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

Khoyratty, S.

Chapter 6: Changes in phenolic, amino acid and carbohydrate contents in axenic *Vanilla planifolia* plant materials – after co-culture with fungal endophytes

Shahnoo Khoyratty[1,2], Young Hae Choi[1], Hippolyte Kodja[2], Tony Lionel Palama[3], Rob Verpoorte[1]

[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 92003-97744Saint Denis Cedex 9, La Réunion, France.

[3] Institut des Sciences Analytiques (CNRS/ENS Lyon/UCB Lyon1), Centre de RMN à Très Hauts Champs, Université de Lyon, 69100 Villeurbanne, France.

ABSTRACT

In previous chapters, endophyte isolation from *Vanilla* plants was described. Their ability to perform one or more steps of the vanillin biosynthesis was also shown *in vitro* but not yet *in vivo*. Here, we aim at characterizing the interaction of the same endophytic fungi with *Vanilla* plant material *in vivo*. To measure the effect of each single endophyte on *Vanilla*, axenic *Vanilla* plants and callus cultures of different ages were produced and infected with the *Vanilla* endophytic fungi *Diaporthe phaseolorum*, *Pestalotiopsis microspora*, or *Hypoxylon investiens* as well as with the *Vanilla* pathogen *Fusarium oxysporum.* To find how to infect the *Vanilla* material in the experiments, the usual *in situ* endophyte mode of transmission towards infection was investigated. As endophytes were isolated only from open flowers, but not from closed buds, horizontal transmission was possible. Therefore fungal infection was performed by immersing plant material in fungal spore solutions, for the co-culture experiments. Using histological, morphological and ^{1}HNMR-based metabolomics analyses, two different stages of calli development were characterized, 15 and 30 days of culture on regeneration medium (Murashige and Skoog macro- and microelements, Morel and Wetmore vitamins, 30 g l^{-1} sucrose, 0.5 mg l^{-1} α-naphthalene acetic acid). Thus, 15 and 30 days old calli as well as axenic plants were used for the fungal co-culture experiments. For *p*-hydroxybenzyl alcohol and *p*-coumaric acid (both vanillin precursors), the highest level was observed in 30 days old calli cultures co-cultured with *Fusarium oxysporum* and *Hypoxylon investiens* respectively. Overall, it seems that the fungal pathogen *F. oxysporum* does not differ much from endophytes in its effects on plants and calli. Apparently the different plant materials give a different response to co-culturing with an endophyte. Among the tested endophytes, *Hypoxylon investiens* and *Pestalotiopsis microspora* induced highest levels of metabolites associated to steps on the metabolic pathways related to vanillin biosynthesis in the plant. However, as vanillin is produced in pods, and not in the plant itself, further studies are needed to be able to conclude on any effect on the vanillin biosynthesis itself.

Published article, during the time of this thesis, in connection to preparing the calli material used here, complementing the work here: " Kodja H., Noirot M., Khoyratty S., Limbada H., Verpoorte R., Palama T.L. (2015) Biochemical characterization of embryogenic calli of *Vanilla planifolia* in response to two years of thidiazuron treatment. *Plant Physiology and Biochemistry* 96:337-44 "

In preparation for publication as: " Changes in phenolic, amino acid and carbohydrate contents in axenic *Vanilla planifolia* plant materials – after co-culture with fungal endophytes "

6.1 BACKGROUND

The relationship between a fungal endophyte and a host plant is clearly defined thus: a fungal endophyte inhabits a host plant without causing any symptoms visible to the eye (Dutta et al. 2014), whereas pathogenic fungi cause visible symptoms in the host plant. Fungal endophytes reside in the internal tissues of living plants (Aly et al. 2011) and no fungal mycelium is visible on the surface of the host plant.

What is less clear in the definition is whether the relationship is symbiotic (some endophytes produce plant hormones such as auxins, and gibberellins that stimulate growth and development of host plant (Dutta et al. 2014)) or parasitic. In the latter case endophytes do not directly benefit from the plant, but just remain neutral in the relationship (Kogel et al. 2006), though they feed on the plant produced nutrients (e.g. carbohydrates and amino acids), and thus are not directly beneficial to the plant (Spiering et al. 2006). These definitions are not of the most accurate, as little is known of the possible roles of endophytes in a plant‘s interaction with its environment, e.g. plant defense against pests and diseases. This lack of knowledge is mainly due to the fact that only visible symptoms, i.e. with our eyes or via microscopy, form the basis of the definitions. However, the systems biology approaches using the ‘‘-omics’’ as tools, will probably lead to new insights. Because of their permanent presence in a plant, one particularly needs to measure the metabolic changes caused by the endophytes. This includes any changes in the metabolome under stress conditions when comparing endophyte-free plants with endophyte innoculated plants.

We already showed that some *Vanilla* endophytes *in-vitro* cultures are able to do bioconversions of compounds found in *Vanilla* pods. By using a metabolomics approach, the study described in this chapter aims at measuring the metabolic changes in *Vanilla* living material when co-cultured with fungi. Two methods were used. One method was infecting axenic plants with an endophyte, the other was co-culturing a *Vanilla* callus culture with an endophyte. Axenic plants can be obtained by extensive treatment of the plant with systemic fungicides, but that carries the risk of metabolic changes due to the fungicide. The more natural method is to grow axenic plants from tissue culture, and cultivate them on axenic compost. To avoid changes in the plant because of wounding (Garg and Gupta 2010), inoculation was done following the natural horizontal infection via water born spores (Currie et al. 2014).

The other approach was the use of a callus-fungi co-culture. To characterize the interactions between endophytic fungi and host plant cell cultures, both materials can be cultured together (Nawrot–Chorabik 2013). For example, in Zhou et al. (2017) host rice callus was inoculated with mycelium of the fungal endophyte *Phomopsis liquidambari* to study their interaction. Cell cultures of *Vanilla* have been studied in some detail in efforts to produce vanillin in large scale plant cell suspension cultures (Davidonis and Knorr 1991). Precursors of vanillin e.g. *p*-coumaric acid were detected in *Vanilla* callus suggesting that the material could be used for the study of the vanillin biosynthetic pathway (Palama et al. 2010). Moreover small amounts of vanillin were formed after feeding ferulic acid feeding to a *Vanilla* cell culture (Ramagnoli and Knorr 1988). As callus is a typical tissue formed after wounding, it is particularly suited to study the plant response to infections, e.g. with endophytes.

NMR-based metabolomics was used to measure the metabolic changes in the *Vanilla* plant and callus material after co-culture with the endophytes previously used in the bioconversion experiments (ref. Chapters 3-5), i.e. *Hypoxylon investiens*, *Diaporthe phaseolorum*, *Pestalotiopsis microspora*. Additionally, the pathogen *Fusarium oxysporum* was added for comparison. In the work here, metabolite measurements were performed for two phenolics (B, C), two amino acids (A, F) and two carbohydrates (D, E) all connected to the vanillin biosynthesis network (**Figure 1**). Other vanilla flavor compounds on the vanillin biosynthesis pathway, were not detected.

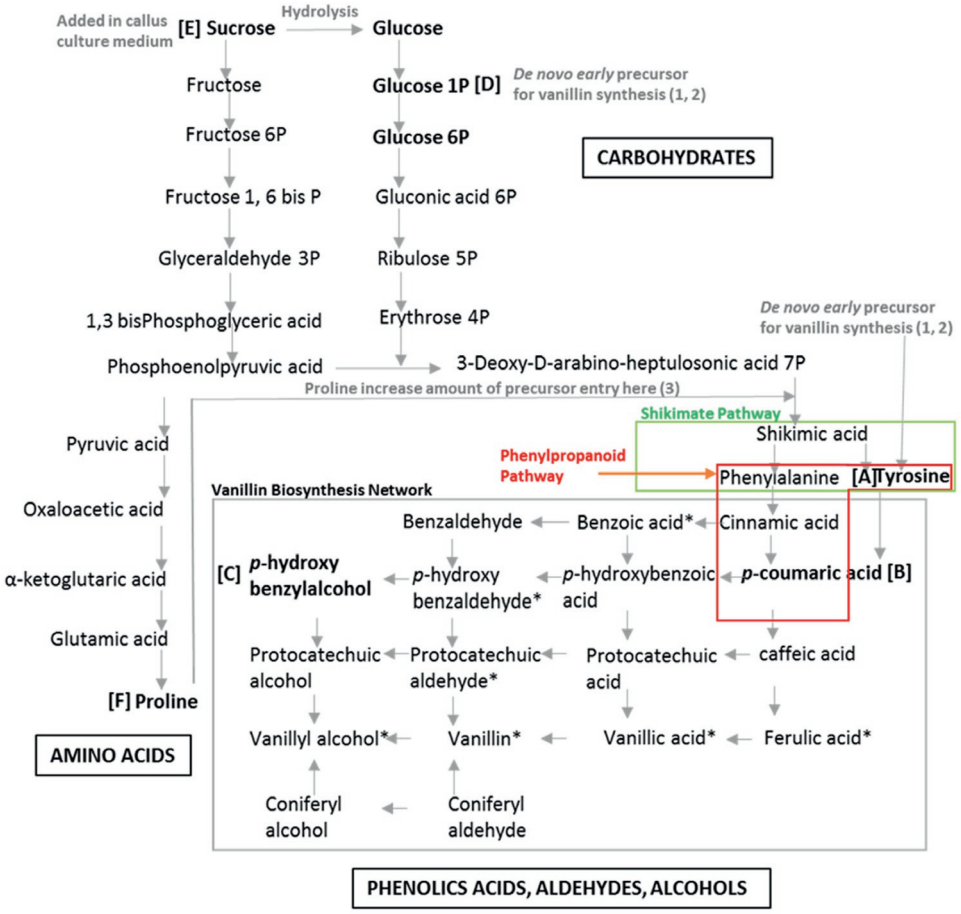

Figure 1 – Metabolic pathway connections from phenolics (B, C) on the vanillin biosynthesis network and amino acids (A, F), carbohydrates (D, E). The pathways were built from KEGG (Kyoto Encyclopedia of Genes and Genomes - http://www.genome.jp/kegg/), Gallage et al. (2014), Korthout and Verpoorte (2007), Havkin-Frenkel and Belanger (2007), Funk and Brodelius (1990). Reactions are reported as unidirectional for simplicity. Additional references – 1: Ni et al. 2015, 2: Hansen et al. 2009, 3: El-Naggar 2012.

Obviously, this model is different from the real process, where glucovanillin is being produced during about 9 months of pod maturation. But to work with axenic pods requires the growth of axenic plants for a period of at least 4 years before the first pods are produced. In the approach used here, we might also get information about the metabolic differences between a pathogenic fungus killing the plant and fungal endophytes of the plant that are capable to survive without showing any visible damage to the plant.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of *Vanilla* material

Seven-months-old green pods of *Vanilla planifolia*, from accession 13B1 Reunion elite flavor, were collected. The protocol established by Palama et al. (2010) was used for pre-sterilization, sterilization of pods, initiation of embryogenic calli and for their maintenance. Calli, which developed from the explant, were transferred to regeneration medium (Murashige and Skoog macro and microelements, Morel and Wetmore vitamins, 30 g l^{-1} sucrose, 0.5 mg l^{-1} α-naphthalene acetic acid). Calli from 15 and 30 days old cultures were used and analyzed at the histological and metabolic level (methods described below) to find if they are at different stages of development. Seven biological replicates were used for each time point (15, 30 days).

Calli were cultured for 240 days on solid regeneration medium in 250 ml Erlenmeyer flasks. Shoots developing from those calli were transferred after micro-cuttings on MS medium supplemented with 20 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 100 mg l^{-1} glutamine, the vitamins calcium panthothenate, nicotinic acid, thiamine hydrochloride and pyridoxine hydrochloride (1 mg l^{-1} each). After four months, *in-vitro* plantlets were obtained.

Compost used for *Vanilla* plant culture in the field was collected at La Vanilleraie (St. Suzanne, Reunion Island). The compost was passed through a sieve to obtain fine compost and was thoroughly mixed to homogenize it. The fine compost was then autoclaved twice and the pH confirmed to be the same as non-autoclaved compost. Magenta™ GA-7 plant culture boxes (Sigma-Aldrich, MO, USA) were autoclaved, and the aseptic fine compost was added to each box. A ¼ MS strength solution was added to the compost. Four months old *in-vitro* plants were then aseptically transferred in the sterile compost in the magenta box. The transfers were made in an aseptic laminar air flow cabinet and with sterilized forceps. Using autoclaved Magenta™ vessel couplers (Sigma-Aldrich), a second box was added onto the first one to allow for the height of the plant as well as sealing the setting against airborne fungal infections. The vessel coupler connections were sealed with parafilm to keep the plant environment sterile. The boxes were then placed in a climate chamber. The light regime was set for 12 h light per day, the temperature was set at 25°C. Every week, fresh ¼ MS strength solution was added to the boxes under aseptic conditions in the laminar air flow cabinet. Every month, the leaf of one plant, not to be used for the infection, was cut and cultured on MS solid medium for four days to ensure that the plants are still aseptic. After three months of growth, the plants were used for the endophyte infection experiment.

To check whether the callus and plants were axenic, both materials were aseptically cultured on Potato dextrose agar (PDA) media (Sigma-Aldrich, MO, USA), the same which was used to isolate the fungal endophytes (Khoyratty et al. 2015). The Petri dishes were incubated at 25°C and checked regularly for growth of fungi for up to 4 weeks.

6.2.2 Fungal infection of *Vanilla* material

To prove whether airborne fungal endophyte transmission is possible, 15 ovaries from both closed and opened flowers were collected at St. André, Reunion Island. Within 3 h after harvest, they were first washed under running tap water for 15 min. The washed material was then dipped for 10 s in 95% alcohol and thoroughly flamed on all sides for 3 s. The ovaries were cut open and placed onto potato dextrose agar (PDA) (Sigma-Aldrich, MO, USA) and incubated at 25°C. The Petri dishes were sealed with parafilm. Petri dishes were checked regularly during 4 weeks for growth of fungi from the ovaries on the plate. The identification of the isolated organisms was performed as reported in Khoyratty et al. (2015).

To mimic natural horizontal transmission, plant material was infected by placing it in a fungal spore solution containing the inoculum solution of one of the fungi to be tested. This was done in triplicate (n=3). The calli were completely covered by the inoculum for 2 min, after which it was transferred to a new culture medium. The plant was removed from the sterile compost, submerged in inoculum solution, then placed on new sterile compost. All operations were performed under aseptic conditions. The control callus and plants were placed in the control solution without fungal spores. Three biological replicates were conducted per fungus. The time period for fungi-*Vanilla* material co-culture was determined based on characteristics of fungal endophyte-host plant interaction according to literature (Spiering et al. 2006) i.e. mycelia which ought no longer be visible on the host surface. Given this time period was five days, fungi-*Vanilla* material co-culture period was kept at 5 days. The material was then collected for NMR analysis.

The fungi chosen in this work for the infection of the plant material were: three endophytes we isolated from *Vanilla* pods: *Pestalotiopsis microspora*, *Diaporthe phaseolorum*, *Hypoxylon investiens* and the pathogen *Fusarium oxysporum* f.sp. *vanillae*. In order to induce sporulation in the fungi, they were all cultured on cornmeal agar (CMA) (Spiering et al. 2006) for 10 days. After culture, the inoculum was prepared by flooding the petri dishes with 1 ml sterile water. The spores were collected with a sterile micropipette. Using a haemocytometer, a spore inoculum was made by serial dilution with 1 X 10^6 spores/ml of water containing 0.01% of aseptic Tween 20. Additionally, water containing 0.01% of aseptic Tween 20 but no spores was used for the control experiments. Three biological replicates were performed per parameter (four fungi and one control).

Calli were co-cultured in liquid ¼ MS strength solution (250 ml Pyrex wide-mouth Erlenmeyer flask, 50 ml of medium) with one fungus per experiment and placed on an agitator at 100 rpm. *In vitro* plants were co-cultured on autoclaved compost.

After five days infection, when endophyte mycelium was no longer visible on axenic plant and calli surfaces, three *Vanilla* tissue (n=3) were aseptically cut in half. This was cultured on potato dextrose agar for 4 days, to check endophyte presence. The same was done with non-infected controls (n=3).

6.2.3 Histological and histochemical analysis

Calli samples cultured for 15 and 30 days were prepared for fixation on glass slides, after which the samples were stained for making pictures using an inverted microscope according to previous methods (Palama et al., 2010).

6.2.4 ^{1}HNMR analysis

Samples were treated with liquid nitrogen and ground to a fine powder using a pestle and mortar followed by freeze-drying. Freeze-dried powder (50 mg) from each sample was transferred to a 2 ml microtube. A volume of 1.5 ml of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.005% trimethylsilylpropionic acid sodium salt (TMSP, w/w) and CD_3OD (1:1) was added to the samples. The mixture was mixed at room temperature for 1 min, sonicated for 20 min and centrifuged at 13,000 rpm for 10 min. An aliquot of 0.8 ml was transferred to a 5-mm NMR tube for analysis.

^{1}HNMR spectra were recorded at 25°C on a 600 MHz Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryo-probe operating at a proton NMR frequency of 600.35 MHz. CD_3OD was used as the internal lock. Each ^{1}HNMR spectrum consisted of 64 scans requiring 5 min and 26 s acquisition time with the following parameters: 0.15 Hz/point, pulse width (PW) = 30° (11.3 µs), and relaxation delay (RD) = 2.0 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low-power selective irradiation at the H_2O frequency during the recycle delay. Free induction decays (FIDs) were Fourier-transformed with LB = 0.3 Hz. The resulting spectra were manually phased and base line corrected and calibrated to TMSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker).

6.2.5 Data analysis

The ^{1}HNMR spectra were automatically reduced to ASCII file. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.30–10.02. The regions of δ 4.70–5.00 and δ 3.28–3.40 were excluded from the analysis because of the residual signal of H_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) was performed with SIMCA-P software (v. 13.0, Umetrics, Umeå, Sweden) using scaling based on the Pareto method. Coordinates of F1 and F2 components were used to identify groups using clustering analysis (Ward aggregation method and Euclidian distances). The metabolite content was calculated from the ^{1}HNMR spectra.

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, tissue type. The dependent variable (continuous factor) was the metabolite amount (X 10^{-5} mol g^{-1} DW). The metabolites were tyrosine, *p*-coumaric acid, *p*-hydroxybenzyl alcohol, glucose, sucrose, proline. The null hypothesis is rejected for a p-value < 0.05.

6.3 RESULTS AND DISCUSSION

6.3.1 Preparation of *Vanilla* material

To study the mutual effects of endophyte and its host plant on their respective metabolomes, the ideal model is to study the changes in the metabolome of pods during maturation. However, that requires a quite long duration of the experiment, i.e. at least 4 years to obtain axenic pods on the plant, followed by 9 months of maturation of the pods after artificial fungal infection. Therefore we decided first of all to look at the more immediate effect of the endophyte on the cells in the plant or in calli. In the plant, different cell types will be involved. To come closest to the first interaction of the plant and the endophyte, we used calli, which per definition is the tissue formed in a plant after wounding. Totipotency – in principle all living plant cell have the full genetic material and are totipotent. In the regeneration process calli change from undifferentiated "stem" cells to differentiated cells. This will result in changes in microscopic and morphological appearance as well as in transcriptome, proteome and metabolome. Therefore experiments were done with two different stages of development, at 15 and 30 days after inoculation on the regeneration medium (**Figure 2**).

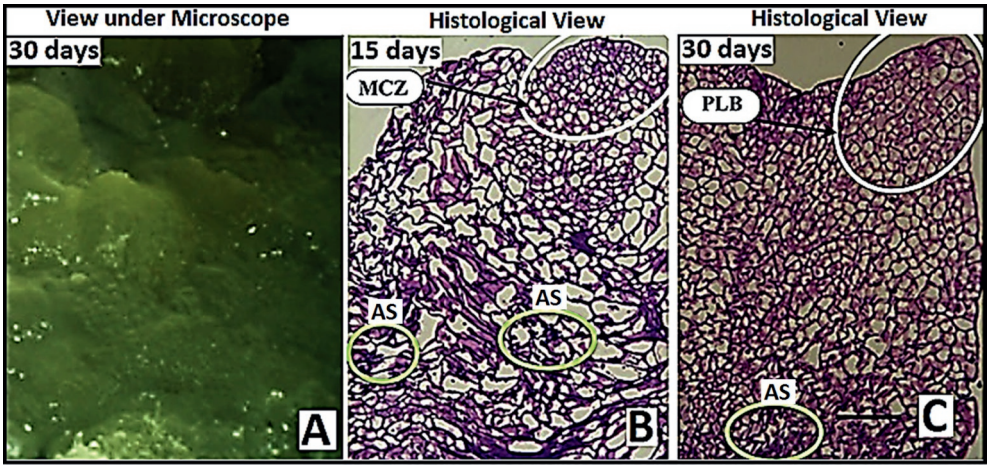

Figure 2 - Microscopic and histological views of calli cultured for 15 and 30 days in regeneration media. [A] Microscope view at 30 days post culture. [B] Histologically stained calli (with periodic acid and Schiff's reagent) from 15 days post inoculation; [C] Ibidem 30 days post inoculation. AS: accumulated starch in the cortex region shown in circle; PLB: Protocorm-like body; MCZ: Mitotic cell zone; bar = 150 µm.

Through microscopy, it was observed that 15 day-old calli contained more mitotic cell zones whereas 30 day-old calli contained more protocorm like bodies, i.e. the former is starting to differentiate. NMR-based metabolic analysis was used to see if the differentiation of the calli affects the metabolome. The principle component analysis (PCA) of the non-treated calli cultures shows that a phenolic alcohol (*p*-hydroxybenzyl alcohol) is higher in 30 day-old calli, whereas sucrose was higher in the 15 day-old calli (**Figure 3, 4**).

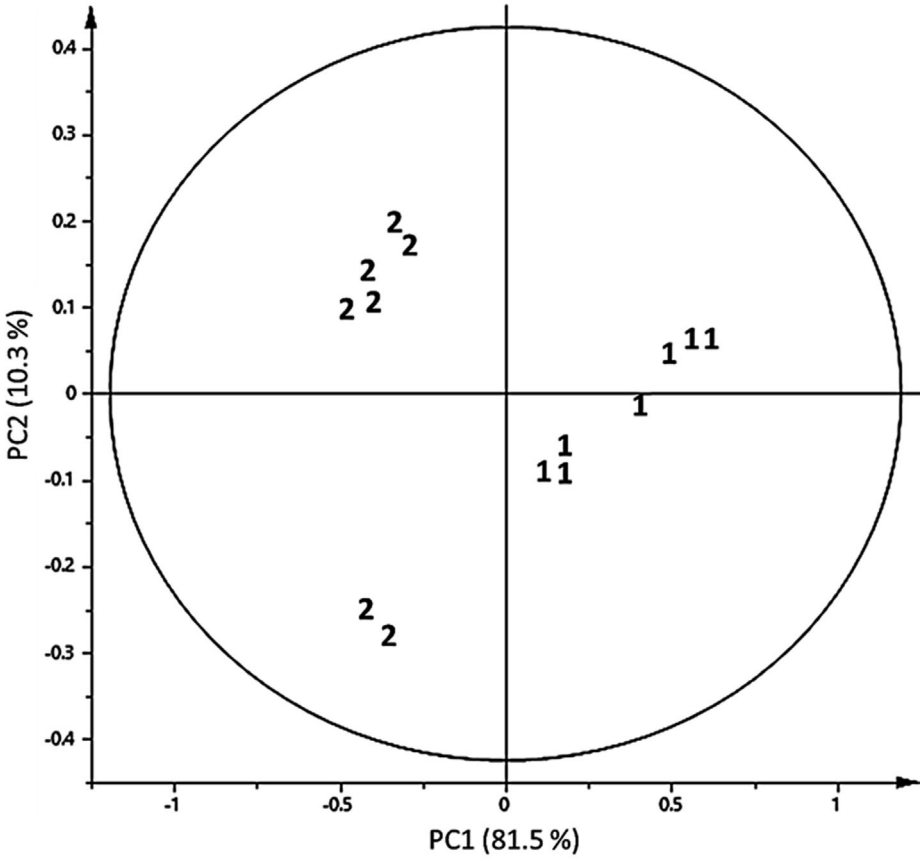

Figure 3 -Principle component analysis of [1]HNMR spectral data of calli cultured on regeneration media for 15 or 30 days using Pareto scaling. Scatter Score Plot of principal components PC1 and PC2, 1 = 15 days old, 2 = 30 days old. Number of replicates was n=7.

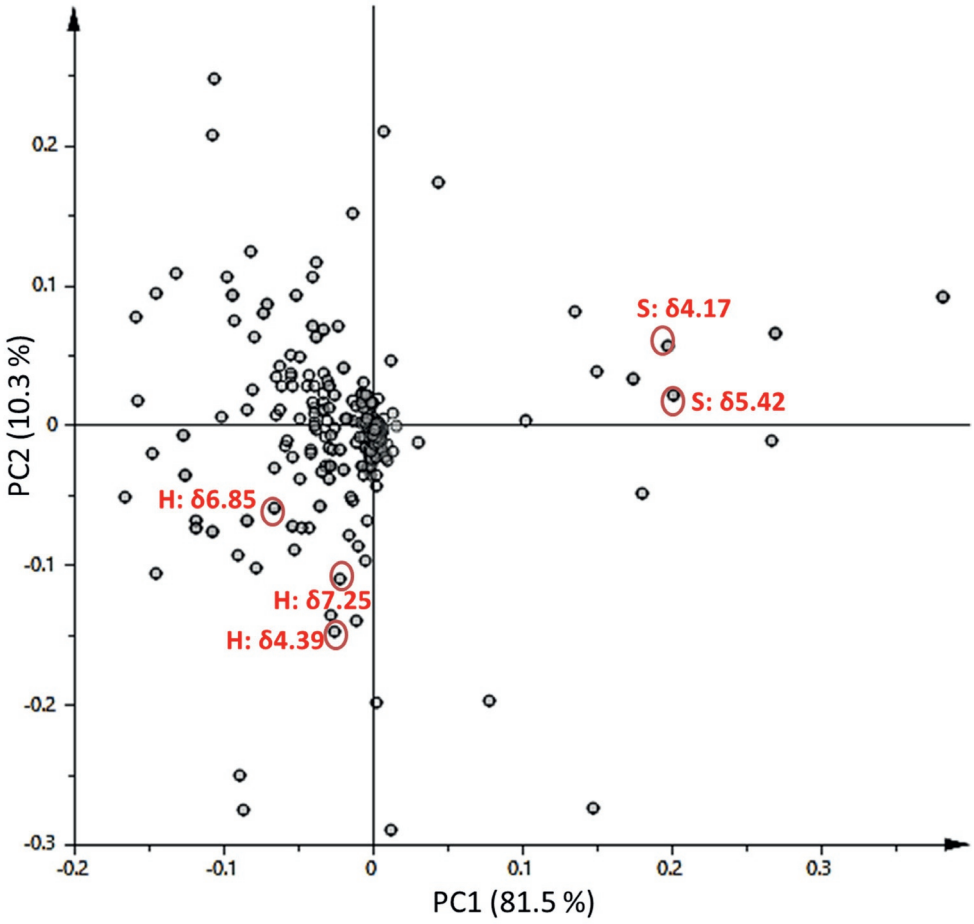

Figure 4 -Principle component analysis of [1]HNMR spectral data of calli cultured on regeneration media for 15 or 30 days using Pareto scaling. Loading Plot shows compounds S: sucrose, H: *p*-hydroxybenzyl alcohol. The chemical shifts for each compound are indicated in the loading plot. Number of replicates was n=7.

The *p*-hydroxybenzyl alcohol is part of the vanillin biosynthetic network. The two types of calli (15, 30 days) were chosen in this work for co-culture with fungal endophytes. Both calli and axenic plantlets were cut and cultured on potato dextrose agar, after which no fungi were observed after 4 weeks. From these results it seems that the *Vanilla* plant material and cell cultures were free of fungi (axenic). Apparently the older calli had lower sugar levels and did produce a phenolic compound at a higher level.

