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# MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

## „Chemodiversity of endophytic fungi from *Psychotria* and *Palicourea* species (Rubiaceae) from a lowland tropical rainforest in Costa Rica"


verfasst von / submitted by

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*"One of the major problems facing the future of endophyte biology is the rapidly diminishing rainforests, which hold the greatest possible resource for acquiring novel microorganisms and their products."*

Strobel, 2003

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# 1. Introduction

Botanical and mycological research is not only historically grouped together, but it is also an inevitable combination of scientific perspectives regarding ecological, nutritional and biochemical investigations in plants. Fungi and plants interact in numerous distinct and fascinating ways. One prominent and well-studied phenomenon is fungal mycorrhiza. Fungi associated with roots connect plants with their offspring and members of other plant species and increase water and nutrient availability by extending the root system (Beiler et al., 2010, Strack et al., 2003). In exchange, plants provide up to one fifth of their carbohydrates from photosynthesis to their associated fungal partners (Wright et al., 1998).

In addition to the close connection of plants and fungi in the rhizosphere, many fungal species are capable to grow entirely inside the tissue of plants. Every plant organ including flowers and seeds can be a suitable habitat. Besides fungi, also bacteria including actinomycetes are found as colonizers of these living niches. The ability of microorganisms to completely reside in a living host plant, for at least a part of their life cycle, without triggering visible infection symptoms characterizes them as endophytes (Petrini, 1991, Wilson, 1995). Growing inside their host, endophytes encounter a niche with reduced environmental stress and sufficient supply of nutrients (Schulz and Boyle, 2005). Presence of endophytic fungi has been reported from higher plants, ferns and mosses, with the highest diversity observed in the tropics (Arnold and Lutzoni, 2007, Arnold et al., 2001, Davis et al., 2003). The adaptation to a life within plants lead to a loss of sexual reproduction in some endophytic fungal species. In order to live on in the next generation, these fungi are distributed within or on the plant seeds and pollen (Hodgson et al., 2014).

Research on endophytic fungi comprises a wide array of scientific fields. The strong impact of endophytes on plant survival and interaction with the environment has been investigated extensively. In particular, contribution to plant defense mechanisms and increased resistance against pathogens were reported for some prominent and agricultural important species, such as *Theobroma cacao* (cacao tree), *Hevea brasiliensis* (rubber tree), and species of the coffee plant genera *Coffea* sp. (Hanada et al., 2010, Gazis and Chaverri, 2010, Vega et al., 2010). In constant contact and competition with various other fungi and bacteria sharing the limited habitat within their host, fungal endophytes have been highlighted a promising source for the isolation of novel secondary metabolites, often with pronounced biological activities (Aly et al., 2010, Schulz et al., 2002, Strobel, 2003).

The discovery of paclitaxel (Taxol®), an effective medication in cancer therapy, in the fungal endophyte *Taxomyces andreana* isolated from *Taxus brevifolia* (Taxaceae) (Stierle et al., 1993) entailed an extensive research on endophytes from pharmaceutically important plants and their bioactive

metabolites. The indole alkaloid vincristine from *Catharanthus roseus* (Apocynaceae) and the quinoline alkaloid camptothecin from *Camptotheca acuminata* (Cornaceae), both used for cancer treatment, were discovered to be also produced by plant inhabiting fungal endophytes (Aly et al., 2013). Beside these metabolites previously only known from plants, numerous substances with antibiotic activity have been isolated from cultivated endophytes (Mousa and Raizada, 2013).

Fungal endophytes isolated from plant species of the genera *Psychotria* and *Palicourea* (Rubiaceae) growing in Costa Rica are in the focus of this thesis. These two plant genera exhibit an interesting diversity of secondary plant metabolites, e.g. iridoids, alkaloids, terpenoids and flavonoids from specific biosynthetic pathways (Berger et al., 2016, Berger et al., 2012). Phytochemical analysis of these genera is one major topic in our working group. Protocols for phytochemical screenings and databases of pure compounds were already at hand in prior to this thesis. The tight interaction of fungal endophytes with plants and their possible contribution to the biosynthesis of plant secondary metabolites drew interest towards endophytic research.

So far, a variety of endophytic fungi and bacteria were isolated from *Psychotria* (Govinda Rajulu et al., 2013, Lemaire et al., 2012b) and *Palicourea* species *(Cafêu et al., 2005, Souza et al., 2004)*. In continuation of a preliminary screening for the presence of endophytic fungi in *Psychotria* species growing in Costa Rica (Schinnerl, unpublished), a closer look was taken at the richness and capacity of secondary metabolite production of endophytic fungi from eight species from the genus *Psychotria* and the closely related genus *Palicourea*. To the best of my knowledge, this work is the first deeper investigation of endophytic fungal diversity from the selected *Psychotria* and *Palicourea* species.

## 2. Aims and structure

The focus of this thesis lies on the diversity, distribution and secondary metabolite production of endophytic fungi isolated from *Psychotria* and *Palicourea* species, growing near the tropical field station of *La Gamba* in Costa Rica. Cultivated fungi were analyzed by chromatographic methods and pure compounds isolated. Fungal and plant secondary metabolite profiles were compared for similarities and the variation of fungal metabolite production growing on different media highlighted. Furthermore, the chemical composition of fungal guttation droplets was analyzed. In addition to chemical analysis, fungal endophytes were tested for anti-microbial effects on bacterial and fungal test organisms. This work is seen as a pilot study for the development of methods in order to embed fungal endophytes in future research as possible contributors to plant defense mechanisms and secondary metabolite production.

Following hypotheses were addressed:

1. Fungal endophytes occur in all above ground parts of *Psychotria* and *Palicourea* species.
2. Fungal endophytes of *Psychotria* and *Palicourea* species are expected to be members of the genus *Xylaria* (Xylariaceae).
3. Endophytes take part in the accumulation of secondary metabolites in these plant species.
4. Endophytic fungi from *Psychotria* and *Palicourea* produce anti-microbial substances *in vitro*.

This thesis is divided into five parts. Material and methods are described under chapter 3, followed by a protocol of how these methods were used for extraction work in chapter 4. Regarding the diversity of research questions addressed in this thesis, individual results are presented and discussed separately in the chapters 5 to 11. Since endophytic research is highly dependent on isolation and cultivation techniques applied, methods are evaluated in a separate chapter. In chapter 13 a conclusion is drawn and future prospects are highlighted.

# 3. Material and methods

## 3.1 Plant and fungal material

Plant and fungal material was collected in the course of a field trip to the Golfo Dulce Region of Costa Rica in November and December 2015. The tropical lowland forest of the *Piedras Blancas* national park around the field station *La Gamba* of the University of Vienna was selected as an ecologically intact area for collection work (Weber and Baumgartner, 2001). The nearby laboratory facilities were helpful for fast processing of the collected material. In the course of three weeks, 15 individuals of 8 *Psychotria* and *Palicourea* species were successfully sampled. All together 102 fungal strains were isolated and further cultivated. For readability reasons, fungal isolates will be named using a **code**. Further information on each fungal isolate is shown under Appendix 2. Due to recent shifts in *Psychotria* and *Palicourea* nomenclature, current names and synonyms are given in Table 1. In addition to the collections from Costa Rica, one *Psy. carthagenensis* individual growing in the botanical garden of the University of Vienna (HBV) was used for endophyte isolation in preliminary tests.

For each plant individual collected in Costa Rica two specimens as well as leaves, shoots and if present inflorescences were sampled. For the purpose of later phytochemical analysis, the plant material was dried at room temperature using an air dehumidifier. For fungal endophyte isolation whole, apparently healthy leaves, central and basal grown branches and if available fruits were placed in clean plastic bags until further processing. GPS data for collected specimens are listed under Appendix 1. Plant specimens were deposited in the herbarium of the University of Vienna (WU) and one copy each in the national herbarium of Costa Rica (CR).

Table 1. Current names and synonyms of collected *Psychotria* and *Palicourea* species

| **Plant species** | **Synonym** |
|---|---|
| *Palicourea acuminata* (Benth.) Borhidi | *Psychotria acuminata* Benth. |
| *Palicourea eleta* (Sw.) Borhidi | *Psychotria elata* (Sw.) Hammel |
| *Palicourea tomentosa* (Aubl.) Borhidi | *Psychotria poeppigiana* Müll.Arg. |
| *Palicourea winkleri* Borhidi | *Psychotria buchtienii* Standl. |
| *Psychotria carthagenensis* Jacq. * | |
| *Psychotria cooperi* Standl. | |
| *Psychotria pilosa* Ruiz & Pav. | |
| *Psychotria solitudinum* Standl. | |
| *Psychotria tsakiana* C. M. Taylor | |

* *Psy. carthagenensis* was collected in the botanical garden of the University of Vienna (HBV)

### 3.2 Isolation and cultivation of endophytic fungi

Different methods and protocols applied for fungal isolation and cultivation were tested and optimized in preliminary tests. Plant material of cultivated *Psychotria* species of the botanical garden of Vienna (HBV) was collected and processed. Different concentrations of media ingredients and the performance of selective media for isolation was tested as well as the skills necessary for isolation and cultivation trained. Observations of these tests were directly applied to protocols used for collection and cultivation work in Costa Rica and afterwards. The fungus **B17**, isolated from twigs of a well grown and flowering *Psy. carthagenensis* plant cultivated in the green house of the botanical garden, was chosen for first upscale cultivation (fermentation) approaches and extraction.

#### 3.2.1 Media for isolation and cultivation

Fungal growth media containing malt extract, glucose, peptone and deionized water were applied for isolation and cultivation steps. For testing media dependent production of secondary metabolites under fermentative conditions, one semi-artificial peptone medium (SP) was used. Screening for anti-microbial activity was conducted partly on a medium containing yeast extract (MYEA). In order to increase the diversity of cultivable endophytic fungi, selective isolation media were applied as similarly used for the isolation and *a priori* classification of pathogenic fungi (Tsao, 1970). To prevent bacterial growth during isolation and subsequent cultivation the antibiotic chloramphenicol was added. Ingredient concentrations were adapted for different purposes.

The general cultivation media contained 20 g/l glucose, 2 g/l peptone, 12 g/l agar, 100 mg/l chloramphenicol and 20 g/l malt extract (**MEA1**) for fast and 5 g/l malt extract (**MEA2**) for slow cultivation, respectively. For anti-microbial screening, the MEA2 recipe without chloramphenicol (**MEA2-C**) and one with additional 0.5 g/l of yeast extract (**MYEA**) were used. The medium for isolation of endophytes in Costa Rica (**MEA3**) was low in nutrients for slow growth regarding the transportation time: 10 g/l malt extract, 20 g/l glucose, 1 g/l peptone, 12 g/l agar and 100 mg/l chloramphenicol. The liquid medium for fermentative cultivation (**ME**) contained 20 g/l malt extract, 38 g/l glucose, 1.25 g/l peptone and 100 mg/l chloramphenicol. For the semi-artificial solid medium (**SP**) following ingredients were used: 30 g/l glucose, 30 g/l peptone, 12 g/l agar, 100 mg/l chloramphenicol, 0,5 g/l $KH_2PO_4$ and 0,5 g/l $MgSO_4$ (modified from Li et al., 2012). For preservation of fungal cultures by freezing, a liquid cryo medium (**CRY**) composed of 14 % sucrose and 1 % peptone in distilled water was prepared (Engelmeier, 1997).

For the **selective isolation media,** 4 mg/l **benomyl** (**MEA3B**) and 200 mg/l **cycloheximide** (**MEA3C**) were added to the MEA3 recipe, respectively. Benomyl is a thermo-sensitive substance and had to be added to the medium after autoclaving and cooling down to approximately 50 °C (Hutchison, 1990, Summerbell, 1993). A stock solution dissolved in acetone was prepared and added to the medium after sterile filtration through a 0.22 µm syringe filter. Petri dishes were left open under the lamina flow to evaporate excess acetone.

All petri dishes were poured under lamina flow in a sterile working bench. Petri dishes used for isolation in Costa Rica were prepared in Vienna and sealed tightly with rubber tape.

### 3.2.2 Isolation techniques

Fungal endophytes were isolated the same day, in most cases only few hours after plant material was collected. Plant samples were washed under running tap water and obvious dirt, epiphytic mosses and algae cautiously removed. Pieces of approximately 3 cm of shoot and 2 $cm^2$ of leaves including the midrib were prepared for surface sterilization. For the isolation of seed endophytes, seeds were extracted from the fruits and cleaned with tap water and paper tissues. Samples were surface-sterilized by immersion in 70 % ethanol for 1 minute, followed by 5 minutes in 3 % NaOCl solution (household bleach) and again 70 % ethanol for one minute to remove excess hypochlorite solution. Samples were subsequently placed in an empty petri plate to evaporate the remaining ethanol. To check for successful surface sterilization, shoots, seeds and fruits were rolled over and both sides of the leaf cuttings were imprinted on petri dishes containing the isolation medium MEA3, respectively (Petrini, 1984). Petri dishes with imprints were sealed, incubated at room temperature and checked daily for fungal growth. If no fungal colonies developed, surface sterilization was considered successful.

After surface sterilization, margins damaged by NaOCl of leaf and shoot samples were removed. Shoots, fruits and seeds were divided longwise and plated with the cutting side onto the isolation medium. For each collected plant organ several pieces were used for isolation (one per petri dish). Petri dishes were stored in dark at room temperature. Fungal growth was controlled and documented daily as well as all isolates linked to a positive imprint test disposed. Petri dished with emerging fungi were sealed tightly with rubber tape and stored until separation of single fungal strains back in Vienna.

### 3.2.3 Cultivation techniques

Endophytic fungi emerging from plated plant samples were separated by cutting out small agar plugs and placing them on a new medium. Growth was constantly controlled and morphologically distinct strains were separated again until isolates were morphologically homogenous.

Five fungal isolates were assigned to fermentation after first analytical screenings in order to increase the yield of secondary metabolites for preparative isolation of pure compounds. Agar plugs with mycelium were cut from cultures on MEA1 and used to inoculate the liquid (ME) and solid (SP) media. **B17** was cultivated in 800 ml (4x200 ml) ME and 250 ml (2x125 ml) SP medium for 72 days in dark at room temperature. **S1** and **D4** were cultivated in 280 ml ME medium (2x140 ml) in dark at 27 °C for 49 days and additionally in 250 ml medium for 24 days. **C1** and **C4** were grown in 280 ml ME medium (2x140 ml) in dark at 27 °C for 68 days and additionally in 250 ml medium for 43 days.

For long term preservation, samples of all fungal isolates were frozen at -80 °C. Agar plugs were cut out of well-grown cultures and placed in vials filled with cryo medium (CRY). Before freezing, fungi were kept in dark for two days at room temperature.

## 3.3 Agar plug diffusion assay

All fungal isolates were tested for their ability to produce anti-microbial chemicals against bacterial and fungal test organisms. Fungi were grown on MEA2-C or MYEA medium until full colonization of the given medium. Agar plugs of 5 x 5 mm were prepared and transferred to petri dishes covered with test organisms. Results of the screenings were evaluated the next day. Endophytes were tested on *Bacillus subtilis*, *Escherichia coli* DH5α, *Candida albicans* and *Saccharomyces cerevisiae*. The first selected subset of 60 fungal strains was chosen by their ability to produce HPLC-DAD detectable substances in culture and good growth. For the screening of the remaining fungal isolates, *S. cerevisiae* was replaced by *C. albicans*. The change of growth medium from MEA2-C to MYEA was due to a possible increase of secondary metabolite production by the addition of yeast extract to the medium (Zotchev, pers. comm.). Tests were performed in the laboratory of Dr. Sergey B. Zotchev at the Department of Pharmacognosy (University of Vienna).

### 3.4 DNA sequencing and identification

A subset of fungal isolates was identified by sanger sequencing and comparison to the NCBI database by BLAST search. DNA was amplified using the ITS5 forward primer for the fungal isolates C5, D4, E3 and ITS1F primer for the remaining isolates (I8, I9, R5, S1, T2, W8). ITS4 was used as reverse primer. Consensus sequences were generated in SeqMan Pro 14 (DNASTAR®). Fungal DNA was extracted and amplified by Dr. Alexander Urban and sequenced at the Division of Systematic and Evolutionary Botany (University of Vienna).

### 3.5 Analytical methods

Thin layer chromatography (**TLC**) is a commonly used chromatographic tool for the separation and analysis of substance mixtures. Depending on the analyzed sample, different mixtures of organic solvents were applied as mobile phase. Separation was visualized under 254 and 366 nm UV-light for substances with a chromophore and by the application of anisaldehyde spray reagent (85 ml methanol, 10 ml acetic acid, 8 ml $H_2SO_4$, 0.5 ml p-anisaldehyde) and subsequent heating with a hot air gun for substances without chromophore. Pre-coated silica gel 60 TLC aluminum plates (0.2 mm, Sigma Aldrich) were applied for monitoring column chromatography separations and purity of isolated compounds.

High-performance liquid chromatography (**HPLC**) is an advanced analytic tool to separate and analyze substance mixtures. The combination of separation and detection with an UV diode array detector (UV-DAD) provides a characteristic UV-spectrum of the components in addition to their specific retention time. Analysis was performed on an Agilent 1100 series HPLC with a reversed phase column (Hypersil BDS-C18, 250 x 4.6 mm, 5 µm). As mobile phase methanol (**MeOH**) in 10 mM ammonium acetate with a gradient (40 % to 100 % MeOH in 12 minutes) was applied with a flow rate of 1 ml/min and an injection volume of 10 µl. HPLC analysis was used for crude extracts of collected fungal isolates, plant organs and regularly during the isolation process of pure compounds.

Nuclear magnetic resonance spectroscopy (**NMR**) is a technique applied for structure elucidation of pure compounds using information from magnetic properties of atomic nuclei in a strong magnetic field. Pure compounds have been measured by Ing. Susanne Felsinger and results interpreted by Dr. Lothar Brecker at the Institute of Organic Chemistry (University of Vienna). Data regarding structure elucidation is not shown here but will be part of future publications (Hinterdobler et al., in prep.).

### 3.6 Preparative methods

Liquid-liquid extraction (**LLE**) is a basic method for a first separation of complex substance mixtures. To separate culture medium ingredients from the mainly lipophilic compounds of interest of the fermentative cultures, water and chloroform (**$CHCl_3$**) was used.

Size-exclusion chromatography (**SEC**) is one form of column chromatography (**CC**). Sephadex LH 20 was applied as stationary phase and MeOH as isocratic eluent. For fast separation after LLE a column of 50 cm height and 2 cm diameter was used. For a more precise separation a column with 80 cm height and 1.5 cm diameter was chosen.

Medium Pressure Liquid Chromatography (**MPLC**) is in theory a combination of CC and HPLC. The used stationary phase in the column is silica gel with a particle size larger than in HPLC (40-60 µm). MPLC was applied for further separation of LL fractions. The composition of the eluent was changed stepwise from a mixture of petroleum ether (**PE**) and ethyl acetate (**EtOAc**) starting from 10 % EtOAc reaching 100 % for the final elution. The collection of fractions was controlled by UV-VIS detection at 254 nm.

Preparative thin layer chromatography (**PTLC**) was used for final separation of almost pure substances with small amounts (Merck glass plates, silica gel 60, 0.25 mm). As eluent a mixture of 50 % PE and 50 % EtOAc was used. After separation, the single bands were scraped off and the remaining silica gel filtrated.

# 4. Processing of plant and cultured fungal material

## 4.1 Extraction of plant material

Plant organs linked to fungal isolates were analyzed by HPLC for comparison. Between 50 and 65 mg dry sample were pulverized in Eppendorf tubes by freezing in liquid nitrogen and subsequent use of a mixer mill and glass beads. Samples were extracted by an addition of 0.7 ml MeOH and supersonication for 30 minutes. After centrifugation, 0.5 ml of the supernatant were separated and directly applied to HPLC analysis.

## 4.2 Extraction of fungal cultures

Fungal cultures were grown on MEA2 at 27 °C until the media surface was entirely overgrown. One week after the fungi reached the border of the petri dish, samples were taken for HPLC analysis following preliminary tests regarding the start point of griseofulvin production *in vitro* by Viehböck (2015). Surface mycelium and a small layer of agar were scraped off with a sterile scalpel and transferred to Eppendorf tubes. For extraction, 0.7 ml MeOH were added and the samples supersonicated for 30 minutes. After centrifugation, 0.5 ml of the supernatant were separated and directly applied to HPLC analysis. **Guttation droplets** were carefully collected from the surface of the aerial mycelium using pipet tips. For HPLC analysis, droplets were directly dissolved in 0.5 ml MeOH.

## 4.3 Extraction of fungal fermentative cultures

### 4.3.1 Fungal isolate B17

Liquid (ME) and solid medium cultures (SP) were mixed with MeOH, blended and filtrated. The extract from the liquid medium culture was used for pure compound isolation. After evaporation of MeOH, water and $CHCL_3$ were added for LLE. $CHCl_3$ phases of 150 ml were collected 6 times yielding an amount of 184,7 mg dry weight extract. $CHCl_3$ extract was further separated in 18 fractions by SEC (50 x 2 cm column). 34.3 mg of a pure substance (**WH01**) were collected in fraction 3. Fraction 10 was further separated by SEC (80 x 1.5 cm column) in 15 fractions. Fractions 9 to 12 were combined yielding 2.7 mg of a pure substance (**WH02**). Fraction 8 from the first SEC was washed several times with EtOAc resulting in 1.3 mg of a pure compound remaining as precipitate (**WH03**).

### 4.3.2 Fungal isolate D4

Liquid cultures were combined, blended for 3 minutes, ultrasonicated for 30 minutes and directly extracted by LLE 4 times with 200 ml $CHCL_3$. Combined $CHCL_3$ phases yielded 21.3 mg of dry weight extract. Further separation of the substances of interest by MPLC (for eluents see 3.6 Preparative methods) was not successful. Fraction 5 and 6 (together 18.5 mg) were recombined for subsequent SEC separation (80 x 1.5 cm column) which also was not successful. Fraction 6 to 9 from SEC separation were recombined (9.2 mg) and separated using preparative thin layer chromatography with an eluent mixture of PE and EtOAc (1:1). After separation, 3 distinct bands were scraped off and the remaining silica gel filtrated resulting in 3 pure compounds (**WH04** – 2.5 mg, **WH05** – 2 mg and **WH06** – 1.2 mg).

### 4.3.3 Fungal isolates C1 and C4

Liquid cultures of each fungal isolate were combined, blended for 3 minutes, ultrasonicated for 30 minutes and directly extracted by LLE 6 times with 200 ml $CHCL_3$. Combined $CHCL_3$ phases yielded 51.5 mg for C1 and 60 mg for C4 of dry weight extract. Dissolved in MeOH, a precipitate separated at the bottom of the flash in both extracts. The precipitates were washed several times with MeOH for purification (**WH07** from C1 – 12.4 mg, **WH08** from C4 – 3 mg). $CHCL_3$ phase of C1 was further separated by SEC (80 x 1.5 cm column) in 25 fractions. Fractions 3-4, 5 and 13-17 contained pure substances (**WH09** – 6.2 mg, **WH10** – 4 mg and **WH11** – 1 mg).

## 5. Fungal species identification

Sequencing attempts resulted in the identification of nine fungal isolates. BLAST search results with corresponding references are listed under Appendix 3. Consensus sequences were deposited at the NCBI database under the accession numbers KY192275 to KY192283 (Table 2).

Table 2. Identification of fungal isolates

| Isolate | Identification | Host species | Organ | GenBank Accession nr. |
|---|---|---|---|---|
| C5 | *Xylaria* sp. | *Psy. solitudinum* | Leaf | KY192281 |
| D4 | *Xylaria* sp. | *Pal. elata* | Leaf | KY192282 |
| E3 | Xylariaceae | *Pal. acuminata* | Fruit | KY192283 |
| I8 | *Arthrinium arundinis* | *Pal. tomentosa* | Leaf | KY192275 |
| I9 | *Fusarium proliferatum* | *Pal. tomentosa* | Shoot | KY192276 |
| R5 | *Clonostachys* sp. | *Pal. elata* | Shoot | KY192277 |
| S1 | *Fusarium proliferatum* | *Psy. pilosa* | Shoot | KY192278 |
| T2 | *Arthrinium arundinis* | *Psy. tsakiana* | Seed | KY192279 |
| W8 | *Colletotrichum* sp. | *Psy. solitudinum* | Shoot | KY192280 |

Isolate **C5** and **D4** matched with identified *Xylaria* species from GenBank. **E3** shared 99 % similarity with entries belonging to the genera *Nemania*, *Entonaema* and *Xylaria* (Xylariaceae). Members of the Xylariaceae are typically found as endophytes and saprobes in the tropics (Rogers, 2000).

The isolates **I8** and **T2** were identified as *Arthrinium arundinis*. *Arthrinium* species occur as endophytes, human- and phytopathogens and have been proven a good source for novel anti-microbial metabolites (Crous and Groenewald, 2013, Ramos et al., 2010).

**I9** and **S1** were identified as *Fusarium proliferatum,* a typical crop pathogen which is regularly found as an endophyte in healthy leaves of various plant species (Stępień et al., 2011). Like *Fursarium*, also *Colletotrichum* species (**W8**) were found to be present as endophytes in *Theobroma cacao* (Malvaceae) and *Taxus* x media (Taxaceae) (Rubini et al., 2005, Xiong et al., 2013). One *Colletotrichum* species with 99 % ITS similarity was isolated from *Trichilia tuberculata* (Meliaceae) from Costa Rica (GenBank accession number KU204655).

The isolate **R5** was identified as a member of the genus *Clonostachys*. *Clonostachy rosea* was found to be an entomopathogenic fungus of leafhoppers and nematodes (Toledo et al., 2006, Zhang et al., 2008).

## 6. Richness and organ specificity of endophytic fungi

### 6.1 Results

Fungal endophytes were isolated from 15 *Psychotria* and *Palicourea* individuals comprising eight species. Leaves, basal and central shoots, mature and immature fruits and seeds were separately used for fungal isolation. All together 102 fungal strains were collected and further cultivated. Out of 68 petri dishes with the standard isolation medium MEA3, 92 fungal strains were recovered, leading to an **isolation frequency** of 1.35 fungal isolates per plant sample. Out of 18 petri dishes containing benomyl and 20 containing cycloheximide as selective agent, six and four fungal strains were isolated, leading to an isolation frequency of 0.33 and 0.2, respectively. From plant species with two and three sampled individuals, between ten for *Psy. pilosa* and 20 fungal isolates for *Psy. solitudinum* and *Pal. elata* were collected in total. Species with only one sampled individual yielded two to eight separate fungal strains (Table 3). Endophytes were isolated from all sampled seeds.

The proportion of isolates from different plant organs is shown in Figure 1. For *Psy. solitudinum*, *Pal. elata*, *Pal. acuminata* and *Psy. pilosa* between 40 to 65 % (5 to 12) of the isolated endophytes were collected from leaves followed by 25 to 40 % (3 to 8) collected from stems. For *Pal. tomentosa,* 20 % (3) were isolated from leaves and 60 % (9) from stems. Fruits and seeds account for 15 to 20 % (2 to 4 isolates) of the total isolates per plant species.

Table 3. Numbers of isolated endophytic strains and sampled individuals per plant species

| | | Number of fungal isolates | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Host species** | **Individuals** | **Leaf** | **Shoot B*** | **Shoot C*** | **Fruit** | **Fruit I*** | **Seed** | **Total** |
| *Psy. solitudinum* | 3 | 8 | 7 | 1 | | | 4 | 20 |
| *Pal. elata* | 3 | 10 | 7 | | | | 3 | 20 |
| *Pal. acuminata* | 2 | 12 | 2 | 2 | 3 | | | 19 |
| *Pal. tomentosa* | 2 | 3 | 2 | 7 | 2 | | 1 | 15 |
| *Psy. pilosa* | 2 | 5 | 3 | | | | 2 | 10 |
| *Pal. winkleri* | 1 | | 4 | | | 4 | | 8 |
| *Psy. cooperi* | 1 | 2 | | | | | | 2 |
| *Psy. tsakiana* | 1 | | 6 | | | | 2 | 8 |
| | | | | | | | **Total** | **102** |

* Basal (B) shoots, central (C) shoots and immature fruits (I)

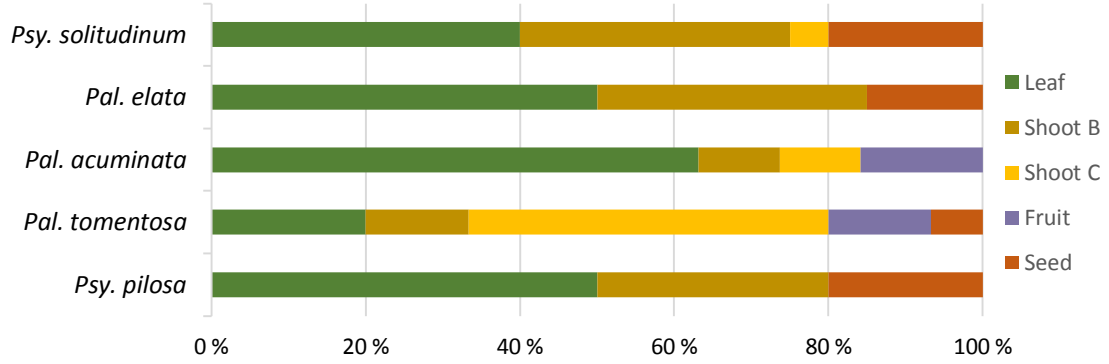


Figure 1. Proportion of fungal isolates from plant organs (leaves, basal (B) and central (C) shoots, fruits and seeds) in percent. Only plant species with more than 10 fungal isolates are shown.

## 6.2 Discussion

Multiple fungal species exist side by side inside the plant tissue, making the repetition of the isolation process necessary to increase the chance of harvesting a high diversity of cultivable strains. Faster growing or more competitive species dealing best with the offered medium, tend to overgrow slower growing or later emerging ones. In order to overcome this phenomenon, selective isolation media containing fungal growth inhibiting substances were applied, as used for the isolation and *a priori* classification of pathogenic fungi (Tsao, 1970). The inhibitory effect of these additives favors a subset of resistant, stress-tolerant or slow growing fungi, increasing the species output.

Beside this technique also the plant fragment size for isolation has influence on the recovered fungal diversity. The reduction of fragment size comes along with an increased cutting border the fungi can grow out of the sample. It is estimated that one leaf fragment of 4 $cm^2$ harbors half of the leaf inhabiting fungal diversity (Gamboa et al., 2002). With the used fragment size and repetition of the process in the present work, 1.35 fungal strains per plant sample were isolated. Gamboa and Bayman (2001) reached an isolation frequency of 1.4 fungal species per sample for *Guarea guidonia* (Meliaceae) with 20 $mm^2$ fragments and increased the frequency to 2.9 by the use of 4 $mm^2$ pieces. The time between collection work in Costa Rica and axenic monoculture is also considered a major factor influencing the isolation frequency. Growing together in one petri plate, representing an artificial environment, more competitive fungi can overgrow others, leading to a less representative fungal composition.

The use of selective media reduced the isolation frequency to a fraction in comparison to the standard medium. The low output indicates a strong suppression of growth for most fungi emerging on the standard medium. Without further identification it is unclear if this method led to an increase

of species diversity. For isolation work in Costa Rica, benomyl and cycloheximide were in prior added to the isolation medium. Benomyl was shown to inhibit ascomycetous fungi but has minor effects on basidiomycetes (Summerbell, 1993). Cycloheximide is an antifungal antibiotic and like benomyl used for fungal classification (Salkin, 1975).

The successful increase of diversity by the addition of selective agents to the isolation medium depends on the fungal species present as endophytes and the processed plant organ. Latter is argued by Bills and Polishook (2000) as a possible result of higher diffusion of the selective agent into small leaf fragments. Despite an increased isolation of fungal endophytes from *Carpinus caroliniana* (Betulaceae) bark disks using cycloheximide medium, the isolation using cycloheximide and benomyl for *Chamaecyparis thyoides* (Cupressaceae) leaves hindered fungal growth and isolation (Bills and Polishook, 1992, Bills and Polishook, 1991). An optimization of the isolation process regarding the plant's properties and different organs might increase the output of cultivable endophytes.

The ubiquity of fungal endophytes in seeds is striking. Vertical transmission of endophytes within plant seeds in contrast to horizontal transmission via spores was highlighted for several herbaceous eudicots. Endophytes found within and on pollen were also collected from seeds, leading to the assumption of an infection of seeds via the pollen tube (Hodgson et al., 2014). Some vertically transmitted fungi have lost their ability to produce spores and thus rely on the distribution via the plant (Steiner et al., 2006; Steiner et al., 2012). Bacterial endophytes collected from *Psychotria* were found to be partly transferred vertically (Lemaire et al., 2012a). The imperfect nature of the vertical transmission phenomenon was shown for grass endophytes. In grasses, fungal infection can be lost within the plant, during seed production and during the germination process (Afkhami and Rudgers, 2008). The early infection of the plant seedling is thought to be a first ecological defense mechanism against pathogens as it was shown for the tropical tree *Theobroma cacao* (Malvaceae) (Hodgson et al., 2014, Arnold et al., 2003).

The expansion of the screening process to anthers and pollen in future studies as well as the controlled infection of *in vitro* cultivated plants with endophytes might give insights in early fungal colonization tendencies and potential protection mechanisms for the seedling.

# 7. Comparative analysis of media dependent secondary metabolite production

## 7.1 Results

Shifts in secondary metabolite production on different growth media was observed in the fungal isolates S1, B17 and C1. **S1**, identified as *Fusarium proliferatum*, which produced three substances with similar UV spectrum but different retention time when growing on MEA2. Cultivated under fermentative conditions in liquid medium (ME), these metabolites were no longer detectable and therefore the extract not further fractionated. The fungus **B17** was analyzed growing in standard liquid fermentative (ME) and solid semi-artificial medium (SP). In total, three pure substances were isolated and purified from B17 (WH01, WH02, WH03). Metabolites WH02 and WH03 were only present in the liquid fermentative medium. WH01 was produced by the fungus in both media (Figure 2).

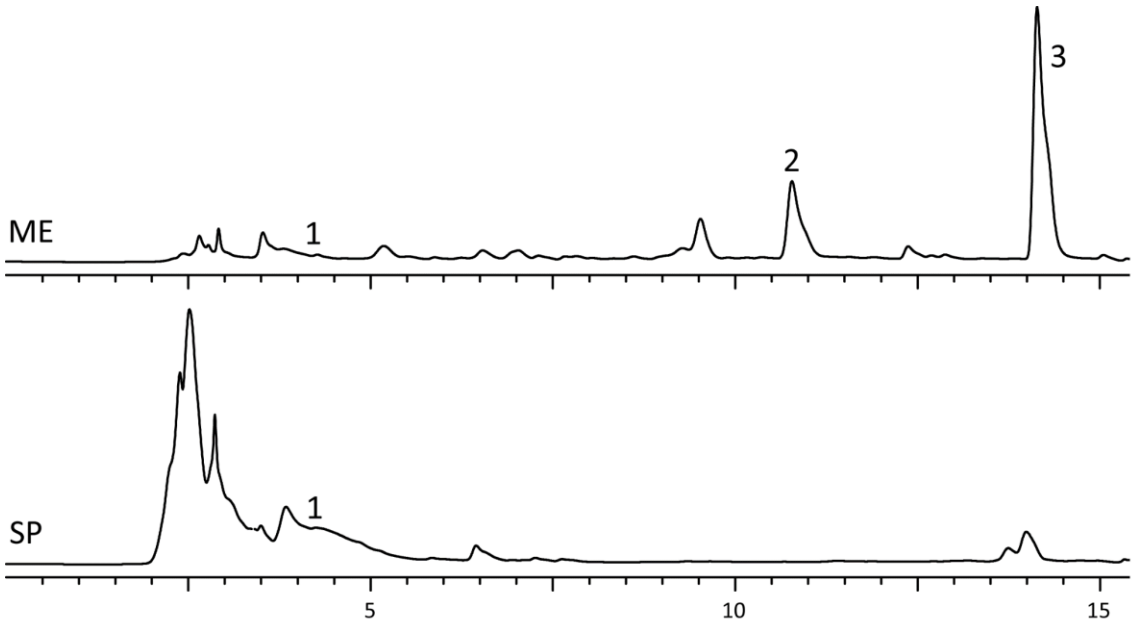


Figure 2. Comparison of HPLC chromatograms at 230 nm of the secondary metabolite profiles of B17 growing in liquid fermentative (ME) and solid semi-artificial medium (SP). The isolated substances WH02 (2) and WH03 (3) were only produced by the fungus growing on ME. WH01 (1) was produced in both media. Pile of peaks on the left represents hydrophilic compounds from the growth medium.

The fungal isolