitted by peaches inoculated with *R. stolonifer* (Liu et al., 2018) and by immature peaches (Bacvonkralj et al., 2014), also showed inhibition of the cell growth of some yeast strains (Liu et al., 2004). Hence, since these compounds were lower in susceptible tissues than in their respective control tissue and were not produced by the fungus itself in the tested conditions, altogether indicates that these compounds may have antifungal activity, and *M. laxa* repressed or inhibited their production as a strategy to infect the fruit. In a study conducted with inoculated pears with either *P. expansum* or *R. stolonifer*, the most effective compounds (i.e., negatively correlated with incidence) reduced or even completely controlled mycelial growth of these pathogens in *in vitro* conditions (Torregrosa et al., 2020). Therefore, future studies could be conducted towards studying the antifungal effect of these compounds and their role as sustainable products for brown rot control.

5. Conclusions

The results from this study demonstrate that the degree of visual brown rot symptoms was associated with the VOCs profile of control and *M. laxa*-inoculated samples. Besides, the *M. laxa in vitro* culture allowed us to discern which of the detected VOCs could also be produced by the pathogen. The different VOCs profile in response to *M. laxa* also sheds light on the different susceptibility to *M. laxa* of the different samples studied herein. Hence, the group of positively correlated VOCs with brown rot (e.g., (E,E)-2,6-nonadienal), some of them shared with the VOCs emitted by *M. laxa in vitro* culture (e.g., alpha-muurolene), was crucial for determining which of them may favor the susceptibility of nectarines to *M. laxa* infection. In turn, negatively correlated VOCs with *M. laxa* development, could be selected as potential antifungal compounds (e.g., (E)-2-decenal and butyrolactone). Overall, the results presented herein improve the knowledge of *M. laxa* infection on nectarines and highlight target volatiles that may serve as potential brown rot control compounds.

6. Declaration of competing interest

- attached document-

7. Author contributions

Marta Balsells-Llauradó: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Data curation. **Rosario Torres:** Conceptualization, Supervision, Project administration, Writing- Reviewing and Editing. **Gemma Echeverría:** Formal analysis, Writing- Reviewing and Editing. **Núria Vall-llaura:** Conceptualization, Methodology, Investigation, Writing- Reviewing and Editing. **Neus Teixidó:** Investigation, Resources, Writing- Reviewing and Editing. **Josep Usall:** Supervision, Funding acquisition, Writing-Reviewing and Editing.

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