6.3.2 Infection methods of *vitro* plants with fungal endophytes

Horizontal transmission of endophytes works by infection with spores spread through the air or through irrigation. Although Chapter 3 reports the presence of fungal endophytes in green pods, there is no indication whether horizontal transmission occurs. Green pods are fruits of the plant. The fruits develop after the pollination of the floral ovary. Horizontal transmission into *Vanilla* pods might thus go via the flowers. The ovary is isolated from aerial exposure when flowers are young with the petals firmly closed. In case transmission is horizontal, no fungal endophytes should grow after isolation from ovaries of closed flowers protected from aerial infection. Horizontally transmitted fungi should only be found in ovaries with opened petals. Indeed five fungal endophytes were isolated (*Fusarium scirpi*, *Nigrospora* sp1, *Delitschia chaetomioides*, *Botryosphaeria ribis* and *Aspergillus fumigatus*) from ovaries with opened petals, whereas none was isolated from the closed flower buds. Thus, these fungi were most likely transmitted horizontally.

Thus as horizontal transmission of *Vanilla* fungal endophytes seems possible, calli and plants in this study were immersed in fungal endophyte spore solutions for inoculation of the *Vanilla* plant material.

6.3.3 Fungi-*Vanilla* material co-culture

Typical for the fungal endophyte-host plant interaction is the symptomless host, and non-visible mycelia growth on the host plant's surface. Axenic plants grown under aseptic conditions and 15 and 30 day-old calli material were co-cultured with the test fungi. For all fungi, upon infection, a thick mycelium mass develops on the surface of *Vanilla* tissue. At five days, the same is observed for the pathogen (*F. oxysporum*). However, at five days, mycelia of endophytic fungi are no longer visible by eye on the surfaces of *Vanilla* plants. To confirm endophyte presence inside these *Vanilla* tissues, all three infected *Vanilla* tissues at five days co-culture were cut in half and cultured on potato dextrose medium. Endophyte growth was observed on the medium originating from the cut plant and the cut calli after four days, showing endophyte presence inside the plant and calli. However, no fungal growth was obtained from the non-infected control *Vanilla* tissue. The five days post infection time was then applied for all fungi tested, for fungal co culture with plants and calli.

6.3.4 Metabolite identification and quantification

Besides the visual inspection for differences between non-infected controls and co-cultured samples infected with fungi, all were subject to NMR-based metabolomics analysis. Metabolites that were identified from the ^{1}HNMR spectra are shown in **Table 1**.

Table 1 - ^{1}HNMR chemical shifts (δ), and coupling constants (Hz) used for the identification of metabolites.

Compound	Chemical shifts and coupling constants
Sucrose	δ 5.41 (1H, d, *J* = 3.6 Hz), δ 4.20 (1H, d, *J* = 7.8 Hz)
Glucose	δ 5.17 (1H, d, *J* = 3.7 Hz), δ 4.56 (1H, d, *J* = 7.9 Hz)
Tyrosine	δ 7.29 (2H, d, *J* = 8.5 Hz), δ 7.09 (2H, d, *J* = 8.5 Hz), δ 3.63 (1H, dd, *J* = 7.9, 5.0 Hz), δ 3.21 (1H, d, *J* = 7.7 Hz), δ 3.18 (1H, d, *J* = 7.7 Hz)
Proline	δ 4.12 (1H, dd, *J* = 9.0, 6.5 Hz), δ 3.51 (1H, dt, *J* = 11.5, 7.0), δ 3.40 (1H, dt, *J* = 11.5, 7.0), δ 2.34 (2H, m), δ 2.07 (1H, m), δ 1.98 (1H, m)
p-coumaric acid	δ 7.61 (2H, d, *J* = 8.6 Hz), δ 7.55 (1H, d, *J* = 16.0 Hz), δ 6.64 (2H, d, *J* = 8.6 Hz), δ 6.31 (1H, d, *J* = 16.0 Hz)
p-hydroxybenzyl alcohol	δ 7.20 (2H, d, *J* = 8.0 Hz), δ 6.89 (2H, d, *J* = 8.0 Hz), δ 4.51 (1H, s)

The raw data of the recorded metabolite amounts are shown in **Tables S1-S3** in the **supplementary data section**. ANOVA analysis was also performed to find differences in metabolite amounts that are significant i.e. not due to random errors. The significance test was based on an alpha value 0.05 i.e. recorded differences in metabolites having *p*-values smaller than alpha are significant.

An example of the partial ^{1}HNMR spectra of 30 day-old calli co-cultured for five days with the fungus *Pestalotiopsis microspora* with detected metabolites in this work is shown in **Figure 5** (**complete spectrum in Chapter 6 - Supplementary Data – Figure S10**). The complete spectra for the different samples are shown in the supplementary data section (**Chapter 6 - Supplementary Data – Figures S1 to S15**).

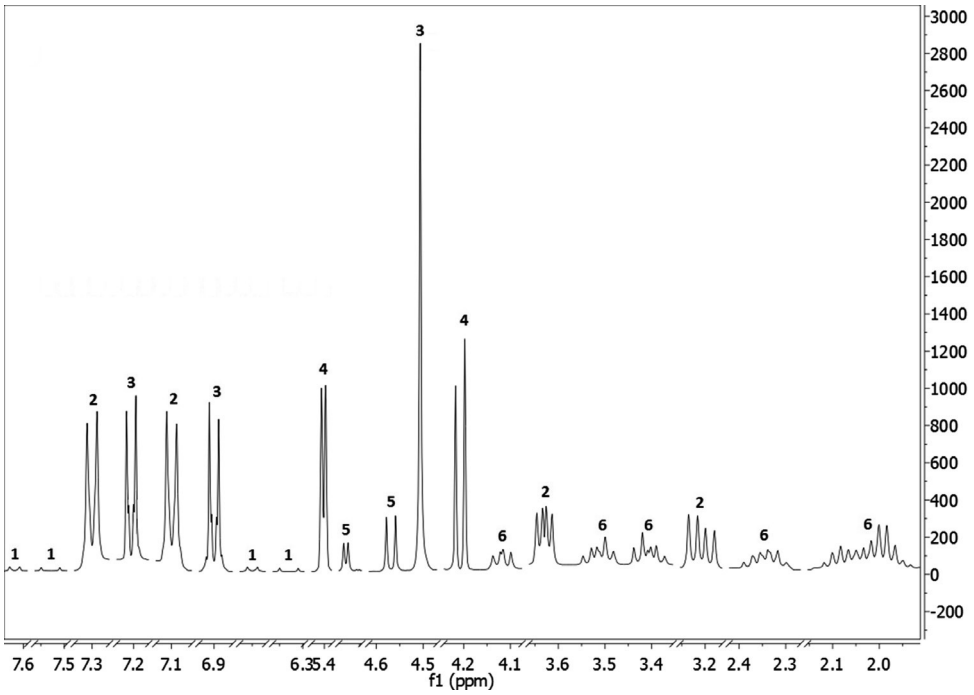

Figure 5 – Partial ^{1}HNMR spectrum (600 MHz, aqueous phosphate buffer pH=6 - CD_3OD (1:1)) of 30 days old calli co-cultured with fungus *Pestalotiopsis microspora* for five days with metabolites detected in this work. Metabolites were identified by comparing the NMR spectra of the extracts with our in-house database with spectra of pure reference compounds recorded under the same conditions. The chemical shifts and coupling constants were then rechecked. The numbers in the figure represent compounds 1: *p*-coumaric acid, 2: tyrosine, 3: *p*-hydroxybenzyl alcohol, 4: sucrose, 5: glucose, 6: proline.

All metabolite quantifications in this work were performed from the integration of ^{1}HNMR signals from the spectra and comparing against the internal standard TMSP. The chemical shifts used, the number of protons at the chosen signal and the proton nomenclature are: sucrose δ 5.40 (1H; H-1'), glucose δ 4.59 (1H; H-1) and δ 5.18 (1H; H-1), tyrosine δ 7.10 (2H; H-3, H-5), proline δ 3.41 (1H; H-5), *p*-coumaric acid δ 6.91 (2H; H-2, H-6), *p*-hydroxybenzyl alcohol δ 7.25 (2H; H-2, H-6).

With regards to vanillin synthesis, the amino acid tyrosine is a known *de novo* early precursor (Ni et al. 2015, Hansen et al. 2009) (**Figure 1**). For this reason, tyrosine content was assessed in the non-infected and infected tissues (**Figure 6**).

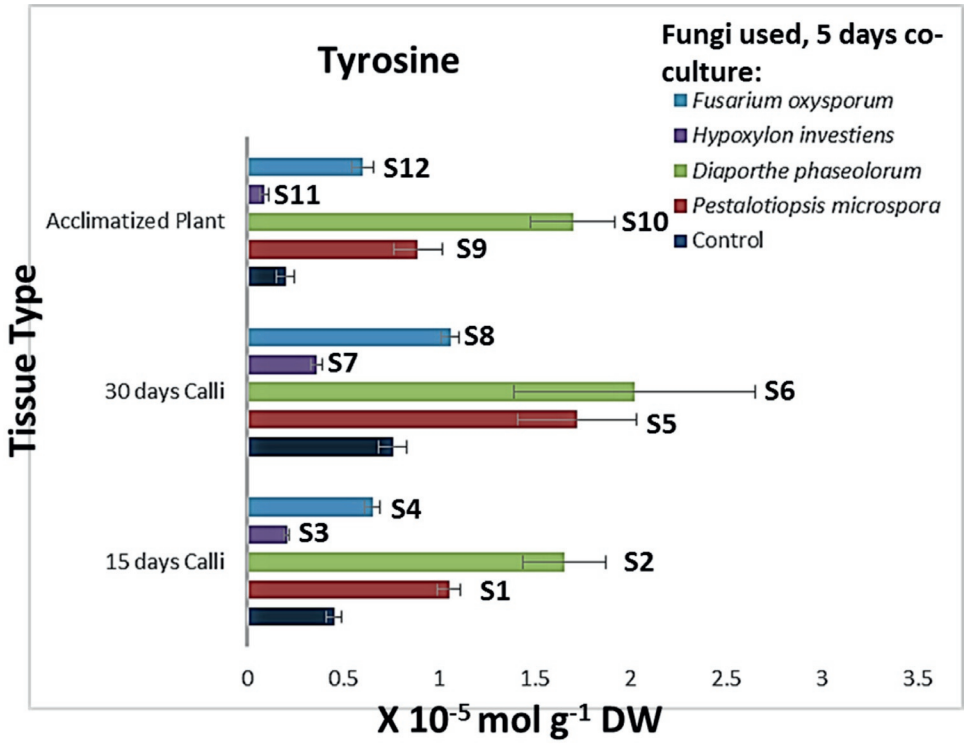

Figure 6 - Amino acid tyrosine content calculated from ^{1}HNMR spectra of extracts of axenic plants or calli (15 or 30 days old) harvested after five days of co-culture with fungi. Non-treated plants and calli were used as controls. The means of metabolites contents were expressed as X 10^{-5} mol g^{-1} dry weight (DW). Compared to non-infected controls, tyrosine amounts that differ statistically significantly across fungal species, are indicated with the following *p*-values/F-values/Total Degrees of Freedom: **S1:** 0.0001/197.5/5, **S2:** 0.0006/91.0/5, **S3:** 0.0005/97.7/5, **S4:** 0.004/34.6/5, **S5:** 0.03/10.1/5, **S6:** 0.03/10.7/5, **S7:** 0.0006/91.8/5, **S8:** 0.008/23.2/5, **S9:** 0.002/41.7/5, **S10:** 0.0007/86.1/5, **S11:** 0.009/22.1/5, **S12:** 0.001/51.9/5. n=3.

Here in **Chapter 6**, the tyrosine amount due to *H. investiens* (HI) is lower than the control while the amounts for all other fungi are higher than the control (non-infected) calli and axenic plant. A similar pattern is observed in **figure 4** of **Chapter 5** where tyrosine accumulation for all fungi, except HI, in vanilla leaf agar medium after five days growth are higher than the control in the medium (without growth).

The non-infected tissue type (the controls) was previously shown to differ significantly if comparing the age of the tissues (**Figures 2, 3, 4**). As such, the use of *Vanilla* tissues of different ages, is justified, given these are not the same tissue type, as shown previously. What is not yet known is whether the infected tissue type (15, 30 days calli, acclimatized plant infected with fungi) had a significant effect on tyrosine and (later) *p*-coumaric acid, *p*-hydroxybenzyl alcohol, glucose, sucrose, proline contents. To answer this question, the controls were not included with the infected tissue in the ANOVA tests to avoid Type 1

errors which are false positives. This is so given the control is known, beforehand, to significantly differ from the infected material from the bar chart results (e.g. **Figure 6**) even when the standard deviation is considered i.e. there is no overlap between controls and infected materials. By comparing data for all infected tissue types only, a *p*-value of 3.2 x E^{-9} was obtained (alpha value was 0.05) showing infected tissue type had a significant effect on metabolite content.

Callus morphology (tissue type) was reported to correlate to total soluble phenols in strawberry although the trend (increase or decrease) depended on the callus development stage (Arnaldos 2001). Whereas in hairy ground cherry and barbatimao calli, total phenolics amounts only increased over time (Bertoncelli et al. 2014). The case of *Vanilla* was thus assessed in the work here. We focused on the *Vanilla* flavor compounds tyrosine and phenolics as major compounds present and both being part of the vanillin biosynthetic network. In Chapter 5 it is reported that HI synthesized vanillin by biotransformation of added free ferulic acid as precursor. In the present experiments neither vanillin nor ferulic acid were detected in any samples suggesting that HI requires *Vanilla* tissue producing free ferulic acid. The integration of free ferulic acid into bound cell wall form (feruloylation) may occur at a slower rate in the pods which makes free ferulic acid more available to the endophyte. Otherwise, the plant competes against the endophyte for free ferulic acid. In any case, the endophyte has the potential to add to the vanillin amount already synthesized by the plant. To overcome the problem of lack of the precursor ferulic acid, Gallage et al. (2014) engineered the gene of a *Vanilla* enzyme capable of biotransforming ferulic acid to vanillin into barley known to contain significantly higher amounts of free ferulic acid than most other plants. This led to the stable overexpression of the enzyme and a significantly higher amount of vanillyl alcohol glucoside formation than the control.

Despite vanillin not being detected, two other possible phenolic precursors of vanillin (*p*-coumaric acid and *p*-hydroxybenzyl alcohol) were observed in our experiments (**Figure 7**). Both, *p*-coumaric acid and *p*-hydroxybenzyl alcohol need a further hydroxylation step and a methylation to get the same phenolic substitution pattern as vanillin (**Figure 1**).

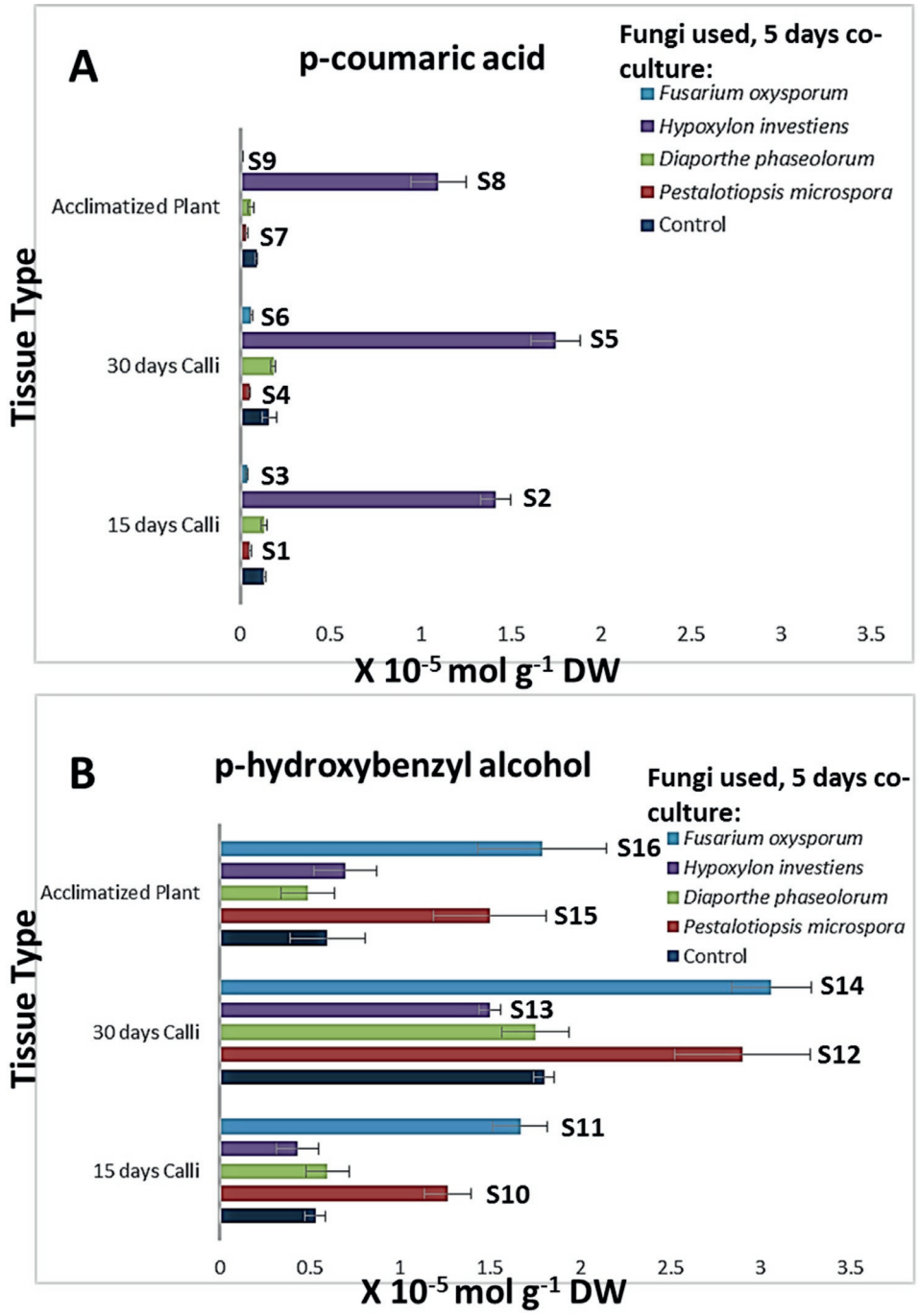

Figure 7 - Phenolics (A-*p*-coumaric acid, B-*p*-hydroxybenzyl alcohol) contents calculated from [1]HNMR spectra of extracts of axenic plants or calli (15 or 30 days old) harvested after five days of co-culture with fungi. Non-treated plants and calli were used as controls. The means of metabolites

contents were expressed as X 10^{-5} mol g^{-1} dry weight (DW). Compared to non-infected controls, phenolic amounts that differ statistically significantly across fungal species, are indicated with the following *p*-values/F-values/Total Degrees of Freedom: **S1:** 7.0 x E^{-5} /288/5, **S2:** 1.2 x E^{-5} /699.1/5, **S3:** 3.3 x E^{-5} /420.5/5, **S4:** 0.005/28.8/5, **S5:** 6.0 x E^{-5} /312.4/5, **S6:** 0.005/28.8/5, **S7:** 0.0006/90.2/5, **S8:** 0.0005/100.1/5, **S9:** 0.0001/220.5/5, **S10:** 0.0008/80.8/5, **S11:** 0.0002/151.9/5, **S12:** 0.02/13.2/5, **S13:** 0.01/18/5, **S14:** 0.0009/76.0/5, **S15:** 0.02/13.0/5, **S16:** 0.01/17.0/5. n=3.

In Chapter 5, the biotransformation of *p*-coumaric acid into *p*-hydroxybenzoic acid by the fungi *D. phaseolorum* (DP), *P. microspora* (PM), and *F. oxysporum* (FO) was described. However, no *p*-hydroxybenzyl alcohol was observed in the control experiments, whereas in the present study (**Figure 7B)** this metabolite was found in untreated tissues. The probable sequence of events in the present experiments is first the tyrosine biotransformation to *p*-coumaric acid followed by the formation of *p*-hydroxybenzoic acid by the fungi PM, and FO (**Chapter 5**). None of these fungi produced *p*-hydroxybenzyl alcohol from the *Vanilla* extracts, which means that this reduction might occur in the plant cells. The amount of tyrosine in the untreated plants and cells was clearly lower than in the FO, DP and PM treated materials, only HI treatment showing the highest *p*-coumaric acid content had lower tyrosine content than the untreated materials. That means that the infection with the fungi results in an increased level of tyrosine and subsequent products.

Looking at the mass balance of these three intermediates (tyrosine, *p*-coumaric acid, *p*-hydroxybenzyl alcohol) that form a linear pathway (**Figure 1**), it seems that the four fungi contain about the same total moles (**Figure 6, 7A, B and Table 2**).

Table 2 – Average recorded metabolite amount from ^{1}HNMR spectra from 15, 30 days calli and acclimatized plants with and without (control) fungus infection. The total of tyrosine, *p*-coumaric acid and *p*-hydroxy benzyl alcohol amounts and the ratio relative to the control is shown for each infected *Vanilla* material and for the non-infected control. The average was calculated from the data shown in the supplementary section.

Metabolite	**Average recorded amount in *Vanilla* 15 days calli after growth with fungus (X 10^{-5} mol g^{-1} DW)**				
	Control (no fungal growth)	***Hypoxylon investiens***	***Diaporthe phaseolorum***	***Pestalotiopsis microspora***	***Fusarium oxysporum***
Tyrosine	0.4	0.2	1.6	1	0.6
p-coumaric acid	0.1	1.4	0.1	0.05	0.04
p-hydroxy benzyl alcohol	0.5	0.4	0.6	1.2	1.6
Total of three metabolites	1	2	2.3	2.3	2.2
Ratio relative to control	1	2	2.3	2.3	2.2
Metabolite	**Average recorded amount in *Vanilla* 30 days calli after growth with fungus (X 10^{-5} mol g^{-1} DW)**				

	Control (no fungal growth)	***Hypoxylon investiens***	***Diaporthe phaseolorum***	***Pestalotiopsis microspora***	***Fusarium oxysporum***
Tyrosine	0.8	0.3	2	1.4	1
p-coumaric acid	0.1	1.6	0.1	0.06	0.06
p-hydroxy benzyl alcohol	1.8	1.6	1.7	2.5	2.9
Total of three metabolites	2.7	3.5	3.8	4.0	4.0
Ratio relative to control	1	1.3	1.4	1.5	1.5
Metabolite	**Average recorded amount in *Vanilla* acclimatized plants after growth with fungus (X 10^{-5} mol g^{-1} DW)**				
	Control (no fungal growth)	***Hypoxylon investiens***	***Diaporthe phaseolorum***	***Pestalotiopsis microspora***	***Fusarium oxysporum***
Tyrosine	0.2	0.1	1.4	0.7	0.5
p-coumaric acid	0.08	1.0	0.08	0.02	0.01
p-hydroxy benzyl alcohol	0.4	0.6	0.5	1.2	1.3
Total of three metabolites	0.7	1.7	2.0	1.9	1.8
Ratio relative to control	1	2.4	2.9	2.7	2.6

The amount of tyrosine in the untreated plants and cells was clearly lower than in the FO, DP and PM treated materials, only HI treatment showing the highest *p*-coumaric acid content had lower tyrosine content than the untreated materials. The fungus DP has the highest tyrosine, HI contains highest in *p*-coumaric acid, whereas FO and PM have the highest level of *p*-hydroxybenzoic acid (higher than controls). This shows that FO and PM are connected with the highest flow through this part of the vanillin metabolic network. This pattern is similar as seen in **Chapter 5, Figure 2**, where HI is the only fungus that produced high *p*-coumaric acid, whereas in case of PM and FO *p*-hydroxybenzoic acid were found as major phenolics.

Glucose is an early *de novo* vanillin precursor for the full biosynthesis of vanillin (Ni et al. 2015, Hansen et al. 2009). Chapter 5 it was shown that that no full *de novo* biosynthesis of vanillin occurred in any of the tested endophytes. An increase in sucrose content is often correlated to an increase in phenolic content in cell cultures (Modarres et al. 2018, Kikowska et al. 2015, Lux-Enrich et al. 2000). Lower sugars in general in plant cell cultures means that the cells will stop growing and enter the stationary phase, which often parallels with the production of secondary metabolites. Callus cultures use the sugars for growth, defense response against microorganisms and general cell maintenance, the same

applies for the fungi that will use the sugars for growth, cell maintenance and possible interactions with the plant cells. Therefore the sucrose and glucose amounts were monitored (**Figure 8**).

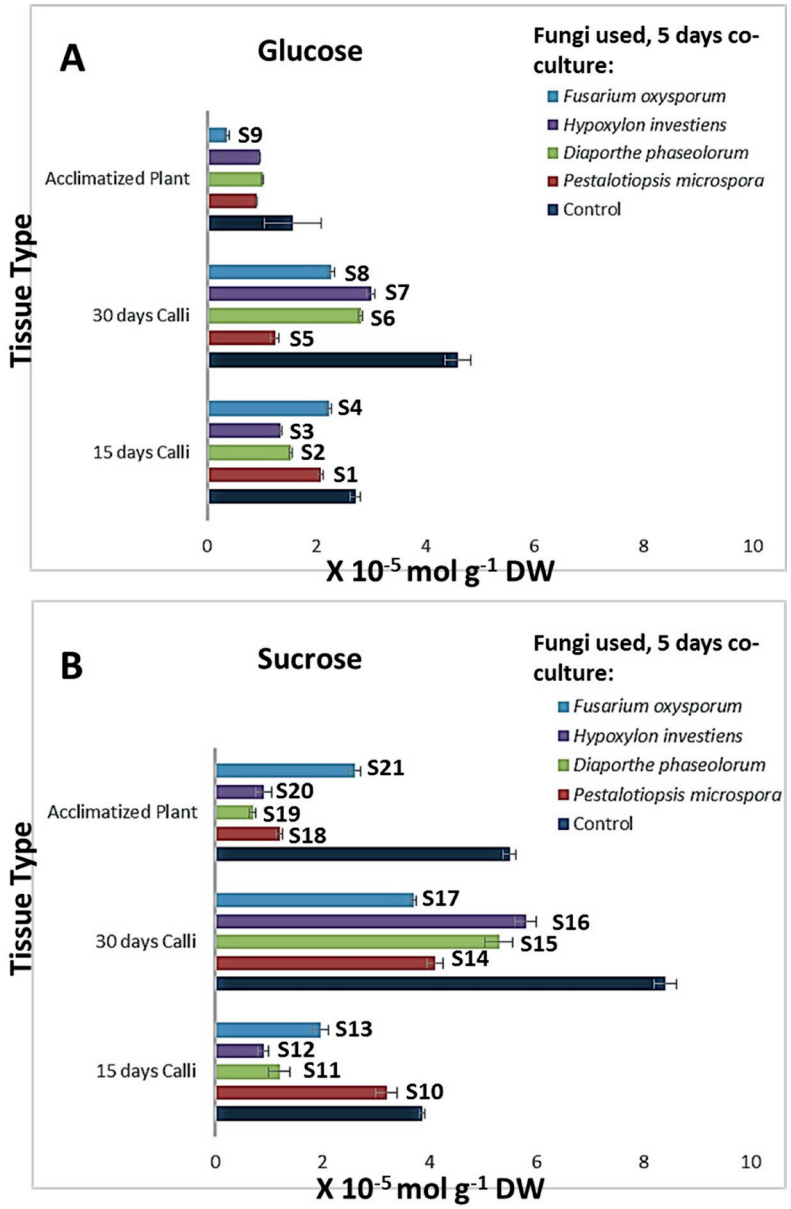

Figure 8 - Carbohydrates (A-Glucose, B-Sucrose) contents calculated from ^{1}HNMR spectra of extracts of axenic plants or calli (15 or 30 days old) harvested after 5 days of co-culture with fungi. Non-treated plants and calli were used as controls, the sugar amounts in the non-infected material is shown. The means of metabolites contents were expressed as X 10^{-5} mol g^{-1} dry weight (DW). Compared to non-infected controls, carbohydrate amounts that differ statistically significantly across

fungal species, are indicated with the following *p*-values/F-values/Total Degrees of Freedom: **S1:** 0.0004/107.2/5, **S2:** 3.2 x E^{-5} /425.2/5, **S3:** 1.8 x E^{-5}/560.9/5, **S4:** 0.001/59.2/5, **S5:** 2.2 x E^{-5}/515.5/5, **S6:** 0.0003/126.0/5, **S7:** 0.0005/101.4/5, **S8:** 0.0001/219.3/5, **S9:** 0.02/14.7/5, **S10:** 0.005/30.7/5, **S11:** 2.4 x E^{-5}/492.3/5, **S12:** 1.5 x E^{-6}/1980.2/5, **S13:** 3.5 x E^{-5}/406.1/5, **S14:** 9.7 x E^{-6}/781.2/5, **S15:** 0.0001/227.7/5, **S16:** 0.0001/213.1/5, **S17:** 3.2 x E^{-6}/1360.2/5, **S18:** 5.5 x E^{-7}/3276.8/5, **S19:** 3.4 x E^{-7}/4147.2/5, **S20:** 2.4 x E^{-6}/1560.1/5, **S21:** 6.0 x E^{-6}/990.1/5. n=3.

Infected *Vanilla* material here showed a higher decrease of the levels of sucrose and glucose present, which makes sense as two organisms are competing for the same carbohydrate source. A similar observation was made in case of grasses after endophyte infection (Chen et al. 2017, Qin et al. 2016). The decrease in carbohydrates amount (**Figure 8**) in infected *Vanilla* tissues is correlated to an increase in phenolics amount (**Figure 7**).

Lettuce inoculation with fungal endophytes increased the amount of proline, a biotic and abiotic stress marker, in the plant (Molina-Montenegro et al. 2016, Omezzine et al. 2014). In both great mullein callus (Al-jibouri et al. 2016) and in rosemary callus (El-Naggar 2012) proline was found to be correlated with an increase in precursor amount entry into the Shikimate and phenylpropanoid pathways (El-Naggar 2012). Thus, proline content in the callus was assessed as per the work here (**Figure 9**).

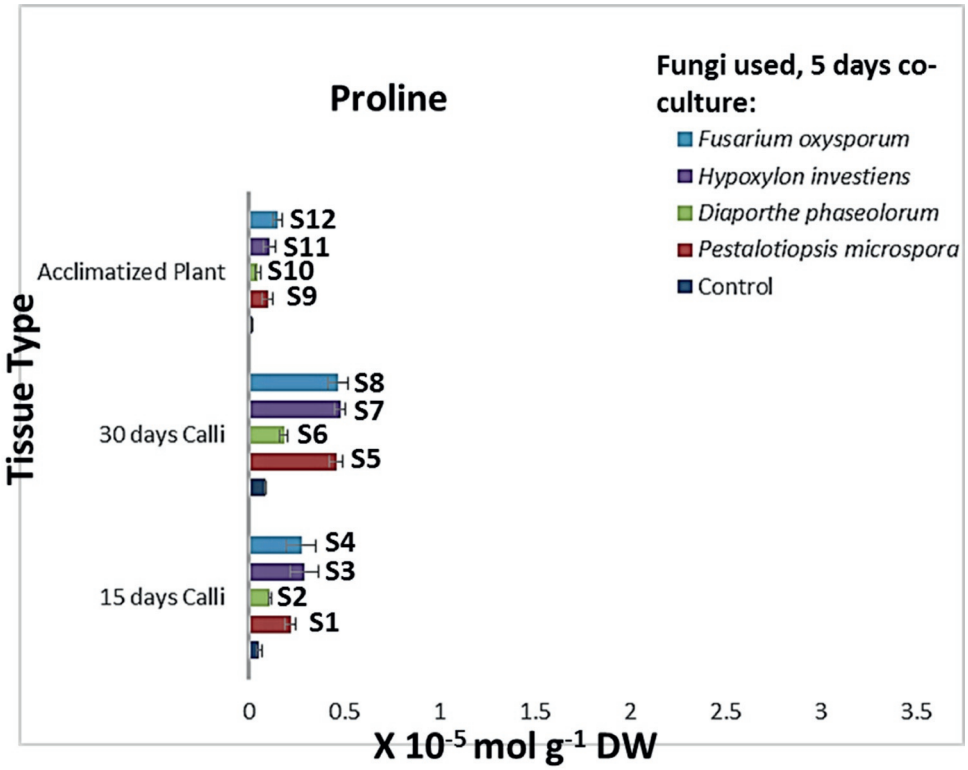

Figure 9 - Amino acid proline content calculated from ^{1}HNMR spectra of extracts of axenic plants or calli (15 or 30 days old) harvested after five days of co-culture with fungi. Non-treated plants and

calli were used as controls. The means of metabolites contents were expressed as X 10^{-5} mol g^{-1} dry weight (DW). Compared to non-infected controls, proline amount that differ statistically significantly across fungal species, are indicated with the following *p*-values/F-values/Total Degrees of Freedom: **S1:** 0.0007/85.7/5, **S2:** 0.003/36.1/5, **S3:** 0.006/27.7/5, **S4:** 0.007/24.8/5, **S5:** 5.5 x E^{-5}/324.2,/5, **S6:** 0.001/56/5, **S7:** 2.5 x E^{-5}/480.1/5, **S8:** 0.0002/148.5/5, **S9:** 0.005/30.7/5, **S10:** 0.01/20/5, **S11:** 0.006/28.1/5, **S12:** 0.002/45/5. n=3.

Fungal infection of *Vanilla* tissue increased proline content. The increase in proline content (**Figure 9**) was correlated to an increase in free phenolics amount (**Figure 7**). The highest increase was recorded for 30 days calli. Funk and Brodelius (1990) reported that elicitation of *Vanilla* cell cultures with elicitor chitosan resulted in lower levels of some free phenolics, but a considerable increase of bound cell wall phenolics. Obviously the difference with our experiment is that here we have the elicited plant cells and the living fungi together. Considering the findings of Funk and Brodelius, the increase of phenolics observed is most likely caused by the fungi.

6.3.5 Comparing overall metabolite contents

Considering the aim of this study, to measure possible effects of fungal endophytes on the metabolome of the host plant, it is clear from this study that the tested endophytes do have a clear effect on the metabolome of the host plant. The definition of endophytes states that an endophyte should not cause any visible symptoms in the host plant. Without defining what is meant by visible symptoms, our results with the endophytes isolated from *Vanilla* would mean that there are clear differences between infected and non-infected plants and calli as shown by NMR-based metabolomics. As the experiments were done with axenic plants not yet bearing any pods, we cannot say much about the effect of endophytes on the *Vanilla* pod metabolome. Where changes occur in the vanillin-related metabolic network is summarized in **Figure 10.** In Chapter 3, the *de-novo* biosynthesis of vanillin by the fungi was shown to be unlikely. Here, we have clear evidence that combination of plant cells and fungi result in the production of some phenolics and tyrosine from the vanillin biosynthetic network, but no production of vanillin was found.

Vanillin is only found in pods, so it was not expected to be present in any of the materials used in this study. At at least the study makes clear that the endophytes may affect phenolic biosynthetic pathways in *Vanilla* plants, and that each endophyte has a different effect on the plant or calli. The question remains as to whether the increased level of the phenolics is through an induction of the plant cells or via biosynthesis in the fungi.

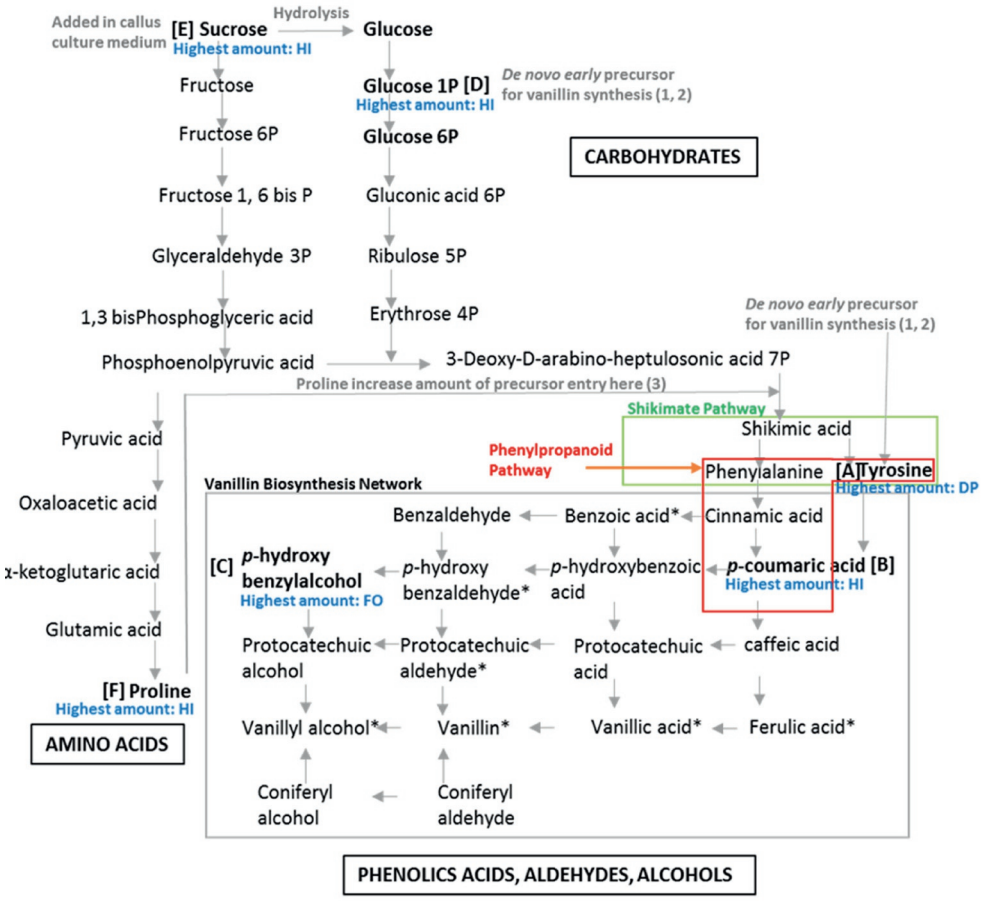

Figure 10 – Metabolic pathways leading to biosynthesis of major metabolites (in bold) identified in living *Vanilla* material after fungal co-culture. The pathways were built from KEGG (Kyoto Encyclopedia of Genes and Genomes - http://www.genome.jp/kegg/), Gallage et al. (2014), Korthout and Verpoorte (2007), Havkin-Frenkel and Belanger (2007), Funk and Brodelius (1990). Reactions are reported as unidirectional for simplicity. Fungi associated with the highest detected metabolite level are indicated: DP – *Diaporthe phaseolorum*, HI – *Hypoxylon investiens*, FO – *Fusarium oxysporum*.

6.4 CONCLUSIONS AND FURTHER WORK

The current work concerns the interaction of two living systems: *Vanilla* material and a fungal endophyte. Gallage et al. (2018) showed that *de novo* vanillin synthesis from fed phenylalanine occurs in *Vanilla* re-differentiated chloroplasts in the pods. However, Gu et al. (2017) showed that microorganisms in the curing added four novel vanillin biosynthetic steps, and thus play a role in vanillin biosynthesis. The fungus HI, isolated during the curing process may thus intervene in vanillin biosynthesis during curing. Further research is required to test this. Also, all tested fungi biotransform tyrosine, yielding potential vanillin

precursors. Endophytes tested here could thus participate, and contribute to the vanillin pathway.

As well as a potential vanillin precursor (**Figure 1**) *p*-hydroxybenzaldehyde contributes to vanilla flavor (Organoleptic property: vanilla, balsamic, sweet (Sigma-Aldrich 2014)). High concentrations of 4-hydroxybenzaldehyde glucoside are known to occur in mature *Vanilla* pods. The fungi tested here were involved in the production of *p*-hydroxybenzyl alcohol, which is abundant in green *Vanilla* pods. However, the biosynthesis of *p*-hydroxybenzaldehyde in different plant species, including *Vanilla*, remains partly unresolved (Gallage et al. 2014). The fungi tested here may significantly affect the *p*-hydroxybenzaldehyde pool.

Ideally, results here should be further confirmed in green pods and, in the case of HI, in pods during the curing process. However, obtaining and maintaining axenic mature *Vanilla* pod material is elaborate and will take at least four years before the axenic plants will carry pods. Moreover, our results concern the effect of co-culture of plants or calli with a single endophyte, but what happens in the plant and pods where probably several endophytes are present is difficult to predict. At this stage no definite conclusions can be drawn from the presented data concerning the role of endophytes in inducing the plant's vanillin biosynthesis. The most pertinent conclusion is that each endophyte has a specific effect on the plant and plant cells and the production of phenolics. However pods, axenic plants, calli from *Vanilla* differ with regards to vanillin synthesis in that only pods are known to contain vanillin precursors. Bringing pod endophytes with living *Vanilla* material together falls short of obtaining vanillin. Vanillin was obtained with the fungus HI in non-living *Vanilla* leaf material when fed with vanillin precursors (**Chapter 5**). The stage of development of the plant cells seems to be important as can be seen from the wide difference between young and old calli, which means that the degree of differentiation seems to play a role. Further research is needed to assess the extent to which observed changes are part of the normal response of *Vanilla* plants to infection with microorganisms. Elicitation of *Vanilla* callus cultures using biotic elicitors, such as methyl jasmonate or cell wall preparations of plant pathogens could be of interest to learn more about the effects of the infection of *Vanilla* plants with endophytes and the effects on the metabolic network in the plant and the fungus. Possibly labeling experiments could be performed to trace the flow from sugars to vanillin biosynthetic network, e.g. by growing calli on a labeled carbon source and eventually infect these calli with non-labeled fungi and determine the labeling in the various phenolics.

6.5 REFERENCES

Al-Jibouri A.M.J., Abed A.S., Ali A.J.A., Majeed D.M. (2016) Improvement of Phenols Production by Amino Acids in Callus Cultures of *Verbascum thapsus* L. American Journal of Plant Sciences, 7:84-91.

Aly A.H., Debbab A., Proksch P. (2011) Fungal endophytes: unique plant inhabitants with great promises. Applied Microbiology and Biotechnology, 90:1829-45.

Arnaldos T.L. (2001) Changes in phenol content during strawberry (*Fragaria×ananassa*, cv. Chandler) callus culture. Physiologia Plantarum, 113:315-22.

Bertoncelli D.J., Oliveira M.D.C., Passos A.I.D., Ariati A.C., Ortolan A.O., Witt E.D.P. (2014) Establishment and content of sugars and phenols in *Physalis* callus obtained from different explants and concentrations of bap and naa. Acta Scientiarum Biological Sciences, 36:27-33.

Chen W., Liu H, Wurihan G.Y., Card S.D., Ren A. (2017) The Advantages of Endophyte-Infected over Uninfected Tall Fescue in the Growth and Pathogen Resistance Are Counteracted by Elevated CO_2. Scientific Reports, 7:6952.

Currie A.F., Wearn J., Hodgson S., Wendt H., Broughton S., Jin L. (2014) Foliar Fungal Endophytes in Herbaceous Plants: A marriage of convenience? In: Verma V.C., Gange A.C. (eds.). Advances in Endophytic Research. Springer, New Delhi, pp.61-84.

Davidonis G., Knorr D. (1991) Callus formation and shoot regeneration in *Vanilla planifolia*. Food Biotechnology, 5:59-66.

Dutta D., Puzari K.C., Gogoi R., Dutta P. (2014) Endophytes: exploitation as a tool in plant protection. Brazilian archives of biology and technology, 57:621-9.

El-Naggar H.M. (2012) Phenylalanine ammonia-lyase (PAL) gene activity in response to proline and tyrosine in rosemary callus culture. African Journal of Biotechnology, 11:159-63.

Funk C., Brodelius P.E. (1990) Influence of Growth Regulators and an Elicitor on Phenylpropanoid Metabolism in Suspension Cultures of *Vanilla planifolia*. Phytochemistry, 29:845-8.

Gallage N.J., Jørgensen K., Janfelt C., Nielsen A.J.Z., Naake T., Dun´ski E., Dalsten L., Grisoni M., Møller B.L. (2018) The Intracellular Localization of the Vanillin Biosynthetic Machinery in Pods of *Vanilla planifolia*. Plant Cell Physiology 59:304–18.

Gallage N.J., Hansen E.H., Kannangara R., Olsen C.E., Motawia M.S., Jørgensen K., Holme I., Hebelstrup K., Grisoni M., Møller B.L. (2014) Vanillin formation from ferulic acid in *Vanilla planifolia* is catalysed by a single enzyme. Nature Communications, 5: 4037.

Garg N., Gupta H. (2010) Isolation and purification of fungal pathogen (*Macrophomina phaseolina*) induced chitinase from moth beans (*Phaseolus aconitifolius*). Journal of Pharmacy and Bioallied Sciences, 2:38–43.

Gu F., Chen Y., Hong Y., Fang Y., Tan L. (2017) Comparative metabolomics in vanilla pod and vanilla bean revealing the biosynthesis of vanillin during the curing process of vanilla. AMB Express 7:116.

Hansen E.H., Møller B.L., Kock G.R., Bünner C.M., Kristensen C., Jensen O.R., Okkels F.T., Olsen C.E., Motawia M.S., Hansen J. (2009) De Novo Biosynthesis of Vanillin in Fission Yeast (*Schizosaccharomyces pombe*) and Baker's Yeast (*Saccharomyces cerevisiae*). Applied and Environmental Microbiology, 75:2765-74.

Havkin-Frenkel D., Belanger F.C. (2007) Application of metabolic engineering to vanillin biosynthetic pathways in *Vanilla planifolia*. In: Verpoorte R., Alfermann A.W., Johnson T.S. (eds.). Applications of Plant Metabolic Engineering. Springer, Dordrecht, pp.175-96.

Khoyratty S., Dupont J., Lacoste S., Palama T.L., Choi Y.H., Kim H.K., Payet B., Grisoni M., Fouillaud M., Verpoorte R., Kodja H. (2015) Fungal endophytes of *Vanilla planifolia* across Réunion Island: isolation, distribution and biotransformation. BMC Plant Biology, 15:142.

Kikowska M., Kędziora I., Krawczyk A., Thiem B. (2015) Methyl jasmonate, yeast extract and sucrose stimulate phenolic acids accumulation in *Eryngium planum* L. shoot cultures. Acta Biochimica Polonica, 62:197-200.

Kogel K.H., Franken P., Hückelhoven R. (2006) Endophyte or parasite-what decides? Current Opinions in Plant Biology, 9:358-63.

Korthout H., Verpoorte R. (2007) Vanilla. In: Berger RG. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. Springer Science + Business Media. Leipzig, pp.203-17.

Lux-Endrich A., Treutter D., Feucht W. Plant Cell, (2000) Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. Tissue and Organ Culture, 60:15–21.

Modarres M., Bahabadi E.S., Yazdi M.E.T. (2018) Enhanced production of phenolic acids in cell suspension culture of *Salvia leriifolia* Benth. using growth regulators and sucrose. Cytotechnology, 70:741-50.

Molina-Montenegro M.A., Oses R., Torres-Diaz C., Atala C., Zurita-Silva A., Ruiz-Lara S. (2016) Root-endophytes improve the ecophysiological performance and production of an agricultural species under drought condition. AoB Plants, 27:plw062.

Nawrot – Chorabik K. (2013) The Use of Interactions in Dual Cultures in vitro to evaluate the Pathogenicity of Fungi and Susceptibility of Host Plant Genotypes. In: Petre M. (ed.) Environmental Biotechnology – New approaches and prospective applications. InTech, Rijeka, pp.287-301.

Ni J., Tao F., Du H., Xu P. (2015) Mimicking a natural pathway for de novo biosynthesis: natural vanillin production from accessible carbon sources. Scientific Reports, 5:13670.

Omezzine F., Ladhari A., Haouala R. (2014) Physiological and biochemical mechanisms of allelochemicals in aqueous extracts of diploid and mixoploid *Trigonella foenum-graecum* L. South African Journal of Botany, 93:167-78.

Palama T.L., Menard P., Fock I., Choi Y.H., Bourdon E., Govinden-Soulange J., Bahut M., Payet B., Verpoorte R., Kodja H. (2010) Shoot differentiation from protocorm callus cultures of *Vanilla planifolia* (Orchidaceae): proteomic and metabolic responses at early stage. BMC Plant Biology, 10:82.

Qin J., Gao Y., Liu H., Zhou Y., Ren A., Gao Y. (2016) Effect of Endophyte Infection and Clipping Treatment on Resistance and Tolerance of *Achnatherum Sibiricum*. Frontiers in Microbiology, 7: 1988.

Ramagnoli L.G., Knorr D. (1988) Effect of ferulic acid treatment on growth and flavor development of cultured *Vanilla planifolia* cells. Food Biotechnology, 2:93–104.

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

Spiering M.J., Greer D.H., Schmid J. (2006) Effects of the Fungal Endophyte, *Neotyphodium lolii*, on Net Photosynthesis and Growth Rates of Perennial Ryegrass (*Lolium perenne*) are Independent of In Planta Endophyte Concentration. Annals of Botany, 98:379-87.

Zhou J., Li X., Chen Y., Dai C. (2017) De novo Transcriptome Assembly of *Phomopsis liquidambari* Provides Insights into Genes Associated with Different Lifestyles in Rice (*Oryza sativa* L.). Frontiers in Plant Science, 8:121.

Chapter 6 - Supplementary Data

The supplementary data here consists of two sections. Section 1 concerns all measured amounts of metabolites detected in this work. Section 2 illustrates ^{1}HNMR spectra.

Supplementary data section 1: measured amounts of metabolites detected in this work

Table S1 – Recorded metabolite amount from ^{1}HNMR spectra from 15 days calli with and without (control) fungus infection.

Biological replicate number	Metabolite	Recorded amount in *Vanilla* 15 days calli after growth with fungus (X 10^{-5} mol g^{-1} DW)				
		Control (no fungal growth)	*Hypoxylon investiens*	*Diaporthe phaseolorum*	*Pestalotiopsis microspora*	*Fusarium oxysporum*
1	Sucrose	3.9	1	1.2	3.4	2
2	Sucrose	3.9	0.9	1	3.2	1.8
3	Sucrose	3.8	0.8	1.4	3	2.1
1	Glucose	2.7	1.3	1.5	2.0	2.2
2	Glucose	2.6	1.3	1.5	2.1	2.2
3	Glucose	2.7	1.3	1.5	2.0	2.1
1	Tyrosine	0.5	0.2	1.5	1	0.6
2	Tyrosine	0.4	0.2	1.9	1.1	0.7
3	Tyrosine	0.4	0.2	1.5	1	0.64
1	*p*-coumaric acid	0.1	1.4	0.1	0.05	0.03
2	*p*-coumaric acid	0.1	1.3	0.1	0.05	0.04
3	*p*-coumaric acid	0.1	1.4	0.1	0.06	0.04
1	*p*-hydroxy benzyl alcohol	0.5	0.3	0.7	1.1	1.5
2	*p*-hydroxy benzyl alcohol	0.5	0.5	0.5	1.4	1.8
3	*p*-hydroxy benzyl alcohol	0.6	0.5	0.5	1.2	1.6
1	Proline	0.06	0.3	0.1	0.1	0.3
2	Proline	0.07	0.2	0.1	0.2	0.2
3	Proline	0.04	0.3	0.1	0.2	0.2

Table S2 – Recorded metabolite amount from ^{1}HNMR spectra from 30 days calli with and without (control) fungus infection.

Biological replicate number	**Metabolite**	**Recorded amount in *Vanilla* 30 days calli after growth with fungus (X 10^{-5} mol g^{-1} DW)**				
		Control (no fungal growth)	***Hypoxylon investiens***	***Diaporthe phaseolorum***	***Pestalotiopsis microspora***	***Fusarium oxysporum***
1	Sucrose	8.4	5.8	5.4	4.1	3.7
2	Sucrose	8.3	5.6	5	3.9	3.6
3	Sucrose	8	6	5.5	4.2	3.6
1	Glucose	4.6	3	2.8	1.2	2.2
2	Glucose	4.2	2.9	2.7	1.1	2.2
3	Glucose	4.2	2.8	2.7	1.1	2.3
1	Tyrosine	0.7	0.3	2.6	1.7	1.0
2	Tyrosine	0.8	0.3	2	1.4	1.0
3	Tyrosine	0.9	0.4	1.4	1.1	1.1
1	*p*-coumaric acid	0.1	1.7	0.2	0.05	0.06
2	*p*-coumaric acid	0.2	1.7	0.1	0.06	0.06
3	*p*-coumaric acid	0.1	1.5	0.1	0.06	0.05
1	*p*-hydroxy benzyl alcohol	1.8	1.5	1.5	2.9	3.0
2	*p*-hydroxy benzyl alcohol	1.8	1.6	1.9	2.6	3.0
3	*p*-hydroxy benzyl alcohol	1.7	1.6	1.7	2.1	2.6
1	Proline	0.09	0.4	0.2	0.4	0.4
2	Proline	0.09	0.5	0.1	0.5	0.4
3	Proline	0.1	0.4	0.1	0.4	0.5

Table S3 – Recorded metabolite amount from ^{1}HNMR spectra from acclimatized plants with and without (control) fungus infection.

Biological replicate number	**Metabolite**	**Recorded amount in *Vanilla* acclimatized plants after growth with fungus (X 10^{-5} mol g^{-1} DW)**				
		Control (no fungal growth)	***Hypoxylon investiens***	***Diaporthe phaseolorum***	***Pestalotiopsis microspora***	***Fusarium oxysporum***
1	Sucrose	5.5	0.9	0.7	1.2	2.6
2	Sucrose	5.3	1.1	0.6	1.2	2.4

3	Sucrose	5.5	1.2	0.6	1.1	2.4
1	Glucose	0.9	0.9	1.0	0.9	0.3
2	Glucose	0.8	0.9	0.9	0.8	0.4
3	Glucose	0.8	0.9	0.9	0.8	0.4
1	Tyrosine	0.2	0.09	1.7	0.8	0.6
2	Tyrosine	0.2	0.1	1.2	0.6	0.4
3	Tyrosine	0.2	0.1	1.3	0.6	0.5
1	*p*-coumaric acid	0.09	1.1	0.06	0.03	0.01
2	*p*-coumaric acid	0.08	1	0.09	0.01	0.01
3	*p*-coumaric acid	0.08	0.8	0.09	0.02	0.02
1	*p*-hydroxy benzyl alcohol	0.6	0.7	0.4	1.5	1.7
2	*p*-hydroxy benzyl alcohol	0.2	0.7	0.7	0.8	1.0
3	*p*-hydroxy benzyl alcohol	0.5	0.4	0.5	1.2	1.3
1	Proline	0.02	0.1	0.05	0.1	0.1
2	Proline	0.03	0.1	0.05	0.09	0.1
3	Proline	0.02	0.1	0.07	0.1	0.1

Supplementary data section 2: [1]HNMR spectra from -0.5 to 10.0 ppm

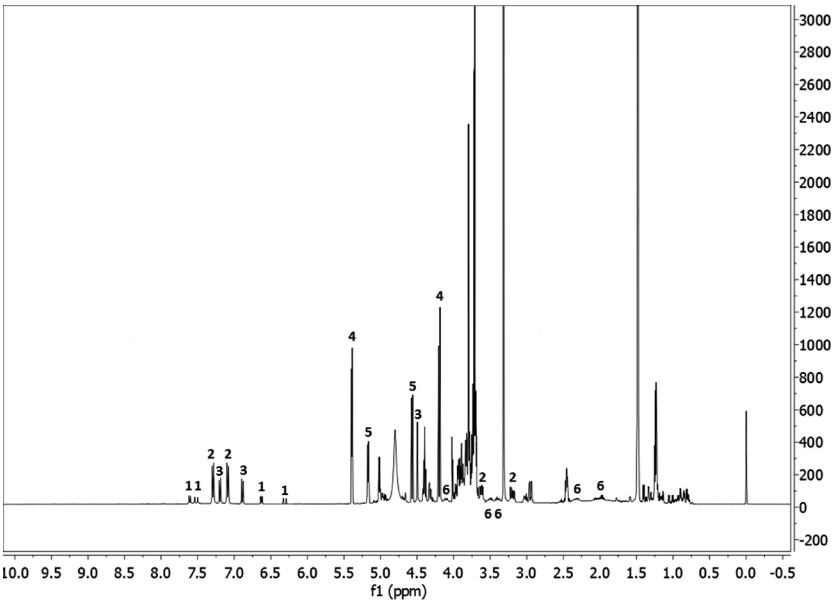

Figure S1 – [1]HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 15 days after culture on regeneration medium then cultured in liquid ¼ MS strength solution (without fungus – the control) for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

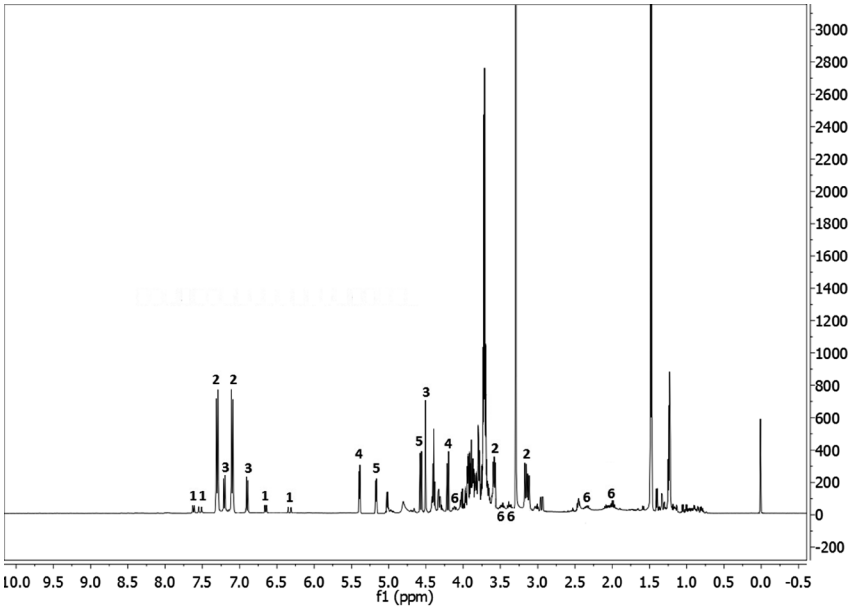

Figure S2 – [1]HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 15 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution

with *Diaporthe phaseolorum* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

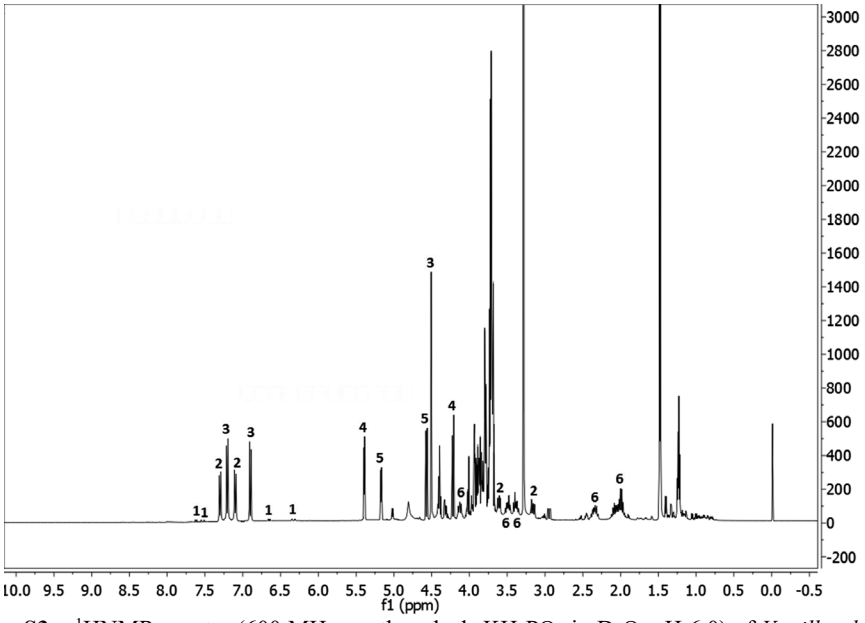

Figure S3 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 15 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution with *Fusarium oxysporum* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

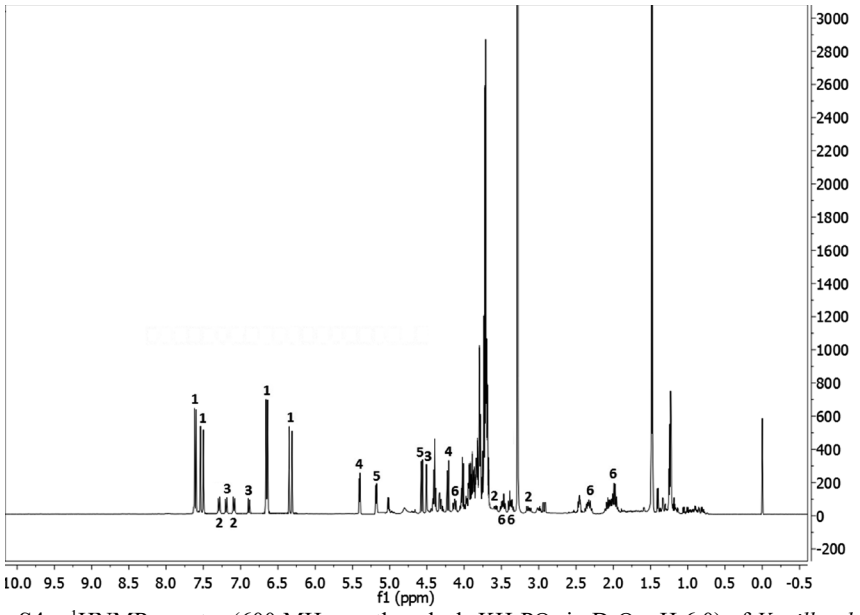

Figure S4 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 15 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution

with *Hypoxylon investiens* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

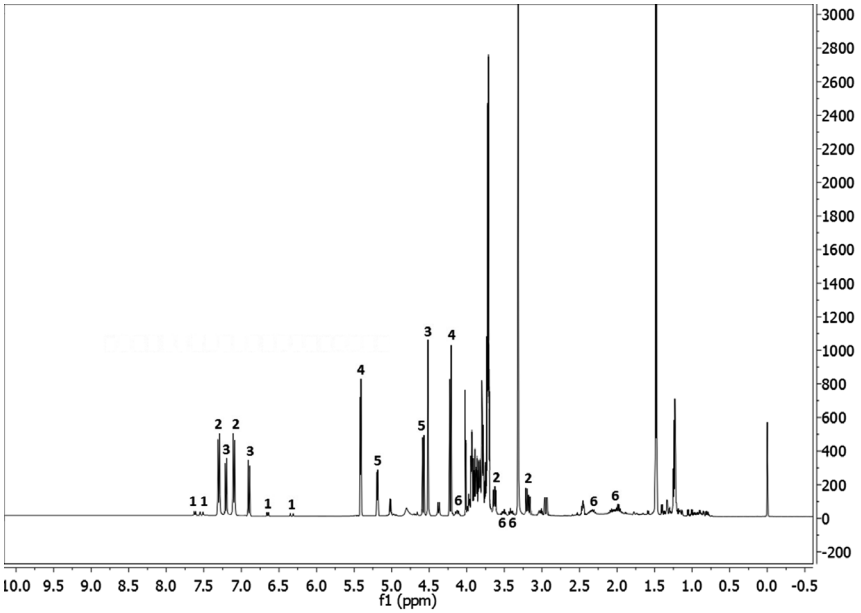

Figure S5 – ^{1}HNMR spectra (600 MHz, methanol- d$_4$-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 15 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution with *Pestalotiopsis microspora* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

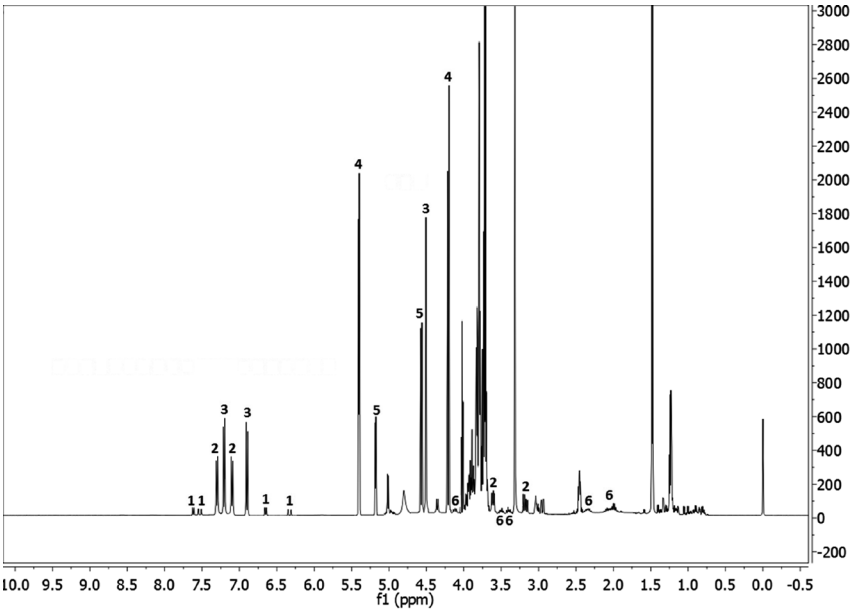

Figure S6 – ^{1}HNMR spectra (600 MHz, methanol- d$_4$-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 30 days after culture on regeneration medium then cultured in liquid ¼ MS strength solution

(without fungus – the control) for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

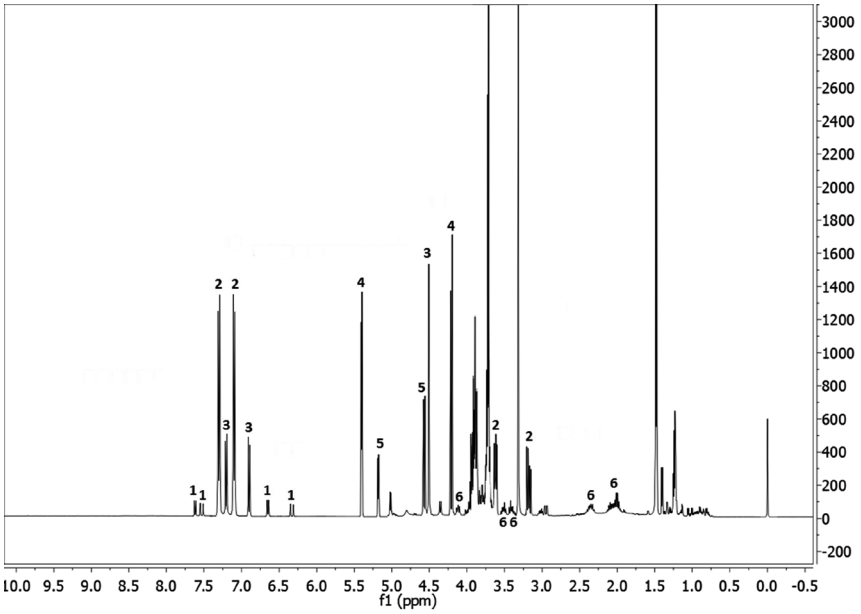

Figure S7 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 30 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution with *Diaporthe phaseolorum* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

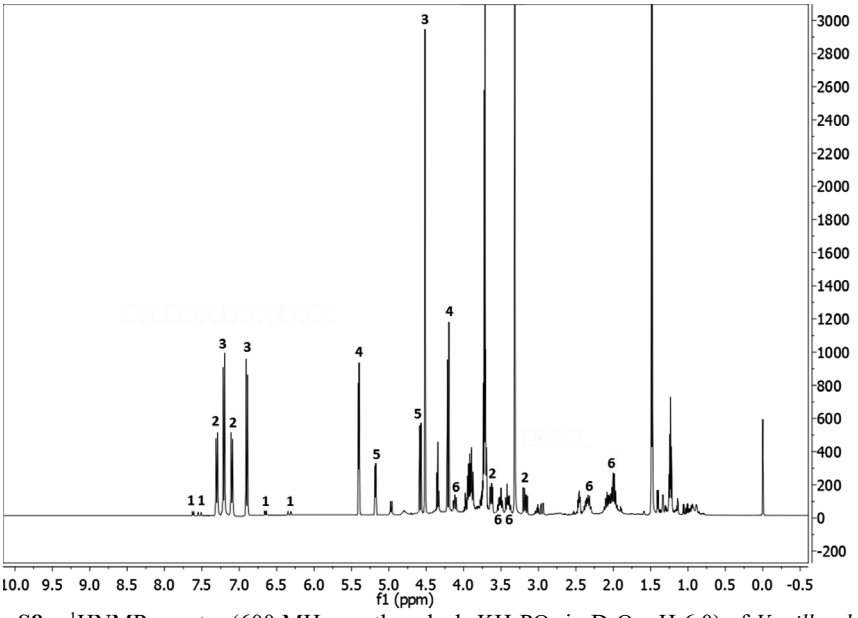

Figure S8 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 30 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution

with *Fusarium oxysporum* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

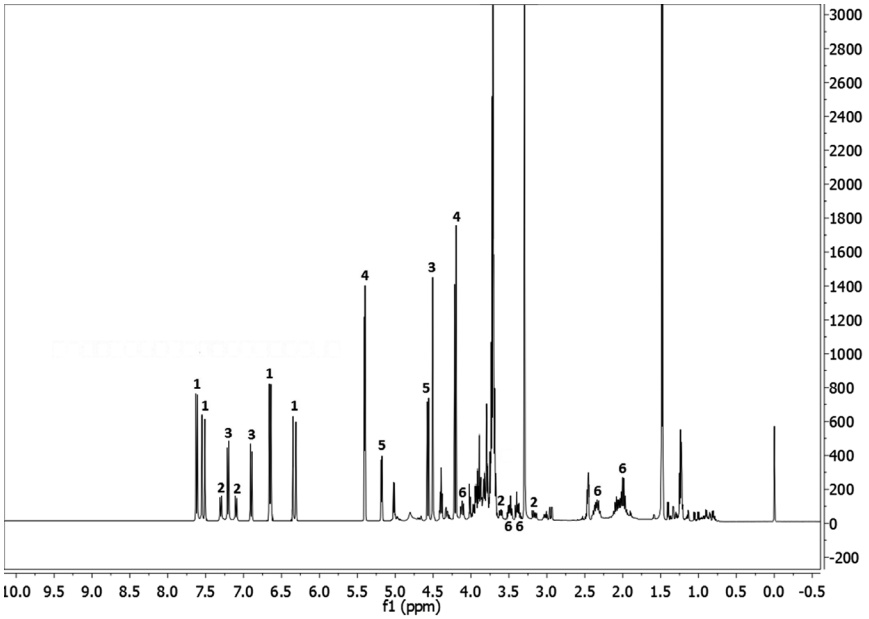

Figure S9 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 30 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution with *Hypoxylon investiens* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

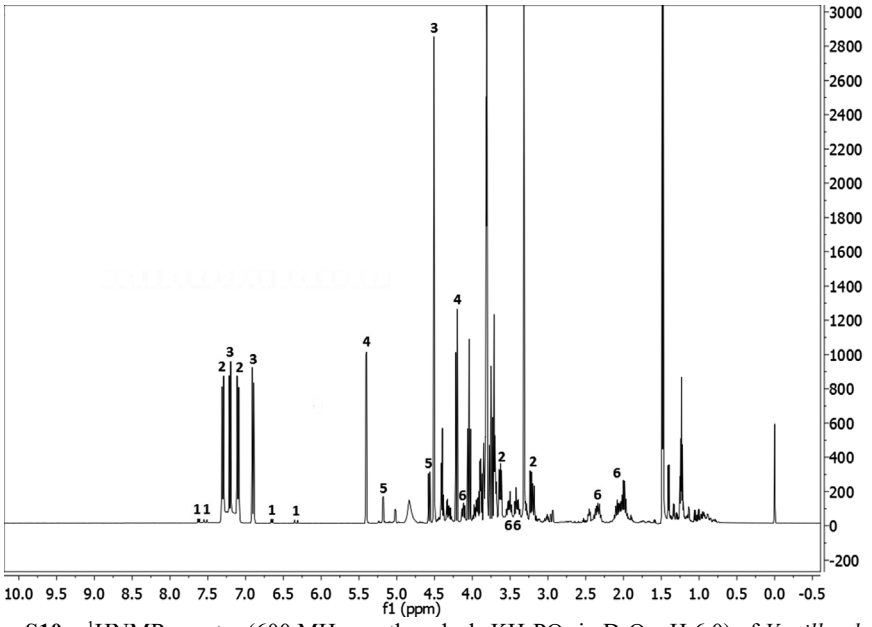

Figure S10 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* callus 30 days after culture on regeneration medium then co-cultured in liquid ¼ MS strength solution with *Pestalotiopsis microspora* for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

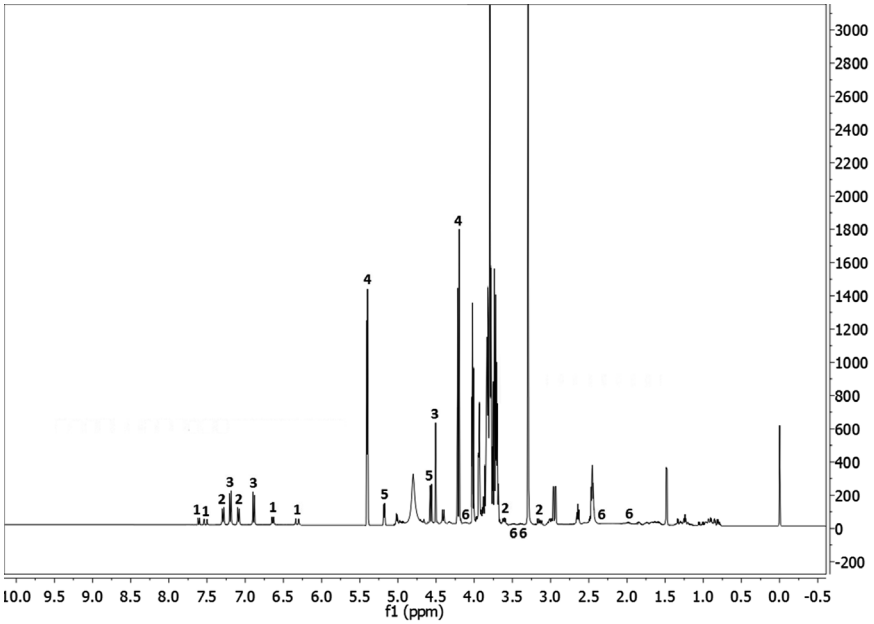

Figure S11 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* axenic plant grown for 3 months on sterile compost then submerged in inoculum solution without fungal spores (the control) returned again for growth on sterile compost for 5 days. Metabolites

detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

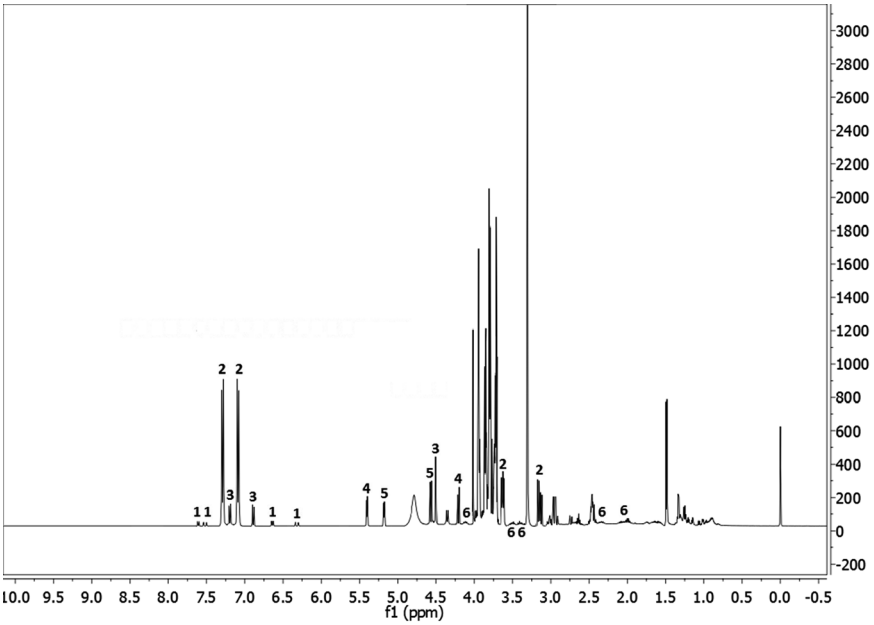

Figure S12 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* axenic plant grown for 3 months on sterile compost then submerged in inoculum solution with *Diaporthe phaseolorum* spores returned again for growth on sterile compost for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

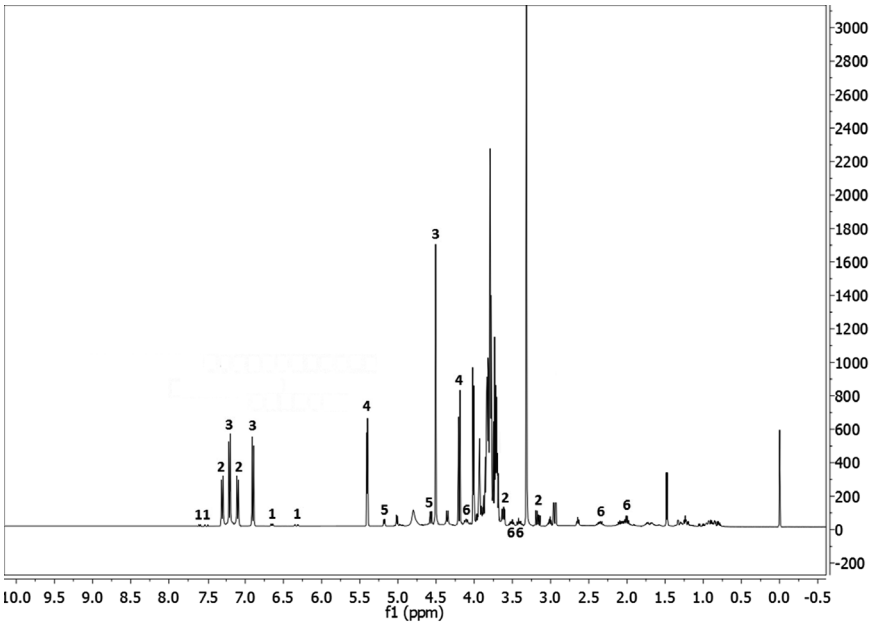

Figure S13 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* axenic plant grown for 3 months on sterile compost then submerged in inoculum solution with *Fusarium oxysporum* spores returned again for growth on sterile compost for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

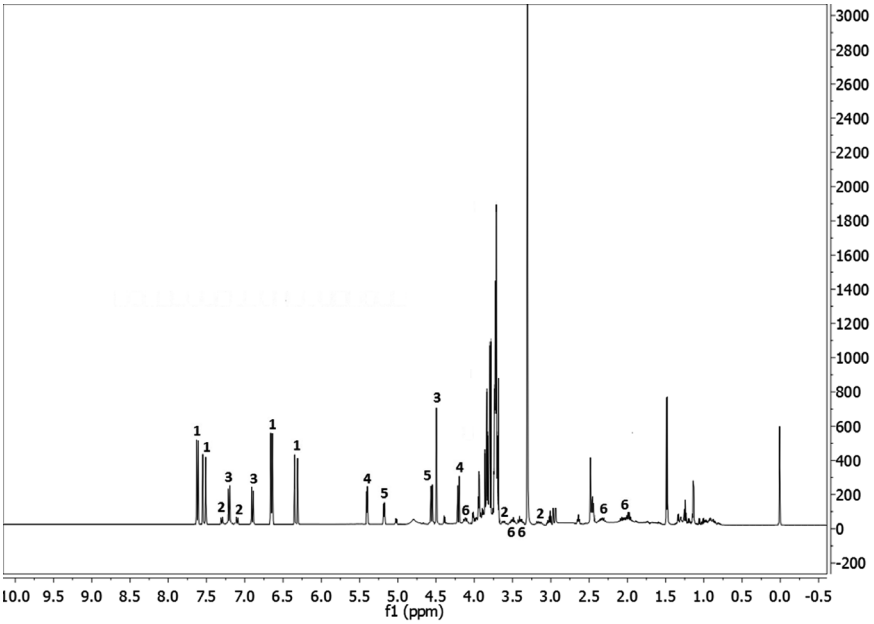

Figure S14 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* axenic plant grown for 3 months on sterile compost then submerged in inoculum solution with *Hypoxylon investiens* spores returned again for growth on sterile compost for 5 days. Metabolites

detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.

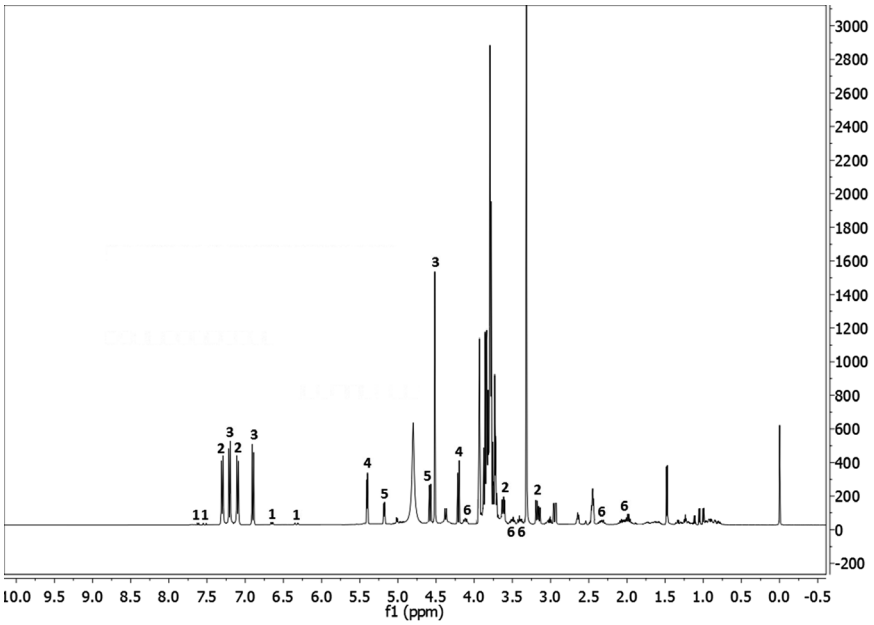

Figure S15 – ^{1}HNMR spectra (600 MHz, methanol- d_4-KH_2PO_4 in D_2O, pH 6.0) of *Vanilla planifolia* axenic plant grown for 3 months on sterile compost then submerged in inoculum solution with *Pestalotiopsis microspora* spores returned again for growth on sterile compost for 5 days. Metabolites detected in the media are: **1:** *p*-coumaric acid, **2:** tyrosine, **3:** *p*-hydroxybenzyl alcohol, **4:** sucrose, **5:** glucose, **6:** proline.