Universiteit
Leiden
The Netherlands

Potential interference of fungal endophytes in Vanilla planifolia on vanilla flavor compounds biosynthesis

Khoyratty, S.

Citation
Khoyratty, S. (2020, October 27). *Potential interference of fungal endophytes in Vanilla planifolia on vanilla flavor compounds biosynthesis*. Retrieved from https://hdl.handle.net/1887/137981

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/137981

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/137981 holds various files of this Leiden University dissertation.

Author: Khoyratty, S.
Title: Potential interference of fungal endophytes in Vanilla planifolia on vanilla flavor compounds biosynthesis
Issue Date: 2020-10-27

Chapter 4: Analysis of volatiles from vanilla pod media after vanilla fungal endophyte growth

Shahnoo Khoyratty[1,2], Robert Verpoorte[1], Young Hae Choi[1], Hippolyte Kodja[2],

[1] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg, 72, 2333BE, Leiden, The Netherlands.

[2] Université de La Réunion, UMR PVBMT, 15 avenue René Cassin, CS 92003-97744 Saint Denis Cedex 9, La Réunion, France.

ABSTRACT

Vanilla flavor includes some 250 components, including a number of volatiles. Various fungi are known to be able to produce such volatiles. So the research question was whether endophytic fungi from vanilla pods may contribute to the vanilla aroma or not. To answer this question two endophytes, *Diaporthe phaseolorum* and *Pestalotiopsis microspora,* previously isolated from green vanilla pods, and one newly isolated fungus from pods after scalding, *Hypoxylon investiens,* were grown on different media. To measure the production by the fungi themselves they were grown on a potato dextrose agar medium. The results were compared with the fungi grown on media containing ground green *Vanilla* pod material or *Vanilla* pod waste after extraction with 40% ethanol (extracted cured pod medium) to isolate the vanilla aroma. After growing the fungi on the media, static headspace GC-MS analysis was performed on the media.

Several volatiles known to contribute to the Vanilla aroma were found in the media: *p*-xylene, α-phellandrene, 3-carene, α-terpineol, *p*-hydroxybenzaldehyde, α-cubebene, β-caryophyllene, vanillin, vanillyl alcohol. All were produced by the fungi growing on *Vanilla* material containing media at levels higher than in the control media without fungal growth. The type of media and the fungal species used contributed significantly to the type and abundance of synthesized volatiles, whereas the growth period had no significant effects. The production of these volatiles could be due to *de novo* biosynthesis by the endophytes, or biotransformation of precursors from the vanilla pod materials.

The fungus *Hypoxylon investiens* (HI) recovered after pod scalding, shows promise for the re-use of extracted cured pods (ECP). It seems that this fungus can convert ferulic acid into vanillin. The results support the hypothesis that endophytes play a role in Vanilla flavor metabolite biosynthesis.

In preparation for publication as: "Khoyratty S., Verpoorte R., Choi Y.H., Kodja H. Analysis of volatiles from vanilla pod media after vanilla fungal endophyte growth"

4.1 BACKGROUND

Vanilla aroma consists of more than 250 taste and aroma components (Kennedy 2015) among them, a number of volatile compounds. The value of vanilla aroma resides in its complexity which does not limit itself to vanillin content (Buccellato 2011). The complex nature is reflected by the diverse organoleptic descriptors used for the vanilla flavor and includes sweet, minty, fruity, spicy, woody, vanilla, balsamic, anise (Burdock 2010, Azeez 2008). Additionally, the presence of non-vanillin volatiles is the reason for a marked preference for natural vanilla preparations over synthetic vanilla flavorings (Reineccius 2005). Volatiles that contribute to the organoleptic properties of vanilla aroma of cured pods have been reported by Toth et al. (2011). These volatiles include chemical classes such as terpenoids, arenes, and phenols (Gu et al. 2015, Zhang and Mueller 2012, Toth et al. 2011, Azeez 2008). And yet, most research on vanilla flavor has focused on phenolics and not much on other vanilla aroma components. Fungi are well known for biotransformations in the production of flavors and fragrances (Molina et al. 2013). For example, *Penicillium digitatum* and *Fusarium oxysporum* can both convert limonene into α-terpineol.

In the previous Chapter 3 the possible role of fungal endophytes in the production of non-volatile vanilla flavor compounds was addressed. The formation of volatile aroma compounds in vanilla pods have up to now only been studied for some *Bacillus* species that were isolated from vanilla (Gu et al. 2015). No studies on the production of volatiles by vanilla fungal endophytes have been reported. The objective of this study was to characterize the volatiles formed from vanilla pod material, as pod-based agar medium and after fungal growth, by some fungal endophytes found in vanilla.

Gas chromatography mass spectrometry (GC-MS) is routinely used in laboratories to identify volatile metabolites in samples. To identify the volatile aroma metabolites possibly produced by the endophytes, static headspace GC-MS analysis was used to avoid the problems of making extracts of volatiles from plant material. The advantage of the head space is that one measures the real volatiles above the material, with little risk of decomposition and loss of volatiles during extraction procedures. The disadvantage is that each individual compound will have a different vapor pressure, and that the head space analysis cannot, thus, be used to measure the amount present in the material itself (Hamilton 2012). Moreover the GC-MS has a compound specific detector response that means that quantitation can only be done with calibration curves for each single compound. This implies one can only compare peak height or peak area for each single compound in a relative quantitative analysis. Comparison of peaks of different compounds in a single chromatogram is not possible.

Considering the possible role of endophytes in the curing process, one needs to characterize the whole postharvest processing of vanilla pods (Dignum et al. 2001, 2002). Harvested green vanilla pods undergo several processing steps before being ready for use as the well-known cured vanilla pods. The first step is to put green pods in hot water (65-70^{o}C) for 2 min, a process known as scalding. Roling et al. (2001) assessed microbial diversity at different post-harvest processing stages in Indonesia (Bali). It was found that microbial communities changed the most after this first scalding step. Only some micro-organisms which are heat resistant survived this treatment. Some fungi are heat resistant e.g. the endophyte *Hypoxylon pulicicidum* sp. *nov.* remains viable even in heat-dried herbarium

voucher specimens (Bills et al. 2012). Hence, we tested scalded pods for the presence of survived endophytes, resulting in the isolation *Hypoxylon investiens*. Besides this heat resistant fungus, two others involved in the production of the non-volatile vanilla flavor compounds (chapter 3): *Diaporthe phaseolorum*, and *Pestalotiopsis microspora* were tested for the production of volatiles.

4.2 MATERIALS AND METHODS

4.2.1 Endophyte isolation from mature green pods

The endophytes *Diaporthe phaseolorum* and *Pestalotiopsis microspora* used in the present study were previously isolated and identified (chapter 3).

4.2.2 Endophyte isolation after green pod scalding

Fifteen green pods were collected from St. Rose, the region with the pods said to have the best flavor (Bertrand Côme - La Vanilleraie, *pers*. *comm*.). The pods were all mature eight months old post pollination. The pods were immersed in hot water at 65°C for 3 min (scalding). Endophyte isolation was performed on the scalded pods as described in Chapter 3. Isolation was performed, as before, on potato dextrose agar medium (PDA), 20 g of commercially available powder (Duchefa Biochemie, Haarlem, The Netherlands) was added to 1 L of distilled water, autoclaved and poured. Identification as *Hypoxylon investiens* was performed by spore and mycelium, morphological cultural characteristics. Moreover a polymerase chain reaction (PCR) was performed as before, except β-tubulin primers were used this time. After sequencing, NCBI blast hit was used for identification (National Center for Biotechnology Information, Md., USA).

4.2.3 Volatile emission from medium after fungal endophyte growth

Three fungi were used: *Hypoxylon investiens* (see above), and the previously used *Pestalotiopsis microspora* and *Diaporthe phaseolorum* (Chapter 3). Each fungus was grown as single culture on three types of medium. Two time points were tested: 15 and 30 days after inoculation. The media were: extracted cured vanilla pod agar medium (ECPM) accession 13B1 from St. Andre, green vanilla pod agar medium (GPM) accession 13B1 from St. Andre, and normal potato dextrose agar growth medium (PDA). The extracted vanilla pod material contained the solid waste-product after the pods had been extracted with 40% ethanol to obtain the liquid vanilla extract. After freeze-drying the waste-product was ground into a fine powder (material 1) and added to the medium. For GPM, green pods (eight months post pollination) were freeze-dried and crushed into a fine powder (material 2). For the Vanilla media 15 g of either pod material 1 or 2, were added toghether with 15 g agar (Duchefa Biochemie, Haarlem, The Netherlands) to 1 L of distilled water. This gave the green pod medium (GPM) and the extracted cured pod medium (ECPM). The media were autoclaved to kill native endophytes in the plant tissue before culturing the test endophytes on the media. For PDA, 20 g of commercially available powder (Duchefa Biochemie, Haarlem, The Netherlands) was added to 1 L of distilled water, autoclaved and

poured. The incubation temperature for all cultures was 28±1°C (as in Chapter 3). After 15 or 30 days the fungal biomass was thoroughly scraped off the surface of the media. Then 1.5 g medium was collected and placed in a 23 mm (diameter) x 75 mm (height) 20 mL headspace flat bottom auto-sampler vial (Agilent Technologies, CA, USA) and sealed for GC/MS analysis.

Three fungi were used and all experiments were done in triplicate (n=3). As control, the three different media without fungal growth were also measured in triplicate (n=3). All were measured at two time points, 15 and 30 days after inoculation.

The GC peak area for each identified volatile metabolite was calculated in all chromatograms and compared for all different samples (including replicates).

