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

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Chapter 3: How Fungal endophytes of green *Vanilla planifolia* pods modify flavor metabolite contents in green *V. planifolia* pod agar media

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ABSTRACT

The biosynthesis of vanilla flavor metabolites in *Vanilla* pods is still a matter of debate. Vanilla flavor depends on a large number of compounds. *Vanilla* plants, like all other plants, contain fungal endophyte species. Endophytes are known as being involved in the biosynthesis of secondary metabolites in plants. This study aimed to investigate the potential involvement of *Vanilla* endophytes in the vanilla flavor formation in pods. Endophytes were grown on a medium containing sterilized *Vanilla* green pod material and analyzed with ^{1}HNMR metabolomics and a vanilla flavor compound targeted HPLC method. Endophytes were isolated from *Vanilla* leaves of different age and pods by microbiological methods, and identified at the species level by molecular tools. To gain insight in the overall endophyte effects on vanilla flavor, fungal endophyte species diversity was assessed from plants from different regions of Reunion Island. Thirteen species, i.e. Molecular Organizational Taxonomic Units (MOTUs) were isolated from pods (62% of all 21 isolated endophyte MOTUs). The spent growth media after the growth of pod, or leaf endophytes or a *Vanilla* pathogen showed clear differences in ^{1}HNMR metabolomics analysis, compared to non-spent media (control). In all cases, glucovanillin levels were reduced to a very low level or could not be detected anymore. With the pathogen *Fusarium oxysporum*, most of the vanillin and its glucoside and vanillyl alcohol were catabolized from the spent medium after fungal growth. The endophyte *Pestalotiopsis microspora* converted most of the vanillin into vanillyl alcohol. This compound is known to have a balsamic flavor, a flavor typical for the Bourbon-type *Vanilla* pods. The spent medium after *Diaporthe phaseolorum* growth contained both vanillin and its alcohol in quite high levels.

All isolated endophytic fungi metabolize compounds from *Vanilla* green pods present in the growth medium in different ways. The next step should be a more in depth study of the *de-novo* and biotransformation capacities of the isolated endophytes for Vanilla flavor related compounds, to establish their role in the vanillin biosynthetic network.

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3.1 BACKGROUND

The flavor of *Vanilla* pods from Reunion Island is characterized by a distinct intense balsamic flavor when compared to pods from other regions of the world (Ranadive 2011). It is hence expected that Reunion Island *Vanilla* contains higher amounts of metabolites associated with balsamic flavors, e.g. vanillyl alcohol (Kim et al. 1998) known to be present in *Vanilla* pods (Hoffman and Zapf 2011). Such differences in flavors are sometimes subtle and at other times more pronounced. Several factors could contribute to these differences. The most obvious is in genetic differences, but *Vanilla* is usually propagated vegetatively. Climatic and soil conditions are other important factors. More recently, fungal endophytes that co-exist within the plant (Souza et al. 2011) have also emerged as a possible cause of different metabolomes of individual plants of one species (Ludwig-Müller 2015).

The vanillin biosynthetic network, especially with regards to vanillin precursors that also contribute to flavor, has not yet been fully elucidated (Kundu 2017, Yang et al. 2017, Korthout and Verpoorte, 2007). Contradictory results were reported in studies using different *Vanilla* materials (plants, pods, cell suspensions, callus cultures). Moreover, there is no consensus about the enzymes that catalyze various reactions in the vanillin biosynthesis (Yang et al. 2017). Interestingly, the *de-novo* production of vanillin in a genetically-engineered microorganism, using genes encoding various known enzymes that catalyze the formation of vanillin, has been reported (Ni et al. 2015). Furthermore, there are cases where variable results were obtained with an enzyme supposed to be acting in the vanillin biosynthetic pathway. Gallage et al. (2014) reported an enzyme that catalyzes the formation of vanillin from ferulic acid, but did not catalyze the formation of *p*-hydroxybenzaldehyde from *p*-coumaric acid. Contrary to this fact, Yang et al. (2017) stated that the same enzyme, the same genetic sequence, is unable to synthesize vanillin from ferulic acid but catalyzes *p*-hydroxybenzaldehyde synthesis from *p*-coumaric acid.

The intracellular localization of the vanillin biosynthetic machinery in pods is also a matter of dispute. Gallage et al. (2018) showed that the whole biosynthetic pathway occurs in re-differentiated chloroplasts (phenyloplasts). Havkin-Frenkel and Dixon, however, showed this site to be hairy secretory tissues (trichomes) and vanillin glucosides then accumulate around seeds (Joel et al. 2003). In contrast, vanillin and its glucosides were shown to be absent from seeds (Odoux and Brillouet 2009).

A reason for the contradictory results for vanillin biosynthesis as reported in literature could be different fungal endophyte species residing in *Vanilla* plants and/or in pods before and during the curing process. Such endophytes could be involved, at least partially, in the vanillin biosynthetic network. So far no endophytes have been isolated from *Vanilla* plants, but several microorganisms have been reported to convert precursors into vanillin (Labuda 2011), e.g. the biotransformation of ferulic acid to vanillin by *Pestalotia palmarum* (Rahouti et al. 1989). Furthermore, vanilla aroma does not consist of only pure vanillin; it also includes about 250 other compounds that contribute to its flavor notes (Sharp et al. 2012).

Vanilla planifolia is a slow-growing plant. Hence, the oldest leaf rank 15, for instance, had more time to be exposed to endophytes than the youngest leaf rank 1. The time of exposure and the difference in metabolic composition across leaf ranks in *Vanilla* can influence the endophyte composition (Palama et al. 2010). It is a well-known fact that many secondary

metabolites are transported from the site of biosynthesis to another site in the plant for storage. It is therefore of interest to study endophytes in both mature pods and in leaves of different ages.

Growth rate varies across fungal species. Radial growth rate ($cm.day^{-1}$) is a convenient nondestructive method to assess fungal growth on a solid medium over time (Miyashira et al. 2010).The maximum biotransformation of metabolites present in the growth medium is ensured, by allowing fungi to completely cover the solid medium surface. Based on experiments, the growth period for slowly growing fungal cultures on media is a minimum of 4 weeks (Bosshard 2011, Morris et al. 1996). However, some authors suggest a minimum of 5 days (Bosshard 2011). Moreover, the quantity and diversity of secondary metabolites produced by fungi vary depending on the culture medium composition (VanderMolen et al. 2013). Hence, the medium used is of great importance; for example, the fungus *Penicillium commune* grown on three different liquid media and a solid medium, produced different secondary metabolites (Gao et al. 2017). Proper standardization of the experiments is thus required. We chose the use of solid media because in plants they are also in a solid phase, with a time frame based on full fungal coverage of the petri dish. For isolation of endophytes normal growth media (potato dextrose agar) were used. To study the biotransformation and biosynthesis of vanilla flavor compounds, a solid agar medium only containing freeze-dried, powdered, mature green *Vanilla* pod material as the sole nutrient, was used to grow the endophytes.

The aim of the present study was to isolate and identify endophytes from *Vanilla* plants and pods and study their effect on metabolites from the vanillin biosynthesis pathway, and possibly identify regional differences in endophyte populations. This translates to the following tasks:

- Isolation and identification of endophytes from different plants collected from different regions of Reunion Island. For this, leaves from different leaf ranks and 8 month old mature pods were collected from three different plants per region.

- Biotransformation of green pod metabolites by endophytes when growing the endophytes on a medium containing *Vanilla* mature green pod material, followed by NMR-based metabolomics of the medium.

- When the endophytes are grown on a normal growth medium, the metabolites they produce can be identified. As the character of the formed compounds is unknown, a non-targeted NMR-based metabolomic approach was used for the analysis of the media extracts.

Apart from the isolated endophytic fungi, (Souza et al. 2011) the *Vanilla* pathogen *Fusarium oxysporum* f.sp. *vanillae* was also studied for its biotransformation abilities. This pathogen was isolated from infected roots of *Vanilla* cuttings.

3.2 MATERIALS AND METHODS

3.2.1 Overview of experiments conducted

The work in this chapter is divided into two steps. **Step 1** – Isolation of fungi from pods, and leaf ranks 1, 3, 5, 15 followed by identification of the fungi. **Step 2** - Individual fungi were cultured for 30 days on *Vanilla* green pod agar (GPA) medium followed by metabolic profiling of the spent medium (**Figure 1**).

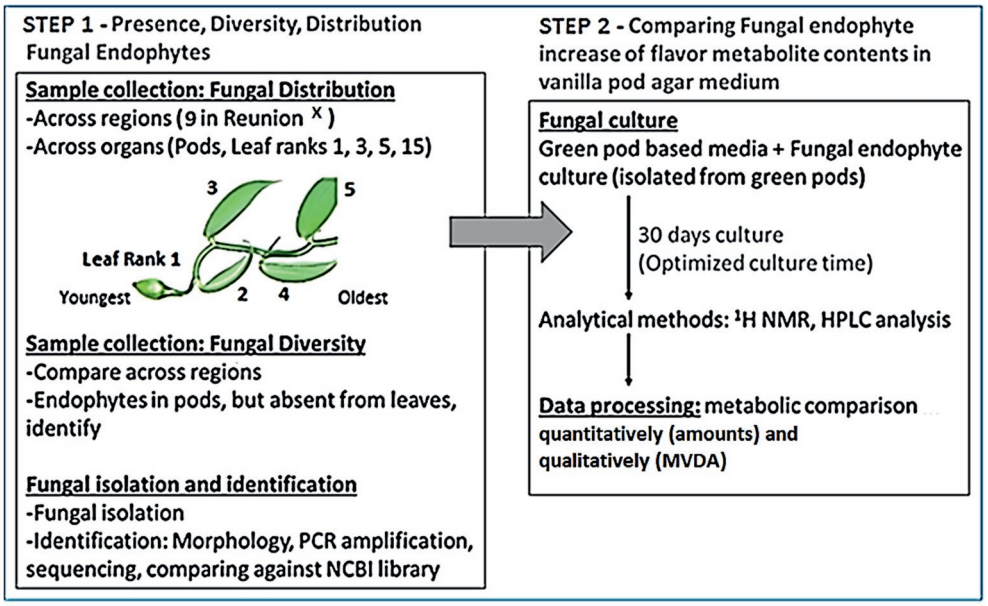

Figure 1 – Work flow to study *Vanilla* endophytes: experiments and analysis conducted. The leaf ranks start from leaf 1 to the oldest leaf 15 on the same branch. Illustration shows leaf ranks 1 to 5. X: Site of collection on Reunion Island: St. Andre (at two plantations), St. Anne (at two plantations), St. Rose, Bois Blanc, Takamaka, Mare Longue, Basse vallée.

3.2.2 Sample collection

To assess fungal diversity, representative samples of leaves and pods of *Vanilla planifolia* plants were collected (**Supplementary data file for collection details, Tables S1-S7**). Fifteen leaves (five replicates from three different plants from different regions) at ranks 1 (the youngest in each sampled plant, see **Figure 1**), 3, 5 and 15 (the oldest in the same plant) and 15 mature pods (8 months mature pods after pollination) from the same plants were harvested from nine *Vanilla* producing sites across Reunion Island (two sites in St. André and St. Anne; one site in St. Rose, Bois Blanc, Takamaka, Mare longue and Basse vallée). One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). The 5 replicates are from different leaves, each on different ranks of the same plant.

3.2.3 Fungal endophyte and pathogen isolation

Within 3 h after harvest, collected samples were first washed with running tap water for 15 min. The washed organs were then dipped for 10 s in 95% alcohol and thoroughly flamed on all sides for 3 s. The latter step was adapted from a protocol of endophyte isolation in *Theobroma cacao* pods by Crozier et al. (2006). To confirm that the surface of the sample

was sterile, the surface was touched onto sterile potato dextrose agar (PDA, Conda-Pronadisa, Madrid, Spain) medium in petri dishes using sterile forceps. Samples with sterile surfaces have no fungal growth on the medium. Then the sample was cut into 0.5 cm x 0.5 cm pieces, placed onto an agar plate (15 g Plant Agar, Duchefa Biochemie, Haarlem, the Netherlands, in 1 L distilled water) and incubated at 25 °C. Petri dishes were checked regularly for growth of fungi for a period of 4 weeks. Fungi that grew from the sterile samples were transferred to a PDA plate, by taking actively growing hyphal tips. Single-spore cultures were prepared in case of sporulating fungi, to ensure the purity of the fungus. A strongly diluted spore suspension (serial dilution, 1:10 for each dilution) was prepared and smeared on a malt agar plate to allow single colonies to develop. Isolates have been deposited at the Fungal Culture Collection of the Muséum National d'Histoire Naturelle (Paris, France). The pathogen *Fusarium oxysporum* f.sp. *vanillae* was isolated with the same procedure as for endophytes except the sample used was from infected roots.

3.2.4 DNA extraction, PCR and sequencing

Fungi were grown on Malt Extract Agar (MA) for 4 weeks. Morphotypes were categorized based on macroscopic and microscopic characteristics of the cultures. Fungal mycelium was collected from representatives of each morphotype grown on MA and genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify rDNA regions in a total volume of 25 µl, with 12.5 µl (50–100 ng) of DNA template, 0.625 units of Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen, Calif., USA), 5 µl of PCR buffer, 0.5 µl of 25 mM dNTPs (Eurogentec, Seraing, Belgium) and 1 µl of each 10 µM primer per reaction tube. The primer sets ITS 4/ITS 5 (White et al. 1990) and LROR/LR6 (Vilgalys, Duke University, Durham, NC, USA) were used to amplify respectively ITS rDNA and the 5' end of the 28 S rDNA from a set of fungi. The elongation factor (EF) oligonucleotide primer set EF-1H and EF-2 T (O'Donnell et al. 1998) was used to amplify a 700 bp portion of the EF-1α gene from *Fusarium* spp. Amplifications were performed on a BioRad DNA engine thermal cycler with the following parameters: a 4 min step at 94 °C, followed by 30 cycles (10 cycles for β-tubulin primer sets (Einax and Voigt 2003)) of 30 s at 94 °C, 30 s at an annealing temperature of either 55 °C (for ITS 4/ITS 5, EF-1H/EF-2 T and β-tubulin primer sets) or 50 °C (for LROR/LR6 primers) and 40 s at 72 °C and then a final 10 min extension step at 72 °C. Additionally, for β-tubulin primer sets only: Denaturation at 94 °C (15 s), annealing at 45–65 °C (30 s), extension at 72 °C (48 s for first round and an additional 3 s for every additional round, for a total of 20 rounds) followed by 72 °C for 6 min. PCR products were purified and then sequenced by Genoscope (Évry, France), on both strands to confirm the accuracy of each sequence. The DNA sequences were assembled using CodonCode Aligner v. 3.7.1. (CodonCode Corporation, MA, USA), checked by visual inspection and edited if necessary. Sequences were deposited in GenBank (http://www.ncbi.nlm.-nih.gov/genbank/).

3.2.5 Taxonomic classification of endophytes

The isolated endophytes were further identified as Fungal Molecular Organizational Taxonomic Units (MOTU) using internal transcribed spacer (ITS) sequences. When a more detailed identification at the species level was not possible with ITS sequences, elongation factor (EF-1α) sequences (*Fusarium* spp. isolates), β-tubulin (*Aspergillus niger*) and 28S sequences were used with the BLAST option at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The best hits were carefully examined to attribute species names. Large-subunit ribosomal RNA

gene (LSU rDNA) sequences have been used for phylogenetic analysis. Sequences were then aligned using ClustalW on MEGA software version 5.10 (Tamura et al. 2011). Alignments included reference sequences from NCBI (www.ncbi.nlm.nih.gov) and the final alignments were edited manually.

3.2.6 Cultivation of endophytes, optimization of incubation time and pathogen for metabolomics analysis

For each experiment, one fungus was grown on sterile *Vanilla* green pod agar medium. Three replicates were made per fungal species (n=3). The control was sterile *Vanilla* green pod agar medium without fungal growth. Fungi which were selected for the experiments are fungal endophytes from pods, leaves and one fungal pathogen. Eight months old green *Vanilla planifolia* pods (accession 13B1 from St. Andre) were freeze-dried and crushed for 3 min into a fine powder (particle size: ~ 5 µm) with a MM 400 mixer mill (Retsch, Haan, Germany). Fifteen g of dried powdered pod material were added to 15 g of agar (Duchefa Biochemie) in 1 L of distilled water. To obtain homogenous media, all media for the experiments were made on the same day. Thirty ml was filled into a petri dish (3 cm high, 9 cm in diameter). All were autoclaved and stored at 4 °C in a refrigerator. For each fungal species, three replicates were made. The time needed to cover completely a 9 cm petri dish was considered as the optimal incubation period for the fungus. The cultures were incubated at 28 ± 1°C for the optimized number of days (30) at 45% relative humidity. The fungi were then scraped off the surface of the media, and the remaining media were freeze dried. The mycelial mass obtained was also freeze dried separately.

3.2.7 ^{1}HNMR procedures, quantification and statistical analysis

^{1}HNMR analyses were performed after dissolving 50 mg of the ground freeze dried pod agar media or mycelium in 1.5 mL of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethylsilylpropionic acid sodium salt (TMSP, w/w) and CD_3OD -d_4 (1:1). The mixture was mixed at room temperature for 1 min, ultrasonicated for 15 min, and centrifuged at 13,000 rpm for 10 min. An aliquot of 0.8 mL was used for NMR analysis. ^{1}HNMR spectra were recorded at 25 °C on a 600 MHz Bruker AV 600 (Bruker, Karlsruhe, Germany) spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600.13 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. All NMR parameters were the same as those used by Kim et al. (2010). The resulting spectra were manually phased and baseline corrected and calibrated to TMSP at 0.0 ppm, using MestReNova software (v. 8.0.2, Mestrelab Research S.L, Santiago de Compostela, Spain). Compounds were identified based on the results of previous studies (Palama et al. 2009, Yamaji et al. 2015) or from reference compound measurements. The ^{1}HNMR spectra were automatically reduced to ASCII files. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) 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 MestReNova software (v. 8.0.2, Mestrelab Research S.L, Santiago de Compostela, Spain) with scaling on total intensity. A principal component analysis (PCA) was performed with SIMCA-P software (v. 13.0, Umetrics, Umeå, Sweden) using scaling based on Pareto method.
Metabolite identification was based on chemical shifts and coupling constants references for the metabolites as shown in **Table 1**.

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

Compound	Characteristic signals
Sucrose	δ 5.40 (1 H, d, *J* = 3.6 Hz), δ 4.17 (1 H, d, *J* = 7.8 Hz)
Glucoside A	δ 7.28 (2 H, d, *J* = 9.5 Hz), δ 7.20 (2 H, d, *J* = 9.5 Hz), δ 7.11 (4 H d, *J* = 9.5 Hz), δ 5.03 (1 H, d, *J* = 7.8 Hz), δ 4.67 (1 H, s), δ 4.39 (1 H, s), δ 4.37 (1 H, s), δ 2.20 (1 H, m), 0.92 (1 H, d, *J* = 7.0 Hz), δ 0.86 (1 H, d, *J* = 7.0 Hz)
Glucoside B	δ 1.90 (1 H, m), δ 1.35 (1 H, m), δ 1.10 (1 H, m), δ 0.84 (1 H, d, *J* = 7.0 Hz), δ 0.77 (1 H, t, *J* = 7.5 Hz)
p-hydroxybenzoic acid	δ 7.80 (2 H, d, *J* = 8.8 Hz), δ 6.90 (2 H, d, *J* = 8.8 Hz)
p-hydroxybenzaldehyde	δ 9.75 (1 H, s), 7.85 (2 H, d, *J* = 8.5 Hz), 7.04 (2 H, d, *J* = 8.5 Hz)
p-hydroxybenzaldehyde glucoside	δ 9.84 (1 H, s), δ 7.94 (2 H, d, *J* = 9.0 Hz), δ 7.29 (2 H, d, *J* = 9.0 Hz), δ 5.01 (1 H, d, *J* = 7.8 Hz)
Vanillyl alcohol	δ 6.99 (1 H, d, *J* = 1.5 Hz), δ 6.84 (1 H, d, *J* = 7.5 Hz), δ 6.55 (1 H, dd, *J* = , 1.5, 7.7 Hz), δ 4.52 (2 H, s), δ 3.86 (3 H, s)
Glucovanillin	δ 9.82 (1 H, s), 7.62 (1 H, dd, *J* = 8.5, 2.0 Hz), 7.57 (1 H, d, *J* = 2.0 Hz), 7.34 (1 H, d, *J* = 8.5 Hz), 5.19 (1 H, d, *J* = 7.8 Hz), 3.95 (3 H, s)
Vanillin	δ 9.73 (1 H, s), 7.52 (1 H, dd, *J* = 8.5, 2.0 Hz), δ 7.49 (1 H, d, *J* = 2.0 Hz), δ 7.04 (1 H, d, *J* = 8.5 Hz), δ 3.93 (3 H, s)
Acetic acid	δ 1.91 (3 H, s)
Homocitric acid	δ 3.01 (1H, d, *J* = 16.0 Hz)), δ 2.80 (1H, d, *J* = 16.0 Hz), δ 2.64 (2H, dd, *J* = 9.5, 7.5 Hz), δ 2.41 (2H, dd, *J* = 9.5, 7.5 Hz)

The amounts of metabolites were calculated using the ^{1}HNMR equation as in Turczan and Medwick (1977) as follows: nx = ny (Ix / Iy) (Ny / Nx) where nx : Number of moles of unknown; ny : Number of moles of TMSP; Nx : Number of protons of unknown signal; Ny : Number of protons of TMSP signal; Ix : Integration of peak of unknown and Iy : Integration of peak of TMSP (9 protons).

To find if the means of more than two independent groups differ significantly, a two-way ANOVA was performed using IBM SPSS Statistics for Windows, Version 24.0. (IBM Corp, Armonk, NY, USA). In SPSS, one dependent variable (continuous factor) was the metabolite amount in Green Pod Agar Medium after fungal growth (µmol.g^{-1} DW medium). Two independent categorical variables (fixed factors) were defined as fungal species and metabolite type.The null hypothesis is rejected for a p-value < 0.05. A post hoc comparison of the mean scores, using Tukey's HSD test, was calculated.