4.2.4 Static headspace GC/MS analysis

An amount of 1.5 g of material per sample (0.75 g for cured pods) was analyzed for volatile substances using the Headspace coupled GC-MS method as described by Pérez-Silva et al. (2006) on a Varian 3800 system (Agilent Technologies Inc., CA, USA). The mobile phase carrier gas was Helium with a flow rate of 1 ml/min. The stationary phase was a Varian GC column (30 m x 0.25 mm, film thickness 0.25 µm) VF-5ms. After incubation at 85°C during 10 min under agitation, a gas volume of 1 mL was collected and injected in the GC-MS in split mode (5:1). The on-column injector temperature program was as follows: 60°C to 160°C (temperature ramp 2°C/min) and held at this temperature for 13 min (**Figure 1**). The GC oven temperature program was 60ºC to 170ºC (temperature ramp 2ºC/min). Then from 170ºC to 260ºC (temperature ramp 30ºC/min). The temperature was then maintained at 260ºC for 5 min. The total GC acquisition time was 63 min. The detector and injector temperatures were 260ºC. The mass spectrometric detection (EI at 70 eV) was performed under Full Scan (50 – 300 amu) mode. Detection was performed on a Varian 4000 ion trap. For identification purposes, an n-alkanes (C5–C30) series was run to calculate system independent Kovats retention indices.

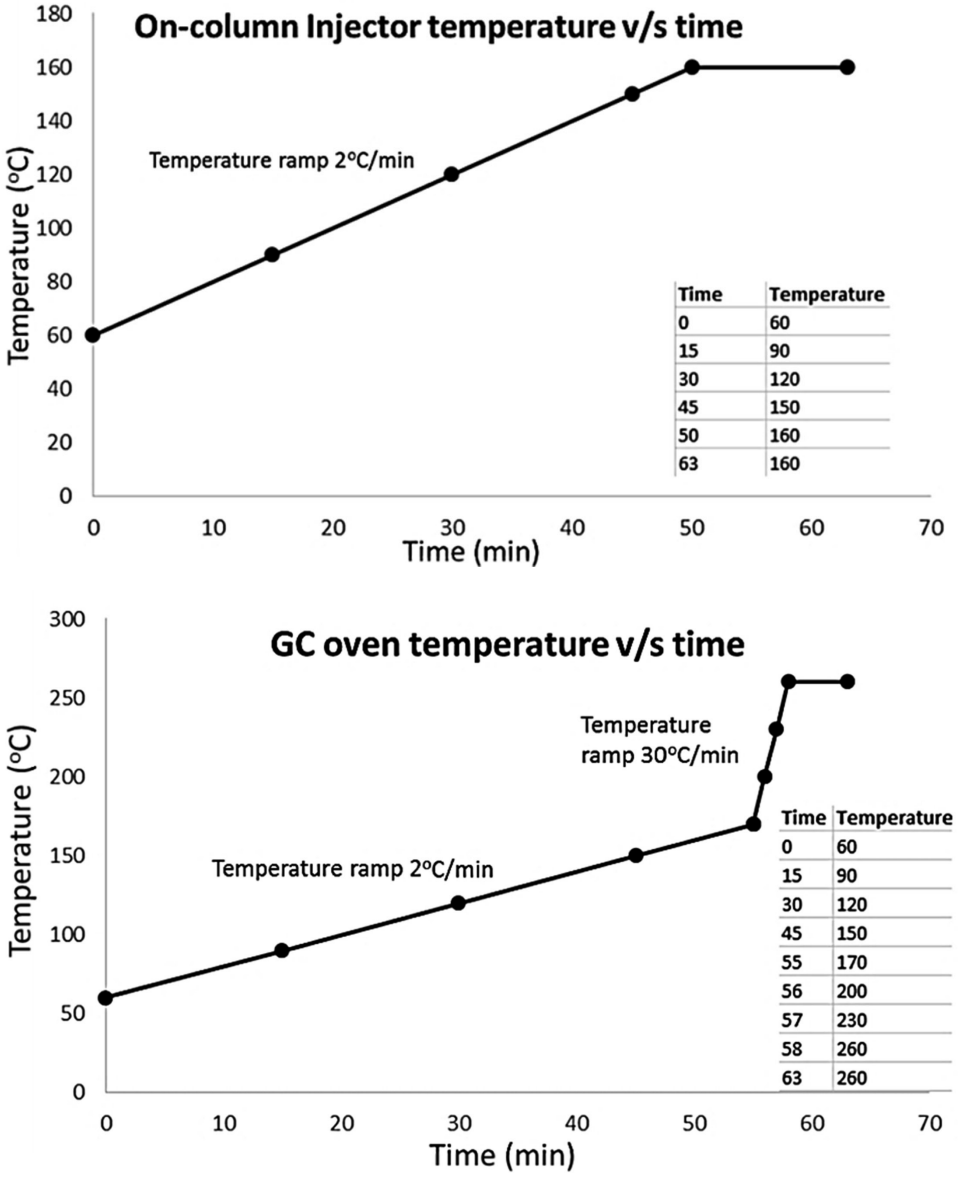

Figure 1 – Temperature programs for GC on-column injector and GC oven.

4.2.5 Identification and comparison of volatiles

The GC/MS chromatograms were viewed, peak area found and comparison were made on MestReNova software (v. 8.0.2, Mestrelab Research S.L, Santiago de Compostela, Spain). The identification was performed through the comparison of retention time indices and fragmentation pattern against the NIST 2014 GC Method and Retention Index Library and

the NIST Mass Spectral Library (NIST 2014, 2011). Library matches were searched for using a linear retention index (RI) from a series of n-alkanes (C5–C30) as reference, and comparing the mass spectra peaks with the NIST 2011 library using software Mass Spectrum Interpreter Version 2 (The National Institute of Standards and Technology, Md., USA).

To find if there is statistical evidence that the means of more than two independent groups differ significantly, a one-way ANOVA was performed using IBM SPSS Statistics for Windows, Version 24.0. (IBM Corp, Armonk, NY, USA). In SPSS, one independent variable (fixed factor) was defined for one one-way ANOVA test, each time. The independent variables were fungal species, media type, growth period. The dependent variable (continuous factor) was the relative metabolite amount. The null hypothesis is rejected for a p-value < 0.05.

4.3 RESULTS AND DISCUSSION

4.3.1 Fungal endophyte isolated after pod scalding only

For 15 pod replicates from St. Rose used for endophyte isolation after scalding, only *Hypoxylon investiens* was recovered from seven pods (47%). This fungal endophyte was only recovered after scalding but not before (Chapter 3). This might be because it is less competitive to utilize artificial media if compared to the other isolated endophytes. Additionally, it may have a slower growth rate than the endophytes isolated in Chapter 3, hence it was not isolated previously. The other previously isolated fungi (Chapter 3) may not have survived the heat treatment while *H. investiens* is heat resistant. Roling et al. (2001) also observed a change in microbial composition after pod scalding.

4.3.2 Volatile emission from media due to endophyte growth only

As mentioned earlier, for GC-MS absolute quantitation, an internal standard and a calibration curve for each individual compound would be required. The use of NMR as described in Chapter 3 has already given extensive quantitative data on the compounds produced by the endophytes. Therefore we here focus on the identification and relative abundance of known Vanilla aroma volatiles (**Table 1**).

Table 1 – Identified volatile metabolites known to contribute to Vanilla flavor/aroma. RI refers to retention index, compared with those in NIST databases using normal alkanes. References here are: **[1]** Sigma-Aldrich 2014, **[2]** Zepka et al. 2014, **[3]** Lim 2012, **[4]** Azeez 2008, **[5]** Burdock 2010, **[6]** Perez-Silva et al. 2006, **[7]** Gu et al. 2015, **[8]** Toth et al. 2011.

Compound in *Vanilla* based media*	Class of compound	Present in cured pod (literature)	Organoleptic properties (literature)	Contribution to vanilla aroma (literature)
p-xylene	arene	+ [8]	Sweet [2]	Sweet [4]
α-phellandrene	monoterpene	+ [8]	Minty [5]	Minty [4]
3-carene	monoterpene	+ [7]	Sweet [5]	Sweet [4]
α-terpineol	monoterpene	+ [8]	Sweet [5]	Sweet [4]

p-hydroxy benzaldehyde	phenolics	+ [6]	Sweet [1]	Sweet [4]
α-cubebene	sesquiterpene	+ [4]	Fruity [3]	Fruity [4]
β-caryophyllene	sesquiterpene	+ [4]	Spicy, woody [1]	Spicy, woody [4]
Vanillin	phenolics	+ [6]	Sweet, vanilla [1]	Sweet, vanilla [4]
Vanillyl alcohol	phenolics	+ [6]	Balsamic, anise [1]	Balsamic, anise [4]

*Vanilla based media: green pod agar media, extracted cured pod media.

The list of metabolites in **Table 1** shows compounds of different chemical classes like arenes, terpenoids and phenolics. They contribute to Vanilla flavor and are present in cured pods. The next step was to identify the volatiles and to determine the relative amounts of these volatile metabolites in the different samples (for full data see **Supplementary data – Part 2 - Figures S1 to S21**). The **Tables S1 to S9** (**Supplementary data – Part 1**) show the average peak size and standard deviation. The significance of the differences in metabolites across fungal endophyte species grown under different conditions and growth periods are shown in **Table 2** (calculations based on the supplementary quantitative data).

Table 2 – One way ANOVA for significance testing (alpha value 0.05) of gas chromatography data across samples from growth period (15, 30 days), media type (extracted cured pod agar medium, green pod agar medium, potato dextrose agar medium), fungal species (*Diaporthe phaseolorum*, *Pestalotiopsis microspora*, *Hypoxylon investiens*).

Investigated parameter	**F-value**	**Total degrees of freedom**	**P-value**	**Conclusion**
Growth period	0.7	647	3.8×10^{-1}	Difference in data sets not significant
Media type	7.2	647	7×10^{-4}	Difference in data sets significant
Fungal species	4.0	647	74×10^{-4}	Difference in data sets significant

Peak size differences between fungal species and between media type are significant and thus due to the experimental parameter, whereas this is not the case for the growth period. Differences connected to the growth period (15, 30 days) will thus no further be considered. For each volatile compound measured, a table (**Tables 3—11**) summarizes the results of **Tables S1 to S9**, showing relative semi-qualitative data. The control Potato dextrose agar (PDA) lacks Vanilla flavor secondary metabolites and its precursors which are present in ECPM and GPM media. Consequently all Vanilla flavor volatiles found after growth of a fungus on the control PDA medium are biosynthesized *de novo* by the fungus. So *p*-xylene, 3-carene were produced *de novo* by the fungi growing on the control medium. The terpenoids 3-carene and β-phellandrene are known fungal volatiles (Schmidt et al. 2016).

Table 3 – Summary (qualitative data) and conclusions of gas chromatography signal size for *p*-xylene (see Table S1 for peak size). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Control**	**Compound: *p*-xylene RT:5.9 Relative Peak Size**						**Medium and Fungus where synthesis occurred**
		Fungus: DP		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	+	++	+	+	+	+	Present from PDA experiments – *de novo* synthesis product Media:All Fungi: All
GPM	Above 0	-	-	++	+	-	+	
ECPM	Above 0	+	++	+	+	++	++	

Table 4 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for α-phellandrene (see Tables S2 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Control**	**Compound: α-phellandrene RT:10.5 Relative Peak Size**						**Medium and Fungus where synthesis occurred**
		Fungus: DP		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – induction or biotransformation product Media: ECPM, GPM Fungi: PM, HI
GPM	Above 0	-	-	++	+	+	+	
ECPM	Above 0	-	-	+	+	-	-	

Table 5 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for 3-carene (see Table S3 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: 3-carene RT:11.2**							**Medium and Fungus where synthesis occurred**
		Relative Peak Size						
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	++	+	0	0	+	0	Present from PDA experiments – *de novo* synthesis product Media: All Fungi: All
GPM	Above 0	-	-	+	-	+	++	
ECPM	Above 0	++	+	++	++	-	-	

Table 6 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for α-terpineol (see Table S4 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: α-terpineol RT:26.2**							**Medium and Fungus where synthesis occurred**
		Relative Peak Size						
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – induction or biotransformation product Media: ECPM, GPM Fungi: All
GPM	0	+	0	+	++	+	+	
ECPM	0	0	0	0	0	+	+	

Table 7 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for *p*-hydroxybenzaldehyde (see Table S5 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: *p*-hydroxybenzaldehyde RT:31.3**						**Medium and Fungus where synthesis occurred**
		Relative Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – biotransformation product Media: ECPM, GPM Fungi: All
GPM	Above 0	+	++	+	+	+	++	
ECPM	Above 0	=	=	=	-	+	+	

Table 8 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for α-cubebene (see Table S6 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: α-cubebene RT:32.9**						**Medium and Fungus where synthesis occurred**
		Relative Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – induction or biotransformation product Media: ECPM, GPM Fungi: All
GPM	Above 0	-	+	-	0	-	+	
ECPM	Above 0	+	0	0	+	0	0	

Table 9 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for beta-caryophyllene (see Table S7 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: beta-caryophyllene RT:37.8**							**Medium and Fungus where synthesis occurred**
		Relative Peak Size						
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – induction or biotransformation product Media: ECPM, GPM Fungi: All
GPM	Above 0	-	++	+	++	+	+	
ECPM	0	0	0	+	0	+	0	

Table 10 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for vanillin (see Table S8 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: Vanillin RT: 38.7**							**Medium and Fungus where synthesis occurred**
		RelativeRelative Peak Size						
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**		
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – biotransformation product Media: ECPM, GPM Fungi: All
GPM	Above 0	++	++	++	++	+	+	
ECPM	Above 0	0	0	0	-	++	++	

Table 11 – Summary (qualitative data) and conclusions of gas chromatography signal size measurements for vanillyl alcohol (see Table S9 for peak surfaces). Three replicates were used for each experimental parameter (n=3). 0 denotes amount not detectable, - denotes amount lower than in control medium, + denotes amount higher than in control medium, = denotes amount similar to that in control medium, ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days and RT denotes retention time.

Medium type	**Compound: Vanillyl alcohol RT:39.3**						**Medium and Fungus where synthesis occurred**
		Relative PeakSize					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**	
PDA	0	0	0	0	0	0	0	Absent from PDA experiments – biotransformation product Media: ECPM, GPM Fungi: All
GPM	Above 0	+	++	+	++	+	++	
ECPM	Above 0	0	0	0	-	++	++	

Apparently the three tested endophytes did not produce vanillin *de novo* on the PDA medium (**Table 10**). In GPM medium after growth with the endophytes, the phenolic volatiles vanillin, vanillyl alcohol and *p*-hydroxybenzaldehyde were detected at a higher abundance than in GPM control (no fungal growth). Thus, a precursor must be present in *Vanilla* pod material (e.g. vanillin glucoside or earlier precursors) for the fungi to synthesize vanillin through biotransformation. This confirms the results as reported in Chapter 3, where ^{1}HNMR analysis showed that the GPM medium after 30 days of growth of *D. phaseolorum* and *P. microspora* contained a higher amount of vanillin and vanillyl alcohol compared to the control GPM medium.

Vanillin, vanillyl alcohol, *p*-hydroxybenzaldehyde abundance shows an increase in ECPM after *H. investiens* growth but not with the other two fungi tested. This increase may be due to *H. investiens* having the ability to biotransform free ferulic acid into vanillin. The ECPM medium might contain higher amounts of free ferulic acid because of the nine months curing process that degrades cell walls (Krushnamurthy et al. 2012) liberating ferulic acid (Thibault et al. 1998) unlike in green pods (of which GPM is made) where the curing process is not yet performed. This additional ferulic acid is biotransformed into vanillin. The ability of *H. investiens* to biotransform ferulic acid into vanillin requires further testing (**see Chapter 5**).

Based on qualitative data (**Tables 3 to 11**), only in the case of α-phellandrene (**Table 4**), the fungi PM and HI synthesized the volatile metabolite while DP did not. α-Phellandrene is known to be synthesized by several *Hypoxylon* spp. (Shaw et al. 2015).

In terms of maximum synthesized amounts (quantitative data), the fungal species effect seems to be specific. The highest amounts of vanillin, vanillyl alcohol, *p*-hydroxybenzaldehyde, α-terpineol, *p*-xylene on ECPM were synthesized by HI (**Table S8, S9, S5, S4, S1 respectively –Part 1 –Supplementary data**). The re-use of waste of the

cured pods after extraction by using HI to obtain Vanilla flavor seems a promising research avenue. However, more experiments should be carried out in this direction. In the case of PDA medium, the highest amounts of the Vanilla flavor metabolites 3-carene, and *p*-xylene were synthesized by DP only (**Table S3, S1 respectively –Part 1 –Supplementary data**).

An important result from this work is that there is a clear difference in volatiles produced by the fungi when grown on the cured vanilla pod agar medium or on the green pod medium. It shows that the endophytes are capable of biotransforming compounds from the pods. However, it is not known whether the endophytes 31 have access to the precursors in the green pods. In the curing process, where the cell integrity is destroyed, diffusion of the metabolites happens and thus the precursors will be available to the endophytes. This makes the role of the endophytes during the curing, of particular interest for further study. *Hypoxylon investiens* is of special interest in this respect as it may increase the vanillin levels during curing by the biotransformation of ferulic acid.

4.4 CONCLUSIONS AND FURTHER WORK

It is clear from the experiments that some of the endophytic fungi isolated from vanilla pods may be involved in the production of volatiles that are known to contribute to the vanilla aroma.

Many fungi can produce terpenes like the ones reported here (Bühler et al. 2000, Busko et al. 2014, Agrawal 2004, Demyttenaere et al. 2001, Shaw et al. 2015). Similarly, vanillin is also produced by other plants than vanilla (unicorn plant, clove, narcissus, hyacinth) but the content is generally lower than in vanilla plants (Havkin-Frenkel and Belanger 2016). Hence, what makes vanilla flavor unique is not the individual metabolites but rather the specific combinations in which these occur within vanilla pods.

Vanilla pod fungal endophytes are unique not because of their abilities to produce several volatiles as reported here, what makes them unique is the circumstances in which this production occurs. Their presence inside the plant means that their metabolites are also found in the pods and the endophytes might biotransform plant products, and vice versa. In other words the biosynthetic networks of the plant and the fungi may mix in different ways, with the consequence that differences in endophyte spectra in the pods may affect the aroma. This means that terroir effects through different spectra of endophytes in the plant and the pods may occur.

Extracted cured pods might be re-used for growing endophytes for Vanilla flavor metabolite production. Further quantitative studies are necessary in order to make any assessment of the economic feasibility. The ability of HI to convert ferulic acid into vanillin should be further investigated.

4.5 REFERENCES

Agrawal R. (2004) Flavors and Aromas. In: Arora D.K. (ed.). Fungal Biotechnology in Agricultural, Food, and Environmental Applications. Marcel Dekker, NY, pp.281-287.

Azeez S. (2008) Vanilla. In: Parthasarathy V.A., Chempakam B., Zachariah T.J. (eds.). Chemistry of Spices. CAB International, Oxford, pp.287-311.

Barwick V.J. (1999) Sources of uncertainty in gas chromatography and high-performance liquid chromatography. Journal of Chromatography A, 849:13–33.

Bills G.F., González-Menéndez V., Martín J., Platas G., Fournier J., Peršoh D. (2012) Hypoxylon pulicicidum sp. nov. (Ascomycota, Xylariales), a Pantropical Insecticide-Producing Endophyte. PLoS ONE, 7:e46687.

Buccellato F. (2011) Vanilla in Perfumery and Beverage Flavors. . In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp.235-240.

Bühler B., Schmid A., Hauer B., Witholt B. (2000) Xylene monooxygenase catalyzes the multistep oxygenation of toluene and pseudocumene to corresponding alcohols, aldehydes, and acids in Escherichia coli JM101. The Journal of Biological Chemistry, 275:10085-92.

Burdock G.A. (2010) Fenaroli's Handbook of Flavor Ingredients. CRC Press, FL.

Busko M., Kulik T., Ostrowska A., Goral T., Perkowski J. (2014) Quantitative volatile compound profiles in fungal cultures of three different Fusarium graminearum chemotypes. FEMS Microbiology Letters, 359:85–93.