3.2.8 HPLC-DAD analysis

Freeze-dried pod agar medium (50 mg) was transferred into a 2 ml microtube. A volume of 1.5 ml of MeOH-Water (1:1) was added to the samples. The mixture was mixed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13,000 rpm for 10 min. The supernatant was analyzed with HPLC. The HPLC system was an Agilent CPL 1100 series (Massy, France) equipped with LC Chemstation software, degasser G1322A, binary pump G1312A, autosampler G1313A, thermostated column oven G1316A and diode array detection system G1315B to monitor at all wavelengths from 200 to 400 nm. For the column, a LiChrospher 100 RP-18 (4.6 X 250 mm, 5 µm, 100 Å (10 nm), Merck, Darmstadt, Germany), protected with a guard column LiChroCART 4–4 (Merck), was used at 35 °C. Gradient elution was performed with solution A: HPLC grade water:acetic acid

(0.1%) (pH 3.3), and solution B: HPLC grade MeOH-H_2O(1:9) with 0.1% acetic acid (pH 3.3), delivered at a flow rate of 1.0 mL/min (**Table 2**).

Table 2 - Solution gradient over time.

Time (min.)	%B
0	70
16	67
30	0
35	100
40 (end)	100

The injection volume for the extract was 30 µl. Detection was made by ultraviolet diode array detector (UV-DAD). The wavelength of detection was set at 280 nm. An HPLC library was made with *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillyl alcohol, vanillin, glucovanillin. HPLC quantification was performed for these metabolites using calibration curves.

3.3 RESULTS AND DISCUSSION

3.3.1 Endophyte isolation and identification

To establish a connection between *V. planifolia* fungal endophytes and their potential ability to affect vanilla flavor metabolite content, endophytes were cultured on media containing green *Vanilla* pod material. To avoid microbial contamination from the pod material, the media were autoclaved before use. Though endophytic fungi do not grow on the surface of *Vanilla* pods on plants, they grow profusely on the surface of *Vanilla* pod agar medium. Visible mycelial growth, on the plant surface has been observed for some endophytes with latent pathogenic traits (*Epichloe typhina* in grasses), while in case of pathogens, like in this study, *Fusarium oxysporum* f.sp. *vanillae,* can grow abundantly on the surface of infected living pods. In total, 23 fungal endophyte strains, and one pathogen, were isolated and identified (**Table 3**).

Table 3 – Identification of fungal endophytes isolated from *Vanillla* leaves and pods. The endophytes and one pathogen (*F. oxysporum* f.sp. *vanillae*) were identified on the basis of internal DNA sequences in different Molecular Organizational Taxonomic Units (MOTU), using NCBI BLAST of 28S, ITS rDNA, EF-1α or β-tubulin sequences.

MOTU number	Fungal species
MOTU1	*Fusarium proliferatum*
MOTU3	*Fusarium oxysporum* (Endophyte)
MOTU4	*Acremonium implicatum*
MOTU5	*Purpureocillium lilacinum*
MOTU6	*Phomopsis phyllanthicola*
MOTU7	*Diaporthe phaseolorum*
MOTU8	*Nemania bipapillata*
MOTU9	*Xylaria sp.*
MOTU10	*Pestalotiopsis microspora*
MOTU11	*Colletotrichum gloeosporioides*
MOTU12	*Colletotrichum sp2.*
MOTU13	*Nigrospora sp1.*
MOTU14	*Nigrospora sp 2.*

MOTU16	*Botryosphaeria ribis*
MOTU17	*Guignardia mangiferae*
MOTU18	*Mycosphaerella marksii*
MOTU19	*Penicillium citrinum*
MOTU20	*Aspergillus fumigatus*
MOTU21	*Sarcosomataceae spp.*
MOTU22	*Perenniporia nanlingensis*
MOTU23	*Cunninghamella blakesleana*
MOTU24	*Fusarium oxysporum* f.sp. *vanilla* (Pathogen)

From the results it is clear that most isolations are reproducible, as from 15 replicate materials, most of them yielded the same endophytes. To identify fungal endophytes (MOTUs) that occur only in pods, a comparison between MOTUs isolated from pods and MOTUs from leaves was made (**Table 4**). Specificity of MOTUs for different plant parts is commonly observed in endophyte-plant interactions (Wearn et al. 2012). The reason for this specificity in colonization of the host by different endophyte species is due to differences in the physiology of the plant parts.

Table 4 – Summary of results of Supplementary data section: Endophyte isolation frequencies from different plant parts, collected from different sites. Leaves Rank 1 youngest, rank 15 oldest (**Figure 1 illustration**). MOTUs in bold and underlined are those isolated from pods but not from leaves. Endophytes which were not isolated can either mean there were no fungal endophytes in the sampled organ or there may be fungal endophytes in the sampled organ but are not isolable under laboratory conditions.

Organs	**number of times isolated x isolated MOTUs codes**		**Total per region (MOTUs may repeat across region)**
	Pods	**Leaf Ranks 1, 3, 5, 15**	
Region: St. Andre	14x MOTU1	39x MOTU1	7 MOTUs
	12x MOTU7	7x MOTU9	
	14x MOTU11	11x MOTU12	124 isolated fungi from St. Andre
Site 1		13x MOTU13	
Site 2		14x MOTU19	
Region: St. Anne	6x MOTU1	16x MOTU1	6 MOTUs
	9x MOTU3	9x MOTU5	
	13x MOTU14		77 isolated fungi from St. Anne
Site 1	**14x MOTU20**		
Site 2	**10x MOTU23**		
Region: St. Rose	13x MOTU1	15x MOTU1	5 MOTUs
	12x MOTU4		
	13x MOTU8		81 isolated fungi from St. Rose
	13x MOTU10		
	15x MOTU16		
Region: Bois Blanc	9x MOTU1	None	1 MOTUs
			9 isolated fungi from Bois Blanc
Region: Takamaka	11x MOTU1	14x MOTU1	2 MOTUs
		13x MOTU21	
			38 isolated fungi from Takamaka
Region: Mare Longue	9x MOTU1	7x MOTU1	5 MOTUs
	11x MOTU6	14x MOTU17	
	11x MOTU18	8x MOTU22	60 isolated fungi from

			Mare Longue
Region: Basse Vallee	10x MOTU1	None	1 MOTUs 10 isolated fungi from Basse Vallee
Total per organ	13 MOTUs in all 12 MOTUs unique to pods, 1 MOTU common to pods and leaves 219 fungi from 135 pods	9 MOTUs in all 8 MOTUs unique to leaves, 1 MOTU common to pods and leaves 180 fungi from 540 leaves	**Grand Total:** 399 isolated fungi in all 675 sampled organs 59.1% isolation frequency

The results should be interpreted with care. They represent only facultative endophytes that can be isolated on artificial media in the laboratory (Hardoim et al. 2015). Fungal endophyte isolation frequency, a percentage, is calculated as (Total number of isolated fungi / Total number of sampled organs) X 100. This frequency can be as high as 58% (Russo et al. 2016), 67% (Parsa et al. 2016), 316% (Park et al. 2012) especially from plants from tropical regions. Isolation frequency in this study on *Vanilla* plants from Reunion Island is 59.1% (399/675 X 100).

MOTU 1 *Fusarium proliferatum* is the most widely distributed MOTU in the pods across the studied regions (**Figure 2**). This fungus occurs in both pods and leaves whereas twelve other MOTUs occur in pods only. The pod specific fungi seem more adapted to colonize pods than leaves. In all, 21 MOTUs were isolated. The pods seem to have a slightly higher MOTU diversity than the leaves (**Table 4** - 12 MOTUs from pods only, 8 MOTUs from leaves only). Fungal endophytes from pods differ across regions of cultivation with St Anne (4 MOTUs out of 12 pod unique MOTUs = 33%) and St. Rose (4 MOTUs out of 12 pod unique MOTUs = 33%) having the highest number of different pod unique endophyte MOTUs. Generally, more endophytes were isolated from leaf rank 15 than leaf rank 1 given the older age of the leaf. Hence, more endophytes had time to develop in older leaves given the longer exposure time of such leaf ranks to the environment. Endophyte species isolated here are not unique to *Vanilla* in terms of species. However, relying on only species to judge how unique these are is not enough. The same species may have specialized with *Vanilla* species and affect the metabolome in specific ways not present in other plants.

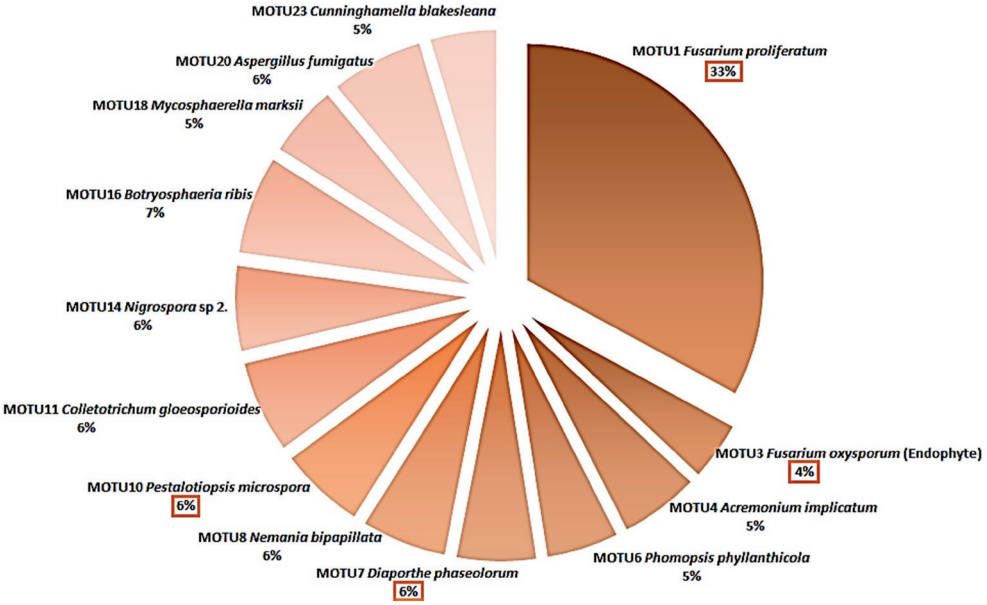

Figure 2 - Percentage of each fungal endophyte species (MOTU) from pods in relation to the total number of fungi isolated from pods only. The following MOTUs are represented in the diagram: **MOTU1** *Fusarium proliferatum*, **MOTU3** *Fusarium oxysporum*, **MOTU4** *Acremonium implicatum*, **MOTU6** *Phomopsis phyllanthicola*, **MOTU7** *Diaporthe phaseolorum*, **MOTU8** *Nemania bipapillata*, **MOTU10** *Pestalotiopsis microspora*, **MOTU11** *Colletotrichum gloeosporioides*, **MOTU14** *Nigrospora* sp. 2, **MOTU16** *Botryosphaeria ribis*, **MOTU18** *Mycosphaerella marksii*, **MOTU20** *Aspergillus fumigatus* and **MOTU23** *Cunninghamella blakesleana.*

3.3.2 Connection between fungal endophytes and vanilla flavor metabolites

The pod is a specific plant organ with a specific chemistry including compounds involved in vanilla flavor. The pod endophytes might be involved in biotransformations of pod metabolites, or produce novel chemicals themselves. Both processes may affect the vanilla flavor from the pods.