Demyttenaere J.C., Van Belleghem K., De Kimpe N. (2001) Biotransformation of (R)-(+)- and (S)-(-)-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry, 57:199-208.

Dignum M., Kerler J., Verpoorte R. (2002) Vanilla curing under laboratory conditions. Food Chemistry 79:165-171.

Dignum M., Kerler J., Verpoorte R. (2001) Vanilla production: technological, chemical and biosynthetic aspects. Food Research International 17:199-219.

Fang M., Ivanisevic J., Benton H.P., Johnson C.H., Patti G.J., Hoang L.T., Uritboonthai W., Kurczy M.E., Siuzdak G. (2015) Thermal Degradation of Small Molecules: A Global Metabolomic Investigation. Analytical Chemistry, 87:10935-41.

Gu F., Chen Y., Fang Y., Wu G., Tan L. (2015) Contribution of Bacillus isolates to the flavor profiles of vanilla beans assessed through aroma analysis and chemometrics. Molecules, 20:18422-36.

Hamilton (2012) HD-Type Syringe for Reproducible Headspace Gas Chromatography. *Hamilton company*. https://www.hamiltoncompany.com/products/syringes-and-needles/chromatography-syringes/gc-autosampler-syringes/ctc-leap-technologies-gc-autosamplers/ctc-leap-combipal-hd-headspace-syringes (accessed April 14, 2018)

Havkin-Frenkel D., Belanger F.C. (2016) Biotechnological production of vanillin. In: Havkin-Frenkel D., Dudai N. (Eds) Biotechnology in flavor production. 2nd Ed. John Wiley & Sons Ltd, West Sussex, p.168.

Kennedy C.R. (2015) The Flavor Rundown: Natural vs. Artificial Flavors: What's in a Flavor? *Harvard University*. http://sitn.hms.harvard.edu/flash/2015/the-flavor-rundown-natural-vs-artificial-flavors/ (accessed July 29, 2016)

Krushnamurthy A., Shyamala B.N., Naidu M.M. (2012) Vanilla- Its Science of Cultivation, Curing, Chemistry, and Nutraceutical Properties, Critical Reviews in Food Science and Nutrition, 53: 1250-76.

Lim T.K. (2012) Edible Medicinal and Non Medicinal Plants: Volume 3, Fruits. Springer Science, Dordrecht.

Molina G., Bicas J.L., Moraes E.A., Marostica-Jr M.R., Pastore G.M. (2013) Recent developments and industrial perspectives in the microbial production of bioflavors. In: Gupta V.K., Schmoll M., Mazutti M.A., Maki M., Tuohy M.G. (eds.). Applications of Microbial Engineering. CRC Press, FL, pp.122-57.

Pérez-Silva A., Odoux E., Brata P., Ribeyre F., Rodriguez-Jimenes G., Robles-Olvera V., García-Alvarado M.A., Günatac Z. (2006) GC-MS and GC-olfactometry analysis of aroma compounds in a representative organic aroma extract from cured vanilla (Vanilla planifolia G. Jackson) beans. Food Chemistry, 99:728-35.

Reineccius G. (2005) Flavor chemistry and technology. 2nd Ed. Taylor & Francis group, FL.

Röling W.F., Kerler J., Braster M., Apriyantono A., Stam H., van Verseveld H.W. (2001) Microorganisms with a taste for vanilla: microbial ecology of traditional Indonesian vanilla curing. Applied Environmental Microbiology, 67:1995-2003.

Schmidt R., Etalo D.W., Jager V.D., Gerards S., Zweers H., Boer W.D., Garbeva P. (2016) Microbial Small Talk: Volatiles in Fungal–Bacterial Interactions. Frontiers in Microbiology, 6:1-12.

Shaw J.J., Berbasova T., Sasaki T., Jefferson-George K., Spakowicz D.J., Dunican B.F., Portero C.E., Narvaez-Trujillo A., Strobel S.A. (2015) Identification of a Fungal 1,8-Cineole Synthase from Hypoxylon sp. with Specificity Determinants in Common with the Plant Synthases. The Journal of Biological Chemistry, 290:8511–26.

Sigma-Aldrich (2014) Ingredients Catalog: Flavors & Fragrances. Sigma-Aldrich Chemie GmbH, Taufkirchen.

Thibault J., Micard V., Renard C., Asther M., Delattre M., Lesage-Meessen L., Faulds C., Kroon P., Williamson G., Duarte J., Duarte J.C., Ceccaldi B.C., Tuohy M., Couteau D., Van Hulle S., Heldt-Hansen H.P. (1998) Fungal Bioconversion of Agricultural By-Products to Vanillin. LWT - Food Science and Technology, 31:530-6.

Toth S., Lee K.J., Havkin-Frenkel D., Belanger F.C., Hartman T.G. (2011) Volatile Compounds in Vanilla. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp.183-218.

Zepka L.Q., Garruti D.S., Sampaioc K.L., Mercadante A.Z., Da Silva M.A.A.P. (2014) Aroma compounds derived from the thermal degradation of carotenoids in a cashew apple juice model. Food Research International, 56:108–14.

Zhang S., Mueller C. (2012) Comparative Analysis of Volatiles in Traditionally Cured Bourbon and Ugandan Vanilla Bean (Vanilla planifolia) Extracts. Journal of Agriculture and Food Chemistry, 60:10433−44.

Chapter 4 – Supplementary data

This section comprises of two parts. Part 1 concerns tables with calculated average and standard deviation for gas chromatography signal size of volatile metabolites having Vanilla flavor and also present in cured pods, according to literature. Part 2 are gas chromatograms and peak identity with regards to those volatile metabolites. The peak size in chromatograms of part 2 were used for the data presented in tables of Part 1. Additionally, the crude GC data is also presented in this work in a separated file.

Part 1 – Volatile metabolite average and standard deviation

Table S1 – Average gas chromatography signal size measurements for p-xylene. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: *p*-xylene RT:5.9**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	31,941 +/-3,195	**<u>157,148</u>** +/- 15,713	3,473 +/- 348	16,700 +/- 166	4,535 +/- 532	19,323 +/- 2,366
Green Pod Agar Medium	42,396 +/- 4,701	7,656 +/- 848	41,471 +/- 4,673	**<u>125,324</u>** +/- 29,275	51,764 +/- 8,179	23,909 +/- 4289	67,905 +/- 49,243
Extracted Dark Pod Agar Medium	24,481 +/- 916	26,719 +/- 2,224	104,596 +/- 29,145	43,617 +/- 2,537	21,757 +/- 1,199	**<u>129,219</u>** +/- 94,614	124,020 +/- 93,392

Table S2 – Average gas chromatography signal size measurements for α-phellandrene. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: α-phellandrene RT:10.5**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0

Green Pod Agar Medium	5,006 +/- 790	3,018 +/- 477	4,897 +/- 774	**10,157** +/- 6,122	5,516 +/- 871	6,411 +/- 2,289	7,010 +/- 2302
Extracted Dark Pod Agar Medium	3,379 +/- 228	0	0	**16,373** +/- 781	14,247 +/- 1095	0	3,301 +/- 206

Table S3 – Average gas chromatography signal size measurements for 3-carene. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: 3-carene RT:11.2**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	**303,530** +/- 33,726	83,761 +/- 9,288	0	0	36,525 +/- 33,393	0
Green Pod Agar Medium	75,402 +/- 7,430	2,835 +/- 448	6,060 +/- 957	94,424 +/- 79,005	9,377 +/- 1,481	77,738 +/- 26,772	**194,372** +/- 161,831
Extracted Dark Pod Agar Medium	7,005 +/- 3,182	151,463 +/- 10,869	22,090 +/- 18,581	100,979 +/- 2,137	**181,208** +/- 11,954	5,335 +/- 2,110	4,214 +/- 1,508

Table S4 – Average gas chromatography signal size measurements for α-terpineol. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: α-terpineol RT:26.2**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	0	4,867 +/- 486	0	6,599 +/- 2,699	**10,568** +/- 895	5,639 +/- 1,243	6,355 +/- 2,413
Extracted Dark Pod Agar Medium	0	0	0	0	0	4,436 +/- 900	**4,698** +/- 557

Table S5 – Average gas chromatography signal size measurements for *p*-hydroxy benzaldehyde. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: *p*-hydroxy benzaldehyde RT:31.3**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	5,420 +/- 542	6,205 +/- 620	10,280 +/- 541	6,530 +/- 457	9,522 +/- 941	8,127 +/- 2,363	**10,742** +/- 9,745
Extracted Dark Pod Agar Medium	5,342 +/- 255	5,318 +/- 551	5,426 +/- 103	5,408 +/- 540	5,010 +/- 595	**6,133** +/- 1,144	5,699 +/- 1,262

Table S6 – Average gas chromatography signal size measurements for α-cubebene. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: α-cubebene RT:32.9**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	3,298 +/- 329	2,594 +/- 214	**6,388** +/- 535	3,141 +/- 2,729	0	2,891 +/- 152	3,499 +/- 3,140
Extracted Dark Pod Agar Medium	2,061 +/- 1,787	2,955 +/- 280	0	0	**3,187** +/- 503	0	0

Table S7 – Average gas chromatography signal size measurements for beta-caryophyllene. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: beta-caryophyllene RT:37.8**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	4,888 +/- 640	3,122 +/- 312	**9,575** +/- 616	5,828 +/- 876	7,981 +/- 1,261	4,190 +/- 191	4,997 +/- 1,466
Extracted Dark Pod Agar Medium	0	0	0	**3,133** +/- 280	0	2,645 +/- 2,355	0

Table S8 – Average gas chromatography signal size measurements for vanillin. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: Vanillin RT: 38.7**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	29,976 +/- 2,661	704,302 +/- 70,340	**941,634** +/- 65,087	300,771 +/- 2,518	463,053 +/- 37,667	46,737 +/- 11,654	78,772 +/- 11,370
Extracted Dark Pod Agar Medium	3,900 +/- 124	0	0	0	3,865 +/- 728	771,187 +/- 150,290	**1,148,972** +/- 169,684

Table S9 – Average gas chromatography signal size measurements for vanillyl alcohol. Three replicates were used for each experimental parameter (n=3). The standard deviation is indicated. Underlined and bold numbers are the highest recorded values above the control (without fungal growth) for any given metabolite. ECPM denotes extracted cured pod agar medium, GPM denotes green pod agar media, PDA denotes potato dextrose agar medium, DP denotes *Diaporthe phaseolorum*, PM denotes *Pestalotiopsis microspora*, HI denotes *Hypoxylon investiens*, 15D denotes 15 days, 30D denotes 30 days, RT denotes retention time.

Medium type	**Compound: Vanillyl alcohol RT:39.3**						
		Average and standard deviation of Peak Size					
	Control	**Fungus: DP**		**Fungus: PM**		**Fungus: HI**	
		Time: 15D	**Time: 30D**	**Time: 15D**	**Time: 30D**	**Time: 15D**	**Time: 30D**
Potato Dextrose Agar Medium	0	0	0	0	0	0	0
Green Pod Agar Medium	3,524 +/- 538	17,162 +/- 1,056	66,199 +/- 4,721	7,107 +/- 1,646	**177,081** +/- 7,708	4,243 +/- 542	43,534 +/- 31,085
Extracted Dark Pod Agar Medium	3,617 +/- 901	0	0	0	2,671 +/- 695	21,641 +/- 7,169	**30,722** +/- 4,070

Part 2 – GC chromatograms with identified peaks

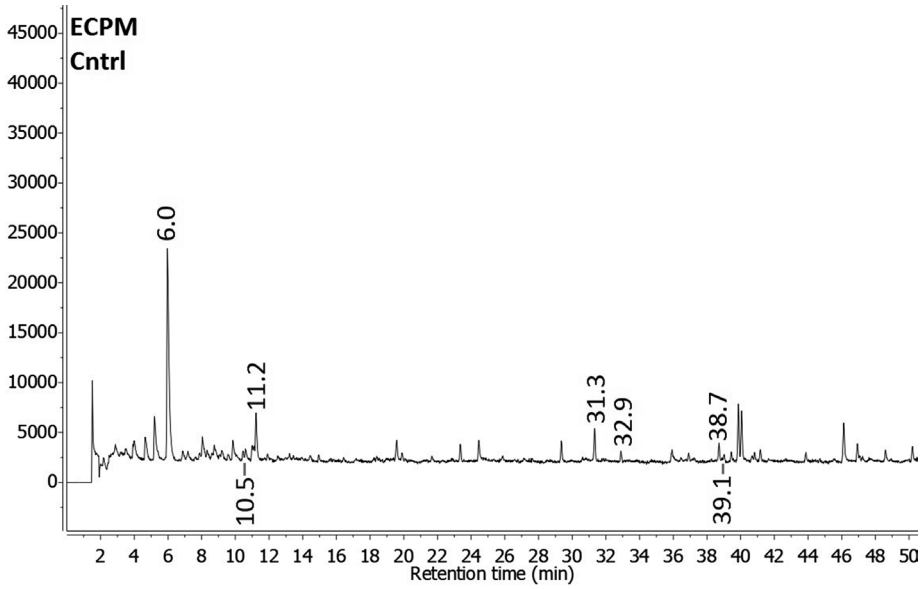

Figure S1 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was control Extracted Cured Pod Medium (ECPM) (No Fungal Growth). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 38.7 - Vanillin, RT: 39.1 - Vanillyl alcohol.

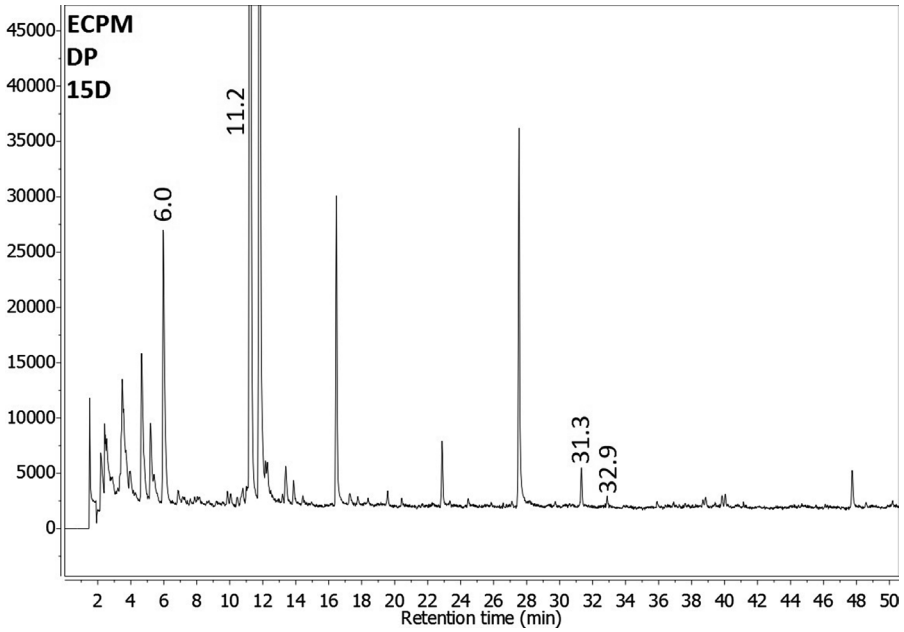

Figure S2 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Diaporthe phaseolorum* (DP) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene.

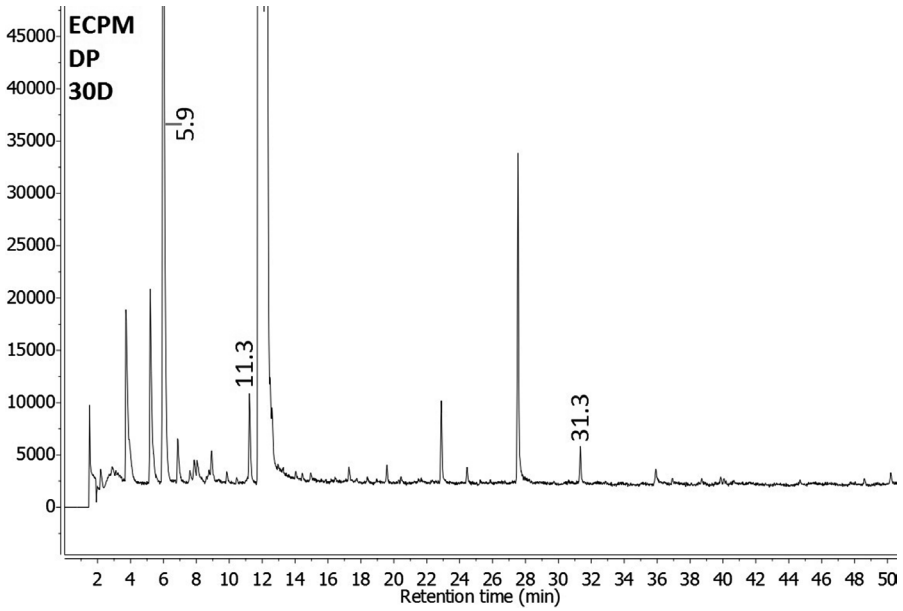

Figure S3 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Diaporthe phaseolorum* (DP) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 11.3 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde.

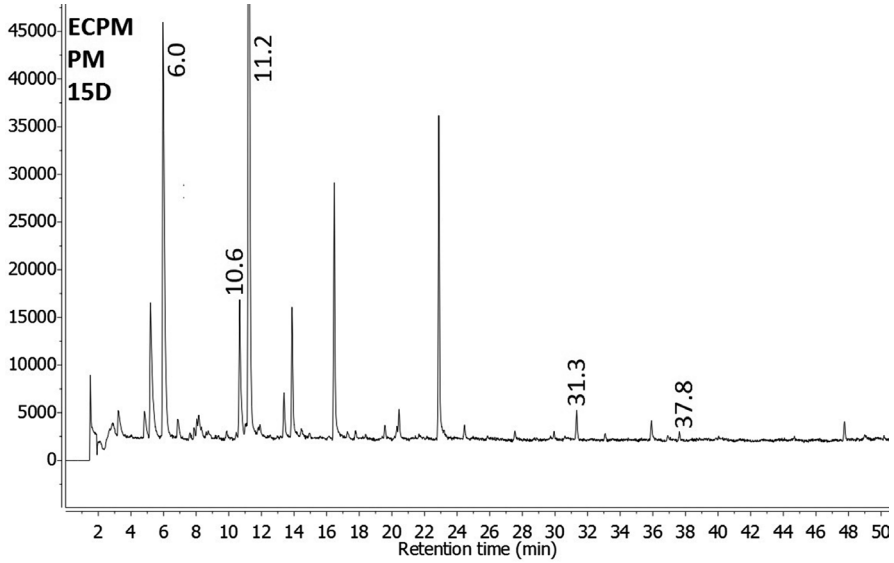

Figure S4 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Pestalotiopsis microspora* (PM) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 10.6 - α-phellandrene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 37.8 - β-caryophyllene.

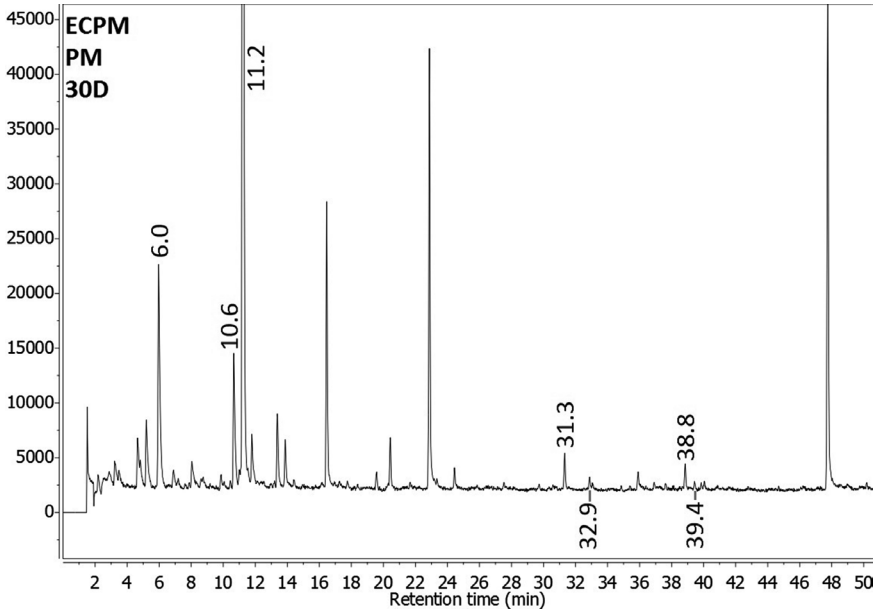

Figure S5 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Pestalotiopsis microspora* (PM) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT:

10.6 - α-phellandrene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 38.8 - Vanillin, RT: 39.4 - Vanillyl alcohol.

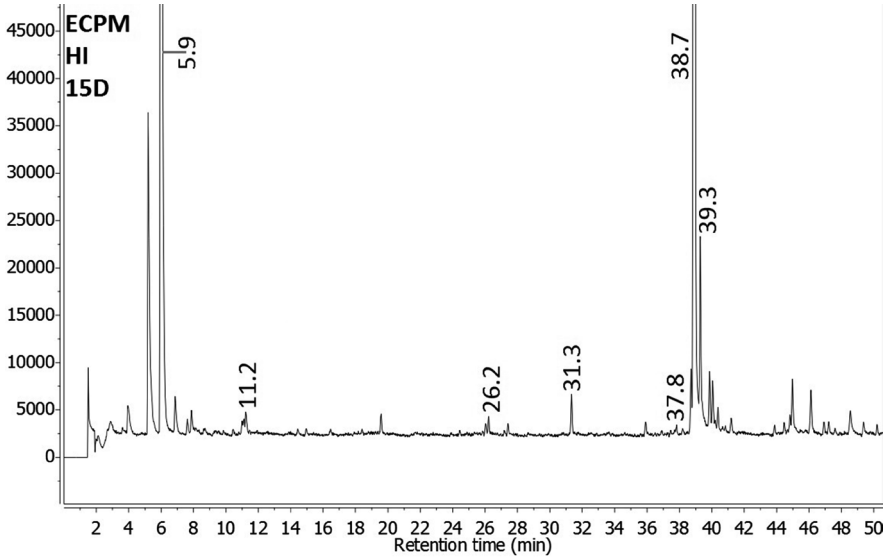

Figure S6 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Hypoxylon investiens* (HI) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 11.2 - 3-carene, RT: 26.2 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 37.8 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

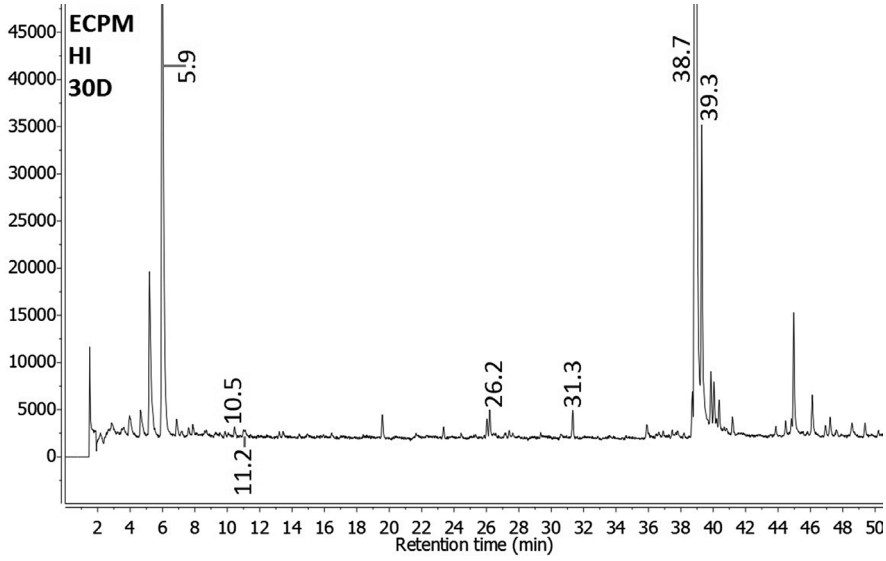

Figure S7 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Extracted Cured Pod Medium (ECPM) on which *Hypoxylon investiens* (HI) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 10.5

- α-phellandrene, RT: 11.2 - 3-carene, RT: 26.2 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

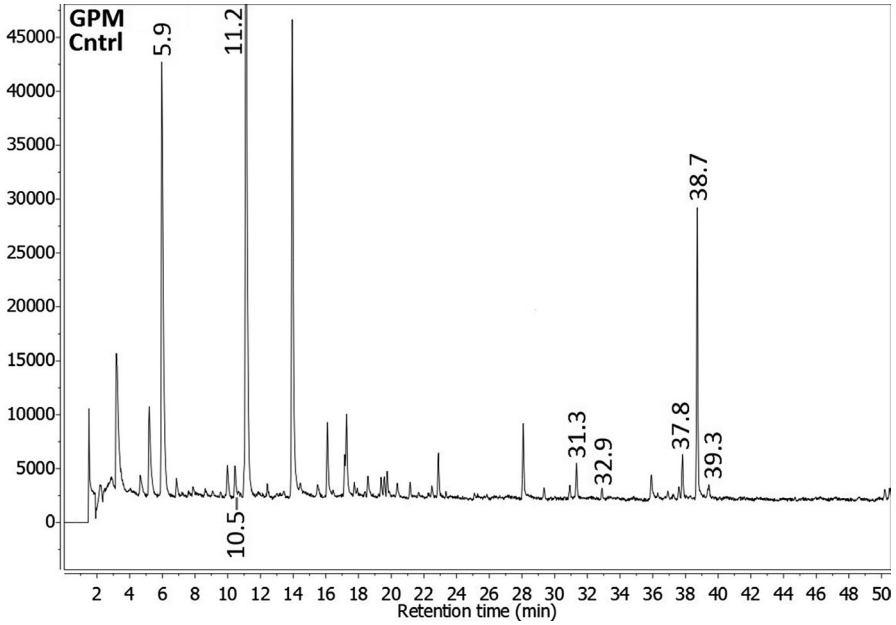

Figure S8 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was control Green Pod Agar Medium (GPM) (No Fungal Growth). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 37.8 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

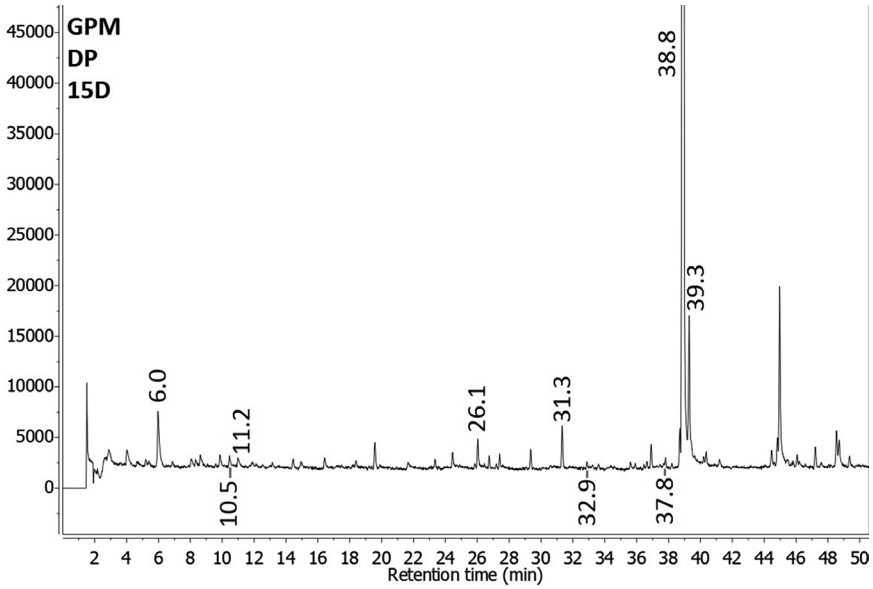

Figure S9 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Diaporthe phaseolorum* (DP) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 26.1 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 37.8 - β-caryophyllene, RT: 38.8 - Vanillin, RT: 39.3 - Vanillyl alcohol.

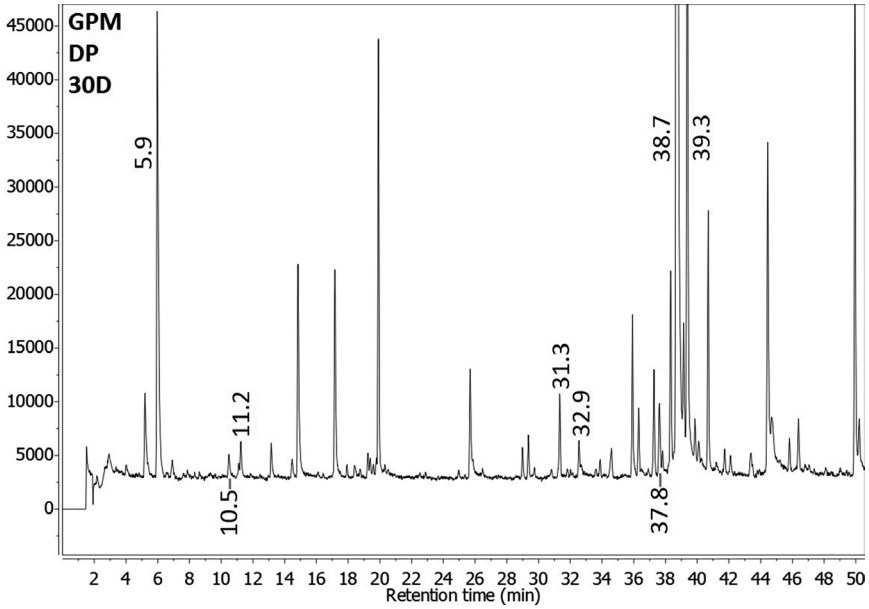

Figure S10 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Diaporthe phaseolorum* (DP) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 37.8 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

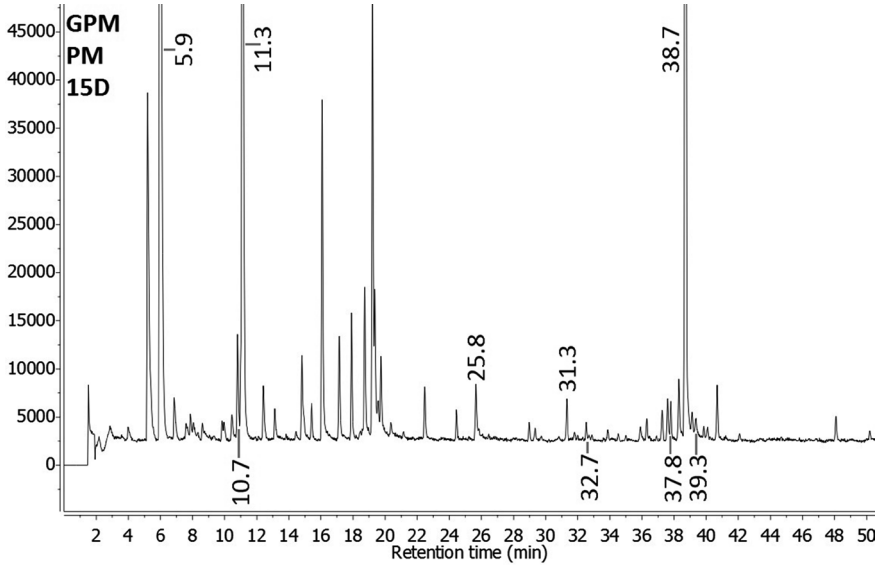

Figure S11 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Pestalotiopsis microspora* (PM) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 10.7 - α-phellandrene, RT: 11.3 - 3-carene, RT: 25.8 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.7 - α-cubebene, RT: 37.8 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

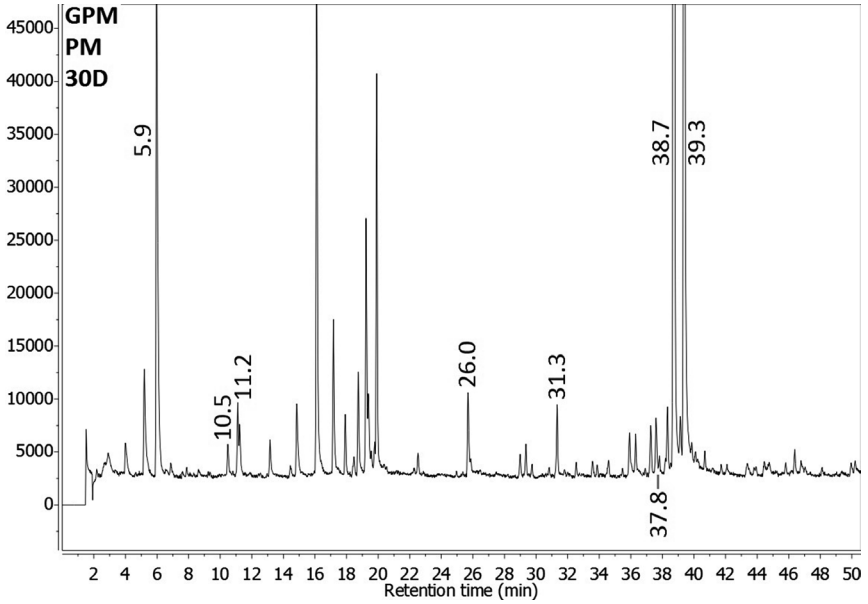

Figure S12 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Pestalotiopsis microspora* (PM) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 5.9 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 26.0 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 37.8 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

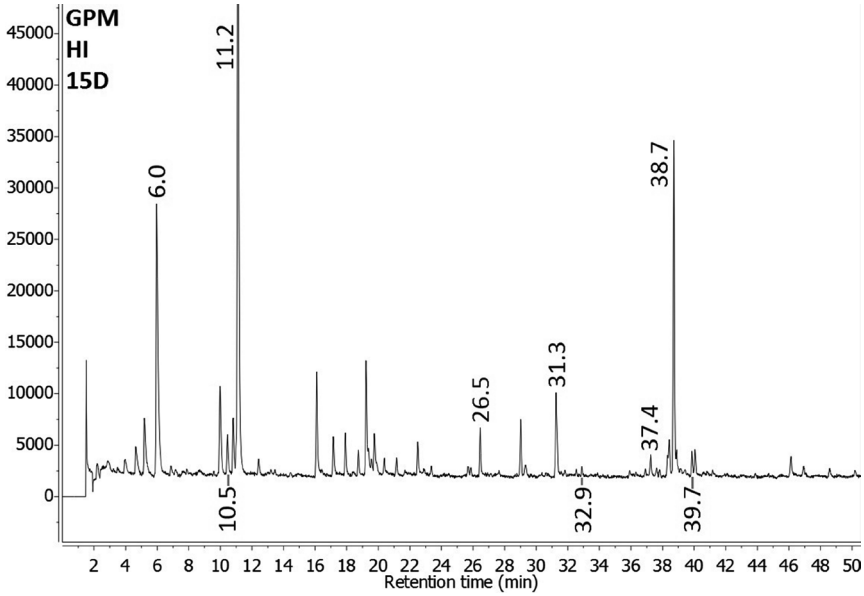

Figure S13 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Hypoxylon investiens* (HI) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 10.5 - α-phellandrene, RT: 11.2 - 3-carene, RT: 26.5 - α-terpineol, RT: 31.3 - *p*-hydroxybenzaldehyde, RT: 32.9 - α-cubebene, RT: 37.4 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.7 - Vanillyl alcohol.

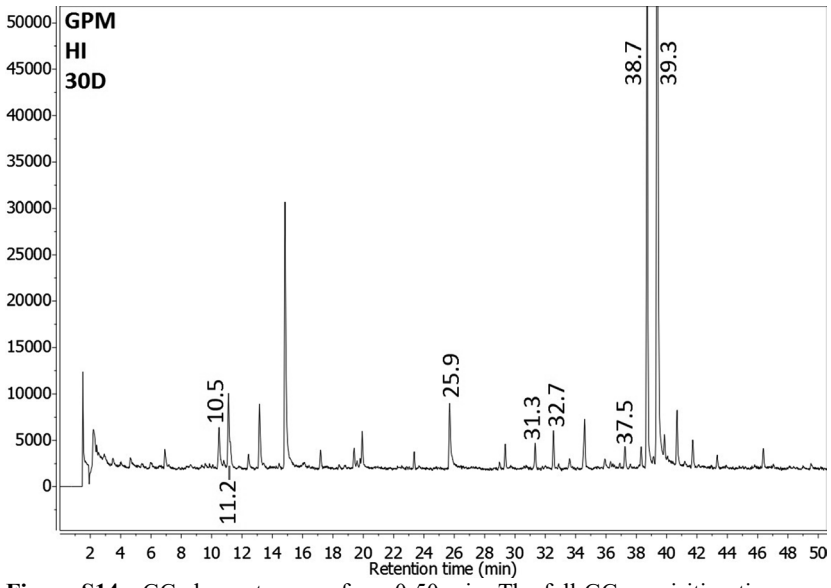

Figure S14 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Green Pod Agar Medium (GPM) on which *Hypoxylon investiens* (HI) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 10.5 - α-phellandrene, RT:

11.2 - 3-carene, RT: 25.9 - α-terpineol, RT: 31.3 - p-hydroxybenzaldehyde, RT: 32.7 - α-cubebene, RT: 37.5 - β-caryophyllene, RT: 38.7 - Vanillin, RT: 39.3 - Vanillyl alcohol.

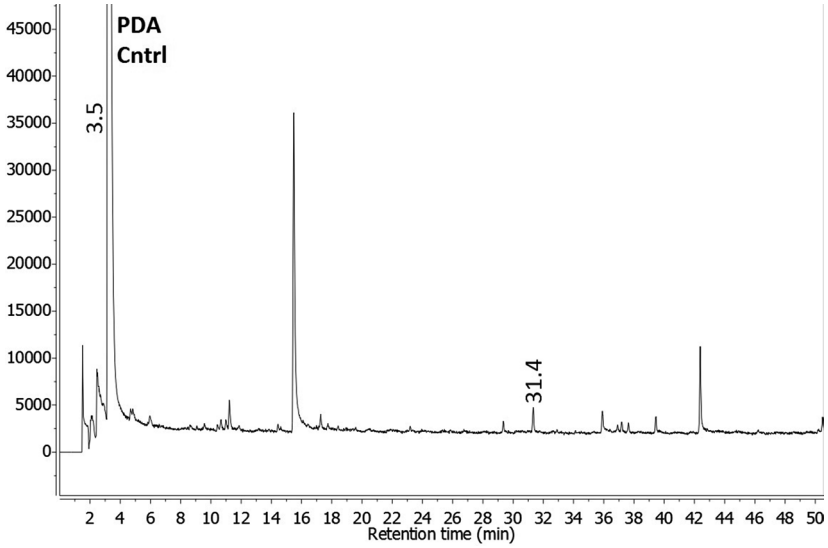

Figure S15 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was control Potato Dextrose Agar Medium (PDA) (No Fungal Growth). Retention times (RT) and associated volatile metabolites are RT: 3.5 - 1-Pentene, RT: 31.4 - Camphene.

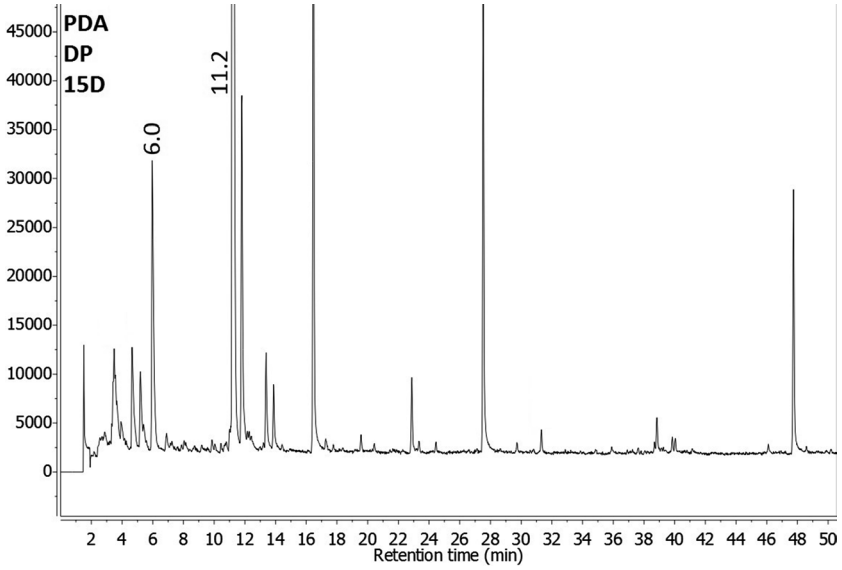

Figure S16 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Diaporthe phaseolorum* (DP) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 11.2 - 3-carene.

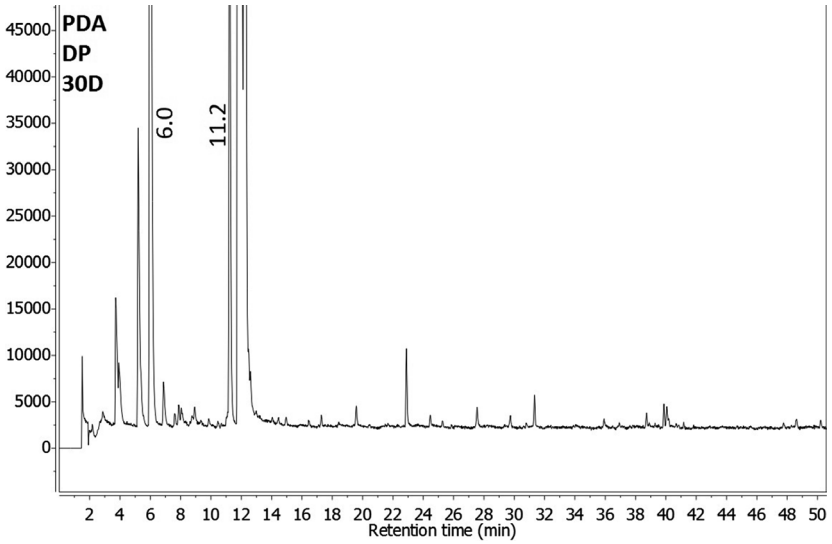

Figure S17 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Diaporthe phaseolorum* (DP) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites are RT: 6.0 - *p*-xylene, RT: 11.2 - 3-carene.

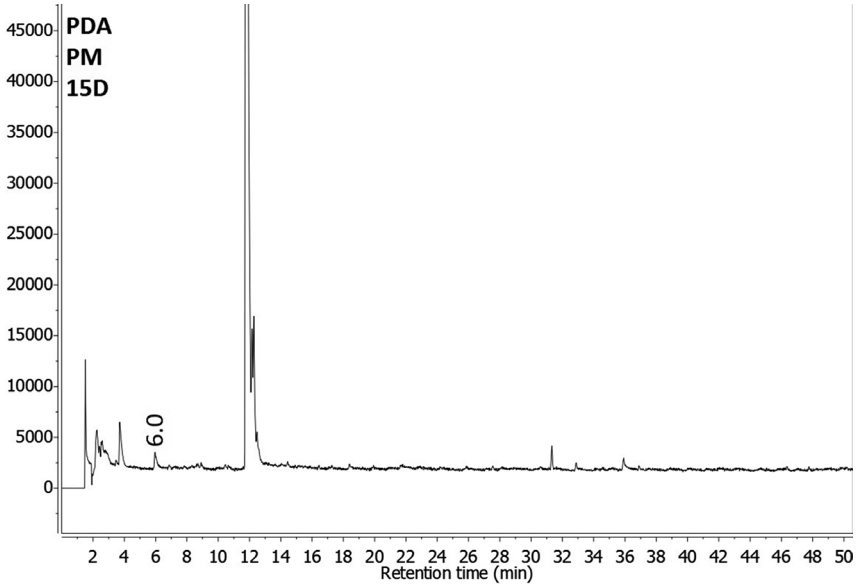

Figure S18 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Pestalotiopsis microspora* (PM) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites is RT: 6.0 - *p*-xylene.

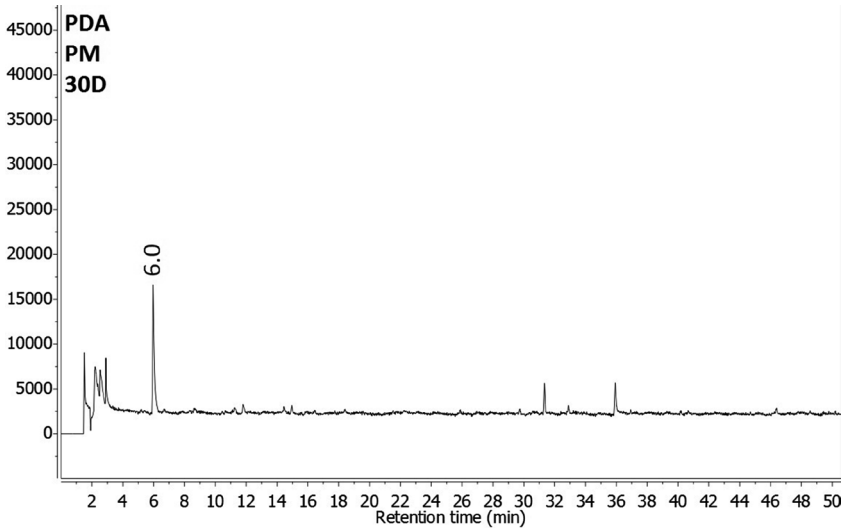

Figure S19 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Pestalotiopsis microspora* (PM) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites is RT: 6.0 - *p*-xylene.

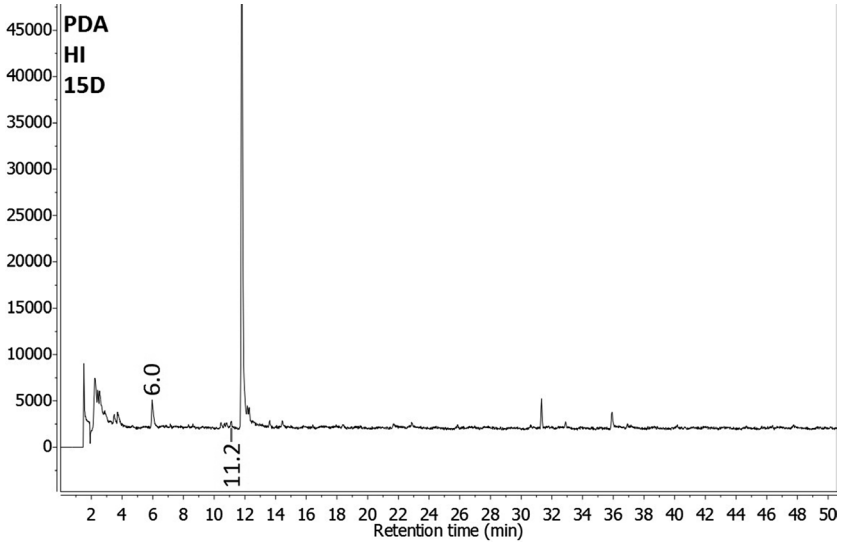

Figure S20 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Hypoxylon investiens* (HI) was grown for 15 days (15D). Retention times (RT) and associated volatile metabolites is RT: 6.0 - *p*-xylene, RT: 11.2 - 3-carene.

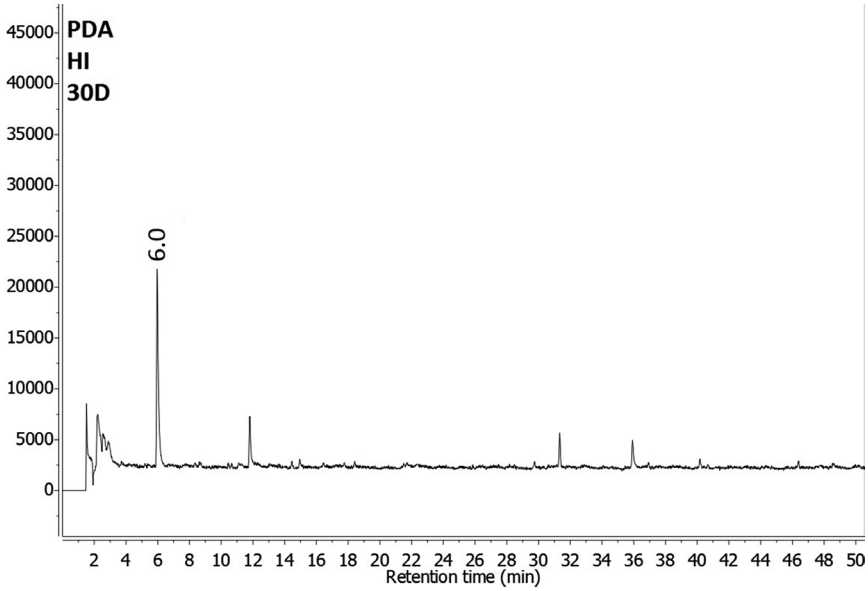

Figure S21 - GC chromatograms from 0-50 min. The full GC acquisition time was 63 min. Sample was Potato Dextrose Agar Medium (PDA) on which *Hypoxylon investiens* (HI) was grown for 30 days (30D). Retention times (RT) and associated volatile metabolites is RT: 6.0 - *p*-xylene.