To study the possible effects of endophytes on the pod chemistry, the fungi were grown on a medium containing only ground freeze-dried and sterilized 8 months old green *Vanilla* pods. This was done to see if the endophytes changed the metabolic profile of the medium, pointing to biotransformation of pod metabolites or for *de-novo* biosynthesis of compounds by the endophyte. A pathogenic microorganism was also part of this study, for comparison, and to see if a common *Vanilla* pathogen might affect the pod chemistry and thus the quality of the pods. As control, the green *Vanilla* pod medium without fungal growth was used. A further control for *de-novo* biosynthesis of compounds by the fungi was to analyze the mycelium of the endophytes after growth on GPA medium.

The incubation period of the fungal culture was optimized, based on the time at which the slowest growing fungus (leaf unique endophyte *Nigrospora* sp.) covers the full medium surface of a 9 cm diameter petri dish (**Figure 3**).

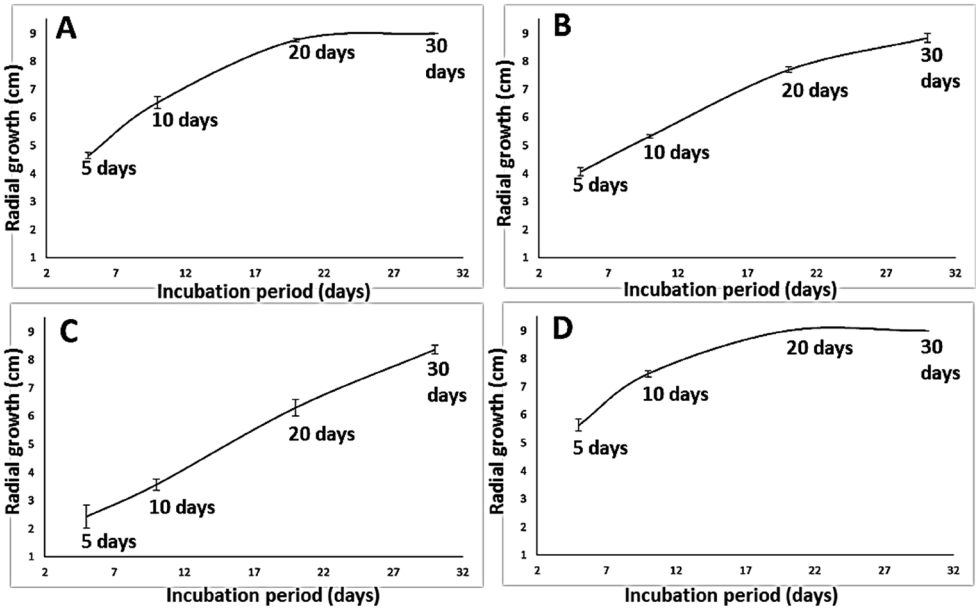

Figure 3 - Radial growth measurements of four of the selected *Vanilla* endophytic fungi grown on *Vanilla* green pod agar medium. Radial growth (cm) of A: *Pestalotiopsis microspora* (pod unique endophyte, MOTU10), B: *Diaporthe phaseolorum* (pod unique endophyte, MOTU7), C: *Nigrospora* sp.1 (leaf unique endophyte, MOTU14), D: *Fusarium oxysporum* f.sp. *vanillae* (*Vanilla* pathogen, MOTU24) on a 9 cm diameter petri dish with *Vanilla* green pod agar medium at different time points after incubation: 5, 10, 20 and 30 days. The standard deviation is shown as a vertical bar (number of replicates, n=3).

The pathogen *Fusarium oxysporum* f.sp. *vanillae* was the fastest growing fungus. All fungi had covered the 9 cm diameter petri dish surface at day 30. This time point was then chosen as the fungal growth period after which the medium was collected for NMR-based metabolomics analysis.

In the next experiment, endophytes isolated from *Vanilla* leaves and pods (**Table 3**) were grown for 30 days on the *Vanilla* green pod agar medium. The medium was analyzed by ^{1}HNMR and the results were subject to principal component analysis (PCA) (**Figure 4**).

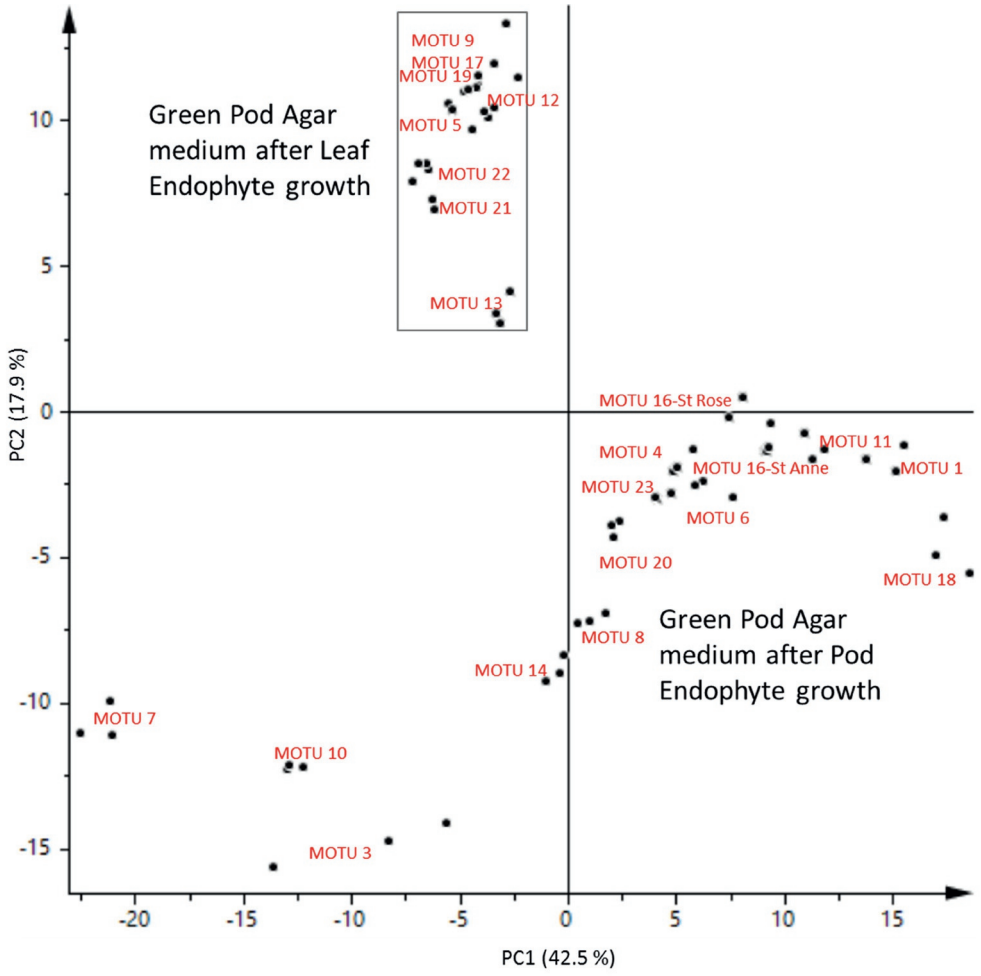

Figure 4 - Scatter Score Plot of principal components 1 and 2 of [1]HNMR spectral data of the green *Vanilla* pod agar medium after 30 days of *Vanilla* leaf and *Vanilla* pod derived fungal endophytes growth. The fungal MOTUs are indicated and represent:

Leaf endophytes MOTU 5- *Purpureocillium lilacinum*, MOTU 9- *Xylaria sp.,* MOTU 12- *Colletotrichum sp2*., MOTU 13- *Nigrospora sp1.*, MOTU 17- *Guignardia mangiferae,* MOTU 19- *Penicillium citrinum*, MOTU 21- *Sarcosomataceae spp.*, MOTU 22- *Perenniporia nanlingensis*;

Green *Vanilla* pod endophytes MOTU 1 (isolated from St. Rose)- *Fusarium proliferatum*, MOTU 3- *Fusarium oxysporum* (Endophyte), MOTU 4- *Acremonium implicatum*, MOTU 6- *Phomopsis phyllanthicola*, MOTU 7- *Diaporthe phaseolorum*, MOTU 8- *Nemania bipapillata*, MOTU 10- *Pestalotiopsis microspora*, MOTU 11- *Colletotrichum gloeosporioides*, MOTU 14- *Nigrospora sp 2.*, MOTU 16-St Rose (isolated from St Rose)- *Botryosphaeria ribis*, MOTU 16-St Anne (isolated from St Anne)- *Botryosphaeria ribis*, MOTU 18- *Mycosphaerella marksii*, MOTU 20- *Aspergillus fumigatus,* MOTU 23- *Cunninghamella blakesleana*.

Metabolites in the medium after growth with all *Vanilla* leaf derived endophytes are clearly separated from those of the pod derived endophytes (**Figure 5**).

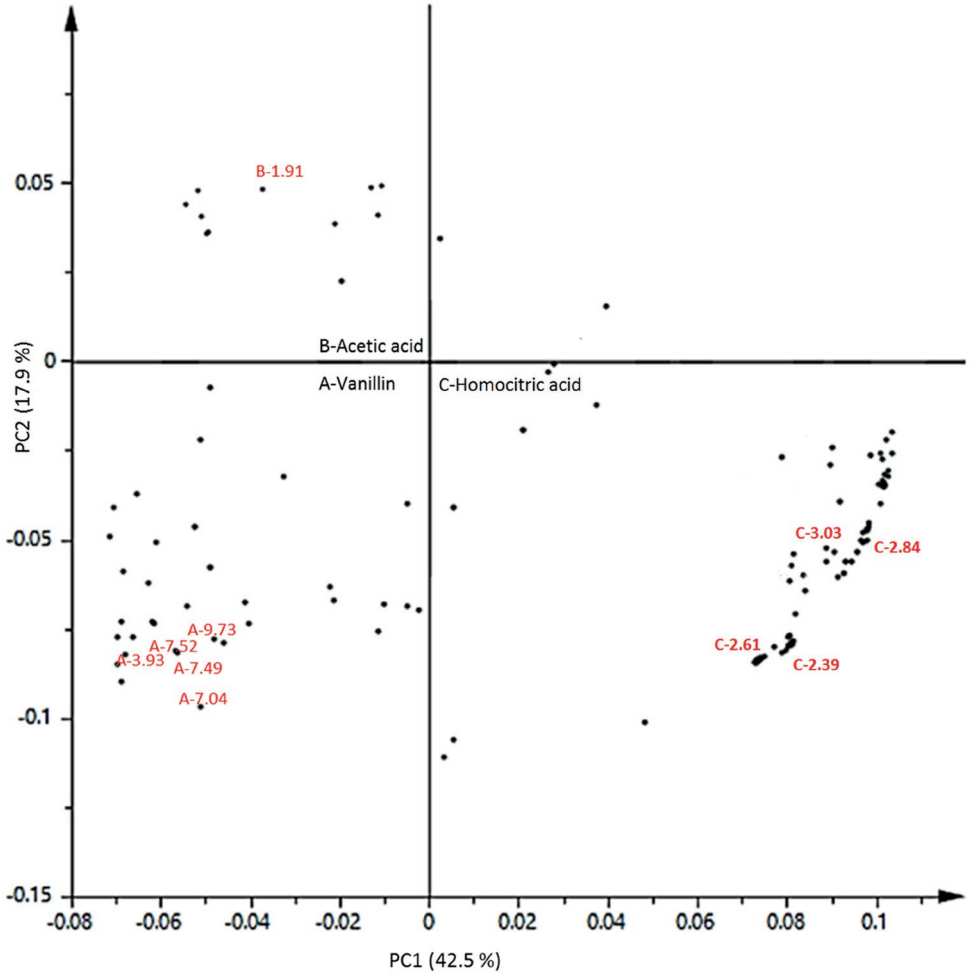

Figure 5 - Scatter loadings plot of principal components 1 and 2 based on ^{1}HNMR spectral data of the green *Vanilla* pod agar medium after 30 days of *Vanilla* leaf and *Vanilla* pod derived fungal endophytes growth. The metabolite chemical shifts are indicated.

In terms of mycelial growth on GPA medium, leaf endophytes tend to grow slower than pod derived endophytes, suggesting an effect of the pod medium on endophyte growth. Phenolics have the ability to inhibit fungal growth (Pizzolitto et al. 2015), though some fungi are able to detoxify phenolics (Zeng and Mallik, 2006). Such an effect might play a role in the slower growth of the leaf derived endophytes in the medium containing ground green pod material, as the pods are rich in phenolics, including vanillin glucoside, i.e. an environment the pod derived endophytes are used to.

Vanillin, the major component of vanilla flavor, was detected in GPA media after growth with pod endophytes MOTU 3, 7, 10 and 14 possibly due to fungal glucosidase activity on glucovanillin present in the GPA media. To find how the pod endophytes differ in their effects on the medium metabolites, the pod endophytes data were subjected to principal component analysis (PCA) (**Figure 6**).

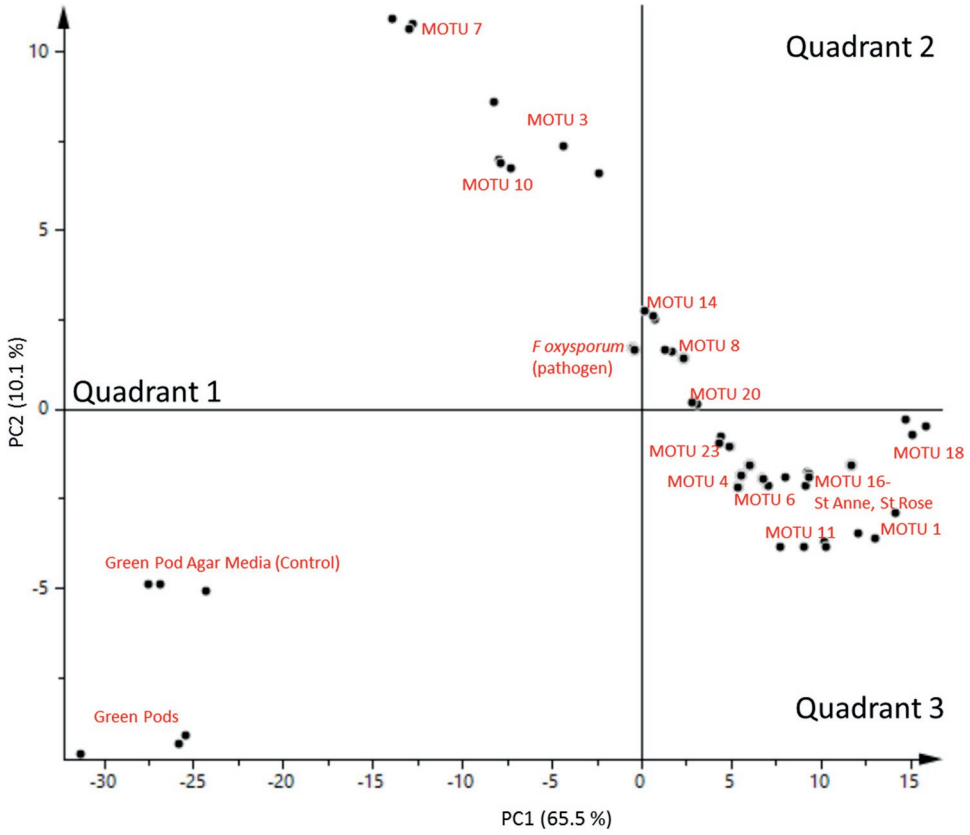

Figure 6 - Scatter Score Plot of principal components 1 and 2 of ^{1}HNMR spectral data of the green *Vanilla* pod agar medium after 30 days of growth of pod fungal endophytes. The MOTUs are: MOTU 1 (isolated from St. Rose)- *Fusarium proliferatum*, MOTU 3- *Fusarium oxysporum* (Endophyte), MOTU 4- *Acremonium implicatum*, MOTU 6- *Phomopsis phyllanthicola*, MOTU 7- *Diaporthe phaseolorum*, MOTU 8- *Nemania bipapillata*, MOTU 10- *Pestalotiopsis microspora*, MOTU 11- *Colletotrichum gloeosporioides*, MOTU 14- *Nigrospora sp 2.*, MOTU 16-St Rose (isolated from St Rose)- *Botryosphaeria ribis*, MOTU 16-St Anne (isolated from St Anne)- *Botryosphaeria ribis*, MOTU 18- *Mycosphaerella marksii*, MOTU 20- *Aspergillus fumigatus*, MOTU 23- *Cunninghamella blakesleana*. The *Vanilla* fungal pathogen *Fusarium oxysporum* f.sp. *vanillae* is also included.

The green *Vanilla* pod agar medium is comparable to green pods in chemical composition as both are in the same quadrant. The results (**Figure 6**) show that all pod fungi caused major changes in the metabolite composition of the green pod agar medium. The

endophytes MOTU 3, 7 and 10 are quite distinct from all others and are present in the same quadrant 1. Also, the pathogen *F. oxysporum* f.sp. *vanillae* is in quadrant 1.

From the loadings plot (**Figure 7**) the following compounds could be identified:

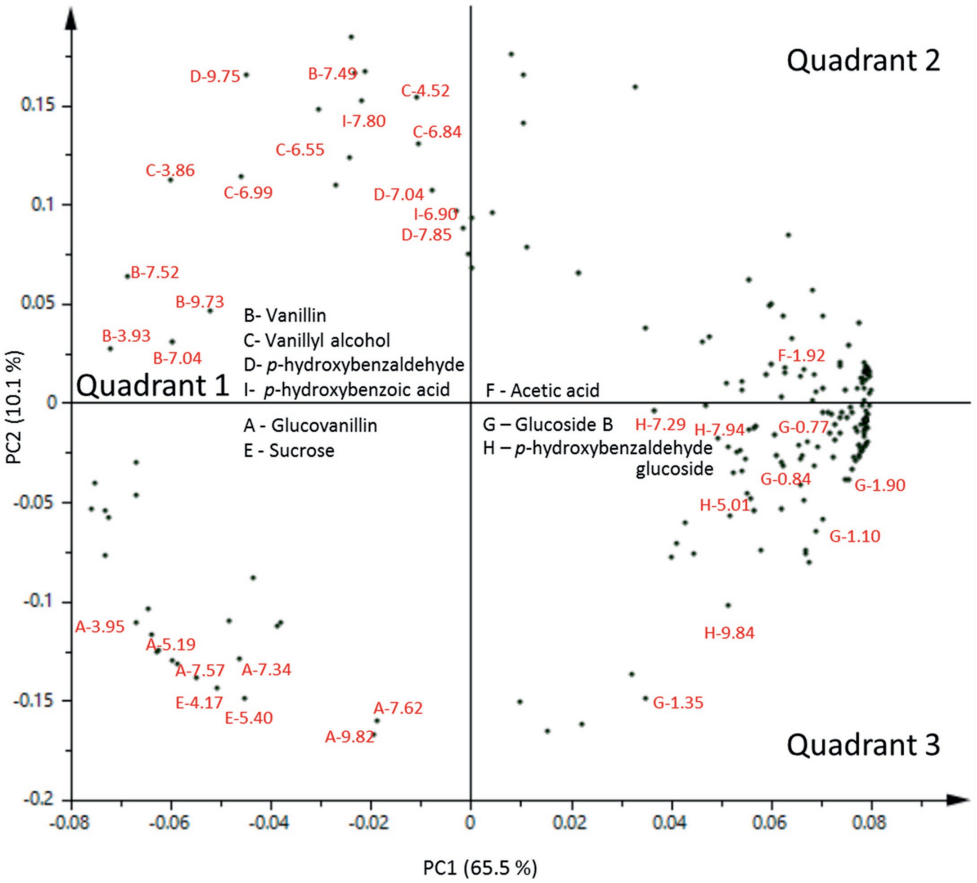

Figure 7 - Scatter loadings plot of principal components 1 and 2 of ^{1}HNMR spectral data of the green *Vanilla* pod agar medium after 30 days of growth of pod fungal endophytes. The metabolite chemical shifts are indicated.

Vanillin, vanillyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, glucovanillin are all major components of *Vanilla* pods and are detected in quadrant 1 side of PC1 of the loadings plot, i.e. where the control medium is present. Obviously sugars are highest in the control medium as the fungi use sugars for growth and energy. Moreover, glucovanillin is highest in the control, showing that all fungi were capable to hydrolyze this compound. But only the spent media of the fungi *D. phaseolorum* (MOTU 7), *F. oxysporum* (Endophyte-MOTU 3) and *P. microspora* (MOTU 10), representing only a total of 15% of all isolated endophytes, are on the negative side of PC1 with high vanillin. Spent media for the other pod endophytes were richer in glucoside B and *p*-hydroxybenzaldehyde glucoside, but had no vanillin derived compounds, thus showing that these compounds were metabolized to

yet unknown compounds. It is unlikely that the missing compounds were absorbed by fungi *D. phaseolorum* (MOTU 7), *P. microspora* (MOTU 10) and *F. oxysporum* (pathogen) given the absence of these from the mycelium (**Figures 8, 9**).

Vanillin is a highly reactive aromatic aldehyde which is toxic to cells (Fleige et al. 2013). Vanillin is thus reduced to the less toxic vanillyl alcohol (Nguyen et al. 2015) by the cells. However, vanillyl alcohol is not the only route to make vanillin less toxic. In bacteria (*Sphingomonas* sp.), vanillin is catabolized into pyruvate as the final product (Caspi et al. 2014). Enzymes in this pathway such as a putative vanillin dehydrogenase (EC 1.2.1.67) catalyzing the oxidation of vanillin to vanillic acid, have been reported from fungi (*Aspergillus niger*) as well (Gong et al. 2016, UniProtKB - A0A100INA5, gene: ABL_06999). Glucovanillin, vanillin, and vanillyl alcohol are absent from the spent medium in quadrant 2 and 3. This means the fungi in quadrant 2 and 3 have degraded vanillin further into products not found any more in the vanillin biosynthesis network. Also *p*-hydroxybenzaldehyde glucoside is found in these two quadrants. This compound might be a metabolite derived from glucoside A and B. The same applies for *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in quadrant 1. The *p*-hydroxybenzaldehyde and its glucoside are among the vanilla flavor components. Apparently the levels of these compounds are affected by the fungi. For all these compounds, further studies are needed to learn if the changes observed are due to *de novo* biosynthesis in the fungus or due to biotransformation of precursors from *Vanilla* pods in the medium.

If vanillin and vanilla flavor components are synthesized *de novo* by the fungi in quadrant 1 (**Figures 6, 7**), they might be abundant in the fungal mycelium. Hence, the mycelium mass of fungi MOTU 7 *D. phaseolorum*, MOTU 10 *P. microspora*, MOTU 24 pathogen *F. oxysporum* f.sp. *vanillae* which were collected from the surface of the growth medium after 30 days of growth were analyzed by ^{1}HNMR. The results were subjected to principal component analysis (PCA) (**Figure 8**).

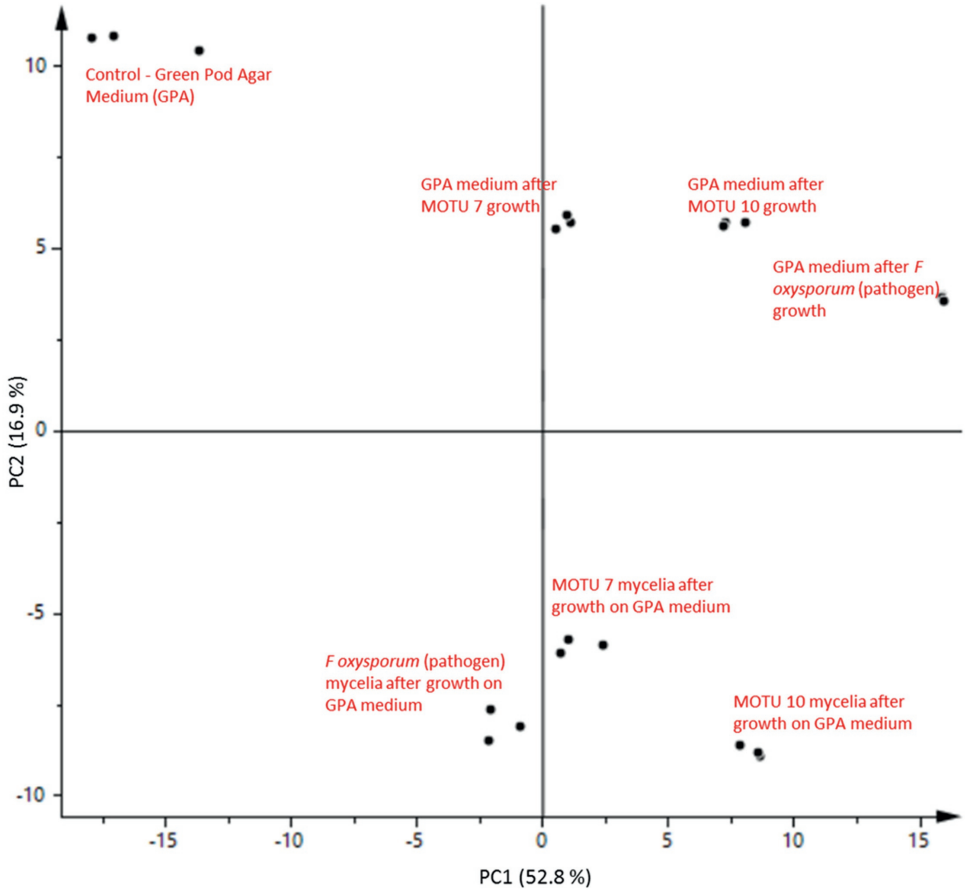

Figure 8 - Scatter Score Plot of principal components 1 and 2 of ^{1}HNMR spectral data of fungal mycelia and of the green *Vanilla* pod agar media after 30 days of growth of pod fungal endophytes. MOTU 10- *Pestalotiopsis microspora*, MOTU 7- *Diaporthe phaseolorum*.

The chemical composition of the fungal mycelia are quite distinct from the green pod agar medium composition after growth with the same fungi. From the loadings plot (**Figure 9**) the following compounds could be identified: homocitric acid, acetic acid, vanillin and its glucoside.

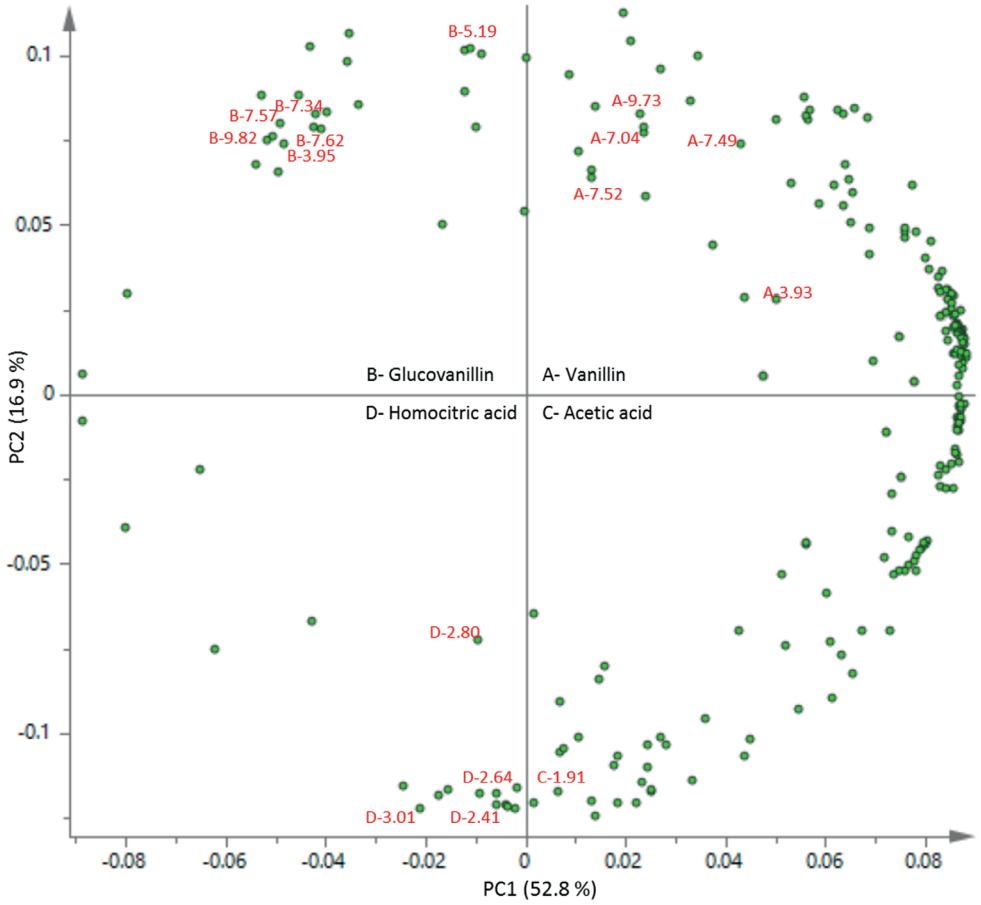

Figure 9 - Scatter loadings plot of principal components 1 and 2 of ^{1}HNMR spectral data of fungal mycelia and of the green *Vanilla* pod agar media after 30 days of *Vanilla* pod derived fungal endophytes growth. The metabolite chemical shifts are indicated.

The absence of vanilla flavor phenolics in the fungal mycelium shows that the fungi are not likely to produce such molecules *de-novo* (**Figure 10**).

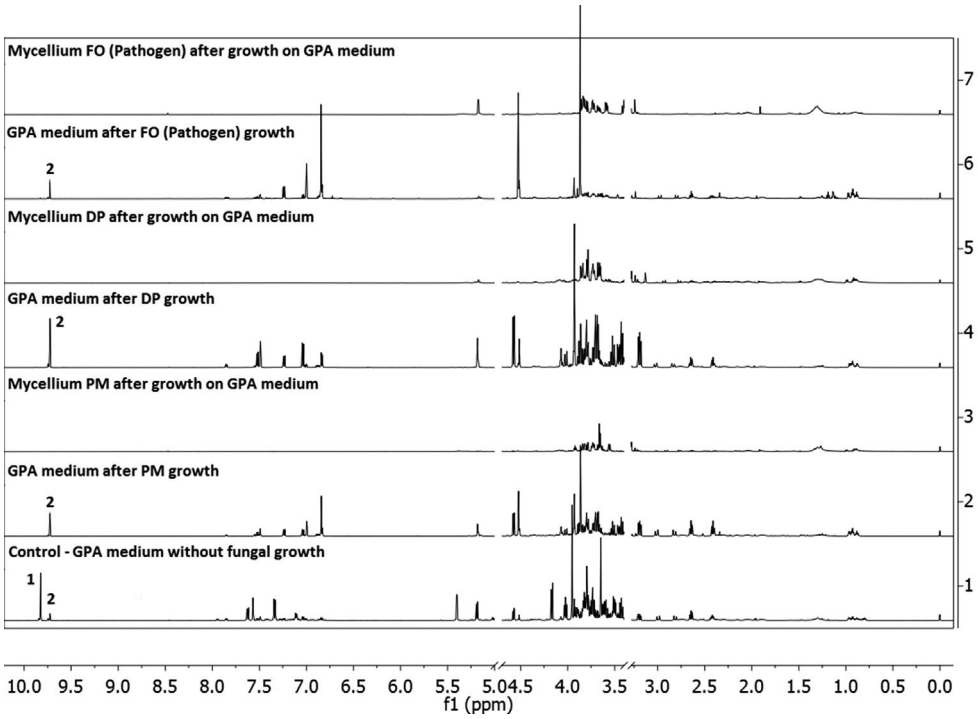

Figure 10 – Alignment comparing seven ^{1}HNMR spectra including the control, the media after fungal growth and the mycelium of the fungi all grown on GPM medium. Abbreviations are: GPM – green pod agar medium, PM – *Pestalotiopsis microspora* (MOTU 10), DP – *Diaporthe phaseolorum* (MOTU 7), FO (Pathogen) – *Fusarium oxysporum* f.sp. *vanillae* (MOTU 24). Assignments: 1 – glucovanillin, 2 – vanillin.

However, these fungi can transform precursors present in the medium, such as glucovanillin, into vanillin and related flavor notes. Still this cannot fully exclude *de novo* biosynthesis. Further, more directed precursor feeding experiments are needed to better understand the metabolic activities of the endophytes.

Among fungal endohytes, the highest amount of recorded Vanilla flavor metabolites were from MOTU 10 and MOTU 7 (**Table 5**) and not from MOTU 3. MOTU 3 was thus not retained for further work in subsequent chapters. This is one reason why the mycelium of MOTU 3 was excluded from the analysis here. Additionally, analysis of fungal mycelium represents preliminary results from which further future experiments can be conducted. Hence, the second reason why MOTU 3 mycelium was not included in the analysis is that this forms part of future research avenues. The results show that there might be a correlation between the biotransformation of vanilla flavor phenolics and the three pod derived fungal endophytes (MOTU 3, 7, 10; ^{1}HNMR of the mycelium of MOTU 3 was not performed), and a *Vanilla* fungal pathogen (MOTU 24 *F. oxysporum* f.sp. *vanillae*). To quantify the differences in metabolite levels in the spent growth media after fungal growth, two methods were used: ^{1}HNMR and HPLC quantification. The ^{1}HNMR quantification has the advantage that the quantitation of all compounds can be done with one common internal

standard. Whereas the more targeted HPLC requires calibration curves for each individual compound. The advantage of the HPLC is that the problem of overlapping signals in the NMR can be solved. Also for minor compounds NMR requires a longer spectral accumulation time than normally applied in NMR based metabolomics. In fact targeted HPLC and non-targeted ^{1}HNMR techniques complement each other well, for studying the metabolome of any organism. The HPLC profiles for the samples are shown (**Figure 11**).

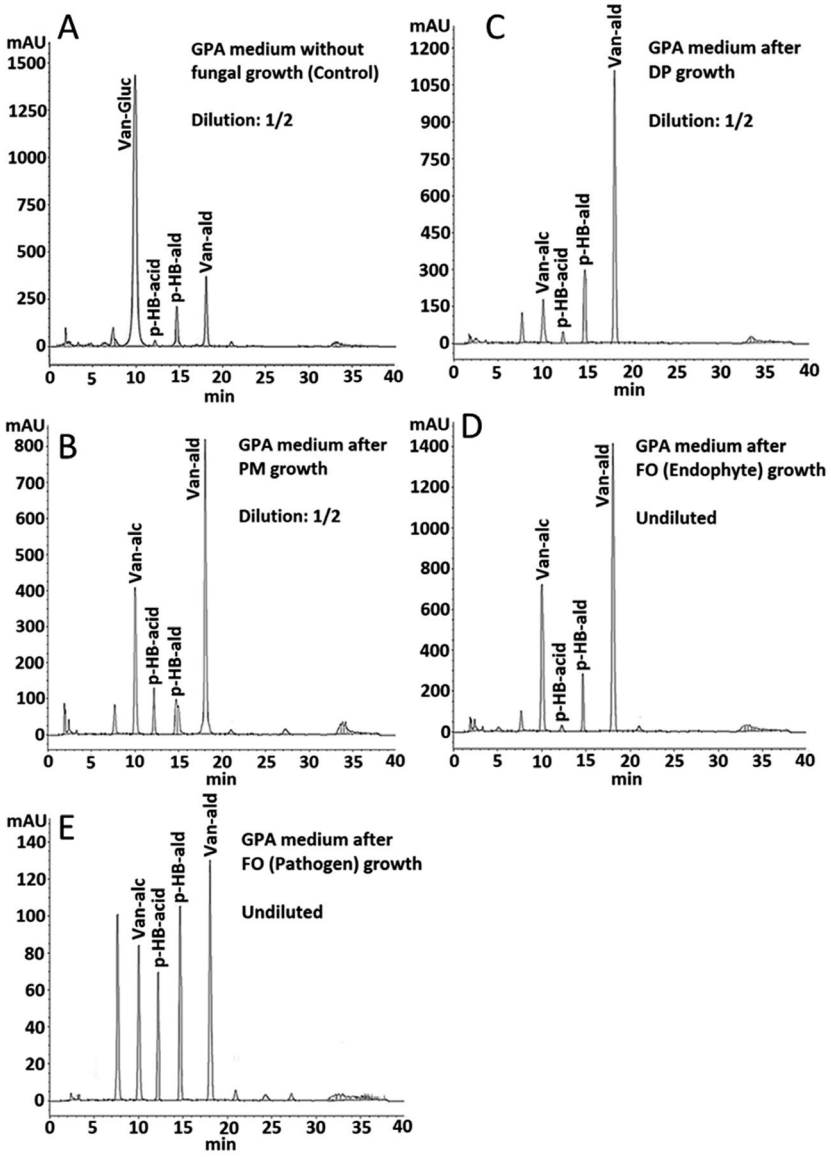

Figure 11 - HPLC profiles of the green pod agar (GPA) media [A] without fungal growth (Control), [B] after growing *Pestalotiopsis microspora* (PM, MOTU 10), [C] after growing *Diaporthe phaseolorum (*DP, MOTU 7), [D] after growing *Fusarium oxysporum* (FO, endophyte MOTU 3), [E]

after growing *Fusarium oxysporum* f.sp. *vanillae* (FO, pathogenic strain). The HPLC was performed with column LiChrospher 100 RP-18 (4.6 X 250 mm, 5 µm, 100 Å (10 nm)), eluents water:acetic acid (0.1%) (pH 3.3) and MeOH-H_2O(1:9) with 0.1% acetic acid (pH 3.3), detection was by UV-DAD. The abbreviations are Van-Gluc: glucovanillin (RT: 9.85), Van-alc: vanillyl alcohol (RT: 10.02), p-HB-acid: *p*-hydroxybenzoic acid (RT: 12.18), p-HB-ald: *p*-hydroxybenzaldehyde (RT: 14.70), Van-ald: vanillin (RT: 18.03). To prevent detector saturation, in cases where glucovanillin or vanillin peaks had absorbance much higher than 1500, the sample solution was diluted by half before injection in the HPLC for quantification. The dilution factor is indicated in the figure.

The calibration curves made for the metabolites glucovanillin, vanillyl alcohol, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and vanillin are shown in **Figure 12**. Using these curves the concentrations of the metabolites were calculated.

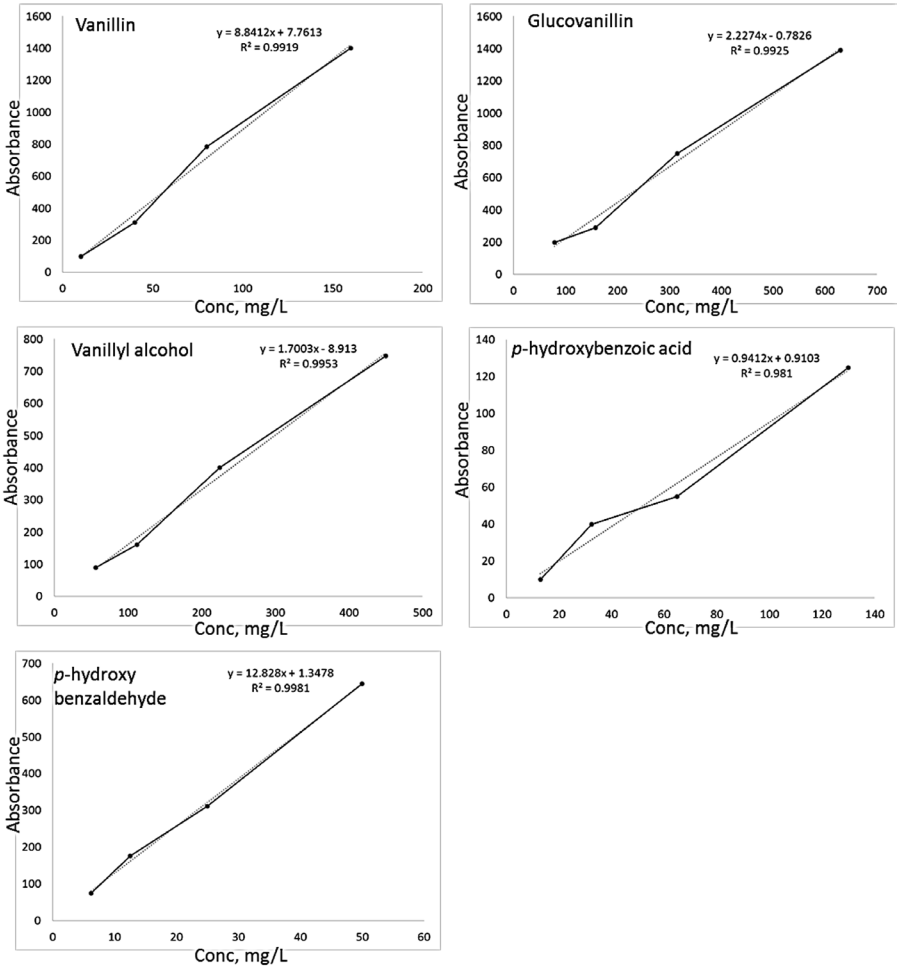

Figure 12 - Calibration curves for metabolite concentration determination by HPLC in samples. The targeted concentration ranges were estimated based on the ^{1}HNMR quantification results. The tested concentration ranges show a generally linear relationship with the absorbance for three independent replicates (n=3).

The fungi *P. microspora* and *D. phaseolorum* stand out for increasing most the levels of vanillin, vanillyl alcohol, *p*-hydroxybenzaldehyde in GPA medium after growth (**Table 5**). These three molecules are involved in vanilla flavor.

Table 5 - Average amounts with standard deviation (n=3) of vanillin and some related compounds in spent green *Vanilla* pod media after fungal growth. * shows the fungus associated with the highest recorded amount for a metabolite.

Metabolite	Metabolite amount in Green Pod Agar Medium after fungal growth in µmol.g^{-1} DW medium									
	Control – No fungal growth		**MOTU10 *Pestalotipsis microspora***		**MOTU7 *Diaporthe phaseolorum***		**MOTU 3 *Fusarium oxysporum* (endophyte)**		**MOTU24 *Fusarium oxysporum* (pathogen)**	
	NMR	**HPLC**	**NMR**	**HPLC**	**NMR**	**HPLC**	**NMR**	**HPLC**	**NMR**	**HPLC**
Vanillin	17.0 +/-0.6	16.3 +/-0.5	37.0 +/-1.6	36.6 +/-0.8	56.0* +/- 11.2	52.5* +/-7.5	32.2 +/-1.4	30.6 +/-1.0	1.9 +/-0.5	1.8 +/-0.8
Gluco vanillin	127.0* +/-2.1	125.3* +/-4.5	0	0	0	0	0	0	0.9 +/-0.2	Masked by Vanillyl alcohol signal
***p*-hydroxy benzoic acid**	7.4 +/-0.9	7.3 +/-1.2	87.9* +/-12.7	66.6* +/-9.8	16.5 +/-4.3	17.3 +/-7.6	8.6 +/-1.0	8.2 +/-0.9	13.3 +/-2.5	12.9 +/-1.8
***p*-hydroxy benzaldehyde**	6.2 +/-0.7	6.7 +/-0.9	3.9 +/-0.5	4.0 +/-0.2	9.0* +/-1.7	9.5* +/-1.7	4.2 +/-1.2	4.1 +/-0.8	1.2 +/-0.3	1.4 +/-0.5
Vanillyl alcohol	15.5 +/-7.0	Masked by gluco vanillin signal	100.0* +/-6.3	96* +/-5.9	38.6 +/-6.6	38.5 +/-4.7	76.3 +/-8.4	76.0 +/-8.4	11.7 +/-2.6	10.1 +/-1.1

A two-way ANOVA was conducted to compare the means, and find the effects of two independent categorical variables (fungal species, metabolite type), on the amount of metabolite in spent green *Vanilla* pod media after fungal growth (dependent variable). Fungal species included five levels, and metabolite type four levels. Metabolite type glucovanillin was removed from the calculations, given it was completely consumed and has zero values in all spent media. All means were statistically significant at the 0.05 significance level. The interaction effect between the two independent variables, was significant, $F(40, 12) = 57.0$, $P = 0.000$.

All M and SD below concern total means and standard deviation. The main effect for fungal species yielded an F ratio of F(4, 40) = 175.6, p = 0.000, indicating a significant difference between *Fusarium oxysporum* (pathogen) F(3, 40) = 4.5, p = 0.008, (M = 7.03, SD = 5.96); *Diaporthe phaseolorum* F(3, 40) = 51.0, p = 0.000, (M = 30.01, SD = 20.20); *Fusarium oxysporum* (endophyte) F(3, 40) = 121.9, p = 0.000, (M = 30.35, SD = 30.10); *Pestalotipsis microspora* F(3, 40) = 224.7, p = 0.000, (M = 57.21, SD = 41.00) and Control F(3, 40) = 3.4, p = 0.027, (M = 11.56, SD = 5.85).

The main effect for metabolite type yielded an F ratio of F(3, 40) = 177.4, p = 0.000, indicating a significant difference between *p*-hydroxy benzoic acid F(4, 40) = 132.2, p = 0.000, (M = 26.77, SD = 32.25); Vanillin F(4, 40) = 46.9, p = 0.000, (M = 28.81, SD = 19.44) and Vanillyl alcohol F(4, 40) = 166.7, p = 0.000, (M = 48.46, SD = 36.17) were significantly associated to changes in metabolite amount, in the spent media. Whereas the detected changes in metabolite amounts in the spent media, was not due to metabolite type *p*-hydroxy benzaldehyde F(4, 40) = 0.9, p = 0.450, (M = 4.90, SD = 2.82).

To find which levels within each independent variable is significant, a post hoc comparison using the Tukey's HSD test was conducted. The results show all mean scores differed significantly, except between *p*-hydroxy benzoic acid and Vanillin, which do not differ significantly (p = 0.707).

Both fungi MOTU 7 *D. phaseolorum* and MOTU 10 *P. microspora* are of interest for further studies. In comparison, MOTU 3 *F. oxysporum* (endophyte) is not exceptional with regards to amounts of accumulated vanilla flavor metabolites and hence not important for further studies. Whereas MOTU 24 *F. oxysporum* f.sp. *vanillae* (pathogen) is important for further studies given it is a pathogen and not an endophyte and allows the comparison of the effects on the vanilla aroma between endophytes and one pathogen.

As our aim was also to see if the endophytes can catabolize vanilla flavor components, we focused on the compounds in the green pod media associated to vanilla flavor. The ratio of these compounds such as vanillin, vanillyl alcohol, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, all occurring in the pods, influences the flavor of the pod.

Apparently each of the isolated endophytes has a different effect on the green pod constituents present in the medium. Additionally, they may produce secondary metabolites themselves. That means that a different spectrum of endophyte species in a plant, may affect the plant metabolome. The isolation of endophytes from different *Vanilla* plants from different regions resulted in different sets of isolates. By no means have these results represented the full microbiome of endophytes of the plants, but at least it shows that it is likely that differences occur. What it could mean for the flavor can be illustrated with the example of vanillyl alcohol. The level of this compound was increased most in the spent medium of the fungus MOTU 10 which also contained a high level of vanillin. It has been shown by gas chromatography-olfactometry (GC-O) that vanillyl alcohol is present in pods at a lower concentration than vanillin, but its contribution to vanilla aroma is as intense as vanillin (Hoffman and Zapf, 2011). According to Ranadive (2011), the Bourbon label, which includes pods from Reunion Island, is characterized by an intense balsamic flavor. "Le Comité d'experts FAO/OMS sur les additifs alimentaires" (2001) states that the organoleptic property of vanillyl alcohol is a balsamic, vanilla-like flavor. MOTU 10 might thus contribute to the balsamic tone in pod flavor and the appreciated high quality of pods from the St. Rose area, where the pods contained *Pestalotipsis microspora* (MOTU 10).

Fusarium oxysporum (endophyte) and *F. oxysporum* f.sp. *vanillae* (pathogen) do not show exactly the same phenolics biotransformation patterns. The results show that the strains of a fungal species may differ in their conversion of phenolics.

3.4 CONCLUSIONS AND FURTHER WORK

Results from this work make a case for endophyte involvement in vanilla flavor. First of all we isolated a number of endophytes from *Vanilla* pods and leaves. The pods generally gave several endophytes, whereas from leaves, the number isolated was much lower. MOTU 3 - *Fusarium oxysporum* (Endophyte), MOTU 7 - *Diaporthe phaseolorum*, MOTU 10 - *Pestalotiopsis microspora* all have a specific effect (increase or/and decrease) on Vanilla flavor compounds in GPA medium. This suggests the participation of the three fungi in modifying Vanilla flavor in *Vanilla* pods. However, further experiments are required with those fungi on living pods on the plants to confirm this. The three fungi originate from different regions of Reunion Island: MOTU 3 from St. Anne, MOTU 7 from St. Andre, MOTU 10 from St. Rose. This perhaps points to a possible regional effect (terroir) of the fungi on Vanilla flavor.

Apparently the different collection sites gave different spectra of endophytes in the pods. The endophytes isolated from pods, as well as a pathogen and a leaf endophyte all showed different catabolism of compounds from *Vanilla* pods present in the culture medium. In some, only about 15% of the total of vanillin and related compounds was left after fungal growth, whereas in others, only glucovanillin was completely hydrolyzed, leaving vanillin and vanillyl alcohol as major components. Infection of pods with the pathogenic *F. oxysporum* f.sp. *vanillae* may cause significant loss of quality.

The presence of a compound in a spent green *Vanilla* pod media in itself is no evidence for any relation with the presence of other related compounds. As mentioned in Chapter 2, the biosynthetic network of which vanillin is part, is still not fully understood, which makes it even more difficult to draw any pertinent conclusions. In fact the findings reported here should be considered as a first screening of *Vanilla* endophytes for a possible role in vanillin biosynthesis. The next step should be a more in depth study of the *de novo* and biotransformation capacities of the isolated endophytes.

MOTU 7 and 10 increased the most Vanilla flavor compounds in GPA media (**Table 5**) and were hence retained for future experiments. MOTU 24 (a pathogen) was also retained for future experiments, to find how far endophytes are unique in their effects (increase and/or decrease) on Vanilla flavor compounds.

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Chapter 3 - Supplementary Data

Table S1 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here St. Andre) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At St. Andre, plants grown in two different environments were sampled: in the under growth and under shade house cultivation. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ which were not isolated under the applied laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
St. Andre (Under Growth)	1	Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Pod	Yes	*Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides*
		Pod	Yes	*Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	2	Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Pod	Yes	*Colletotrichum gloeosporioides*
		Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Pod	Yes	*Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None

		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	3	Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Pod	No	None
		Pod	Yes	*Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Pod	Yes	*Fusarium proliferatum* *Diaporthe phaseolorum*
		Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides* *Diaporthe phaseolorum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
St. Andre (Shade House)	1	Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides*
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Colletotrichum gloeosporioides*
		Pod	No	None
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None

		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Xylaria* sp. *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Xylaria* sp. *Penicillium citrinum*
	2	Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides*
		Pod	No	None
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum* *Colletotrichum gloeosporioides*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Xylaria* sp. *Penicillium citrinum*

		Leaf Rank 15	Yes	*Fusarium proliferatum* *Xylaria* sp. *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Xylaria* sp. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
	3	Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Colletotrichum gloeosporioides*
		Pod	Yes	*Colletotrichum gloeosporioides*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 5	Yes	*Fusarium proliferatum* *Nigrospora* sp 1
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Xylaria* sp. *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Colletotrichum* sp2. *Penicillium citrinum*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Xylaria* sp. *Colletotrichum* sp2. *Penicillium citrinum*

Table S2 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here St. Anne) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At St. Anne, plants grown under two environments were sampled: in the under growth and under shade house cultivation. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ but are not isolable under laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
St. Anne (Under Growth)	1	Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	No	None
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
	2	Pod	No	None
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	No	None
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*

		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
	3	Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	Yes	*Fusarium oxysporum* (Endophyte)
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
St. Anne (Shade House)	1	Pod	Yes	*Fusarium proliferatum* *Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	No	None
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*

		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	No	None
	2	Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Fusarium proliferatum* *Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Fusarium proliferatum* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	No	None
	3	Pod	Yes	*Fusarium proliferatum* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Fusarium proliferatum* *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Cunninghamella blakesleana* *Nigrospora* sp1. *Aspergillus fumigatus*
		Pod	Yes	*Fusarium proliferatum* *Nigrospora* sp1. *Aspergillus fumigatus*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None

		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Purpureocillium lilacinum*

Table S3 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here St. Rose) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At St. Rose, plants grown under one environment were sampled: from the under growth. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ which are not isolated under the applied laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
St Rose (Under Growth)	1	Pod	Yes	*Fusarium proliferatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Acremonium implicatum* *Nemania bipapillata* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None

		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	2	Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Botryosphaeria ribis*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	3	Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora*

				Botryosphaeria ribis
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Pod	Yes	*Fusarium proliferatum* *Acremonium implicatum* *Nemania bipapillata* *Pestalotiopsis microspora* *Botryosphaeria ribis*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	Yes	*Fusarium proliferatum*
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum*

Table S4 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here Bois Blanc) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At Bois Blanc, plants grown under one environment were sampled: from the under growth. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ which are not isolated under the applied laboratory conditions.

Region	**Plant Number**	**Collected organ**	**Infected organ**	**Isolated Fungus**
Bois Blanc (Under Growth)	1	Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None

		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	2	Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	3	Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None

		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None

Table S5 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here Takamaka) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At Takamaka, plants grown under one environment were sampled: in the under growth. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ which are not isolated under the applied laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
Takamaka (Under Growth)	1	Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
	2	Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None

		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
	3	Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	Yes	*Fusarium proliferatum*
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	Yes	*Fusarium proliferatum*
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.
		Leaf Rank 15	Yes	*Sarcosomataceae* spp.
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Sarcosomataceae* spp.

Table S6 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here Mare Longue) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At Mare Longue, plants grown under one environment were sampled: in the under growth. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ but are not isolable under laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
Mare Longue (Under Growth)	1	Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	No	None
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae*
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	No	None
	2	Pod	No	None
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Leaf Rank 1	No	None

		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae*
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
	3	Pod	Yes	*Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	No	None
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Pod	Yes	*Fusarium proliferatum* *Phomopsis phyllanthicola* *Mycosphaerella marksii*
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae*
		Leaf Rank 15	Yes	*Guignardia mangiferae* *Perenniporia nanlingensis*
		Leaf Rank 15	Yes	*Fusarium proliferatum*

				Guignardia mangiferae
		Leaf Rank 15	Yes	*Fusarium proliferatum* *Guignardia mangiferae*

Table S7 – Isolated fungal endophyte in this work, the plant sample of origin (and three replicates) that lead to the endophyte isolation, the region (here Basse Vallee) of origin of the plant samples. One fragment (0.5 cm x 0.5 cm) was retained per pod (disc) or per leaf (leaf blade). At Basse Vallee, plants grown under one environment were sampled: in the under growth. Under ‘ isolated fungus ’, the term ‘ none ’ either means there were no fungal endophytes in the organ or there may be fungal endophytes in the organ but are not isolable under laboratory conditions.

Region	Plant Number	Collected organ	Infected organ	Isolated Fungus
Basse Vallee (Under Growth)	1	Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	2	Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None

		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
	3	Pod	No	None
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	Yes	*Fusarium proliferatum*
		Pod	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 1	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 3	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 5	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None
		Leaf Rank 15	No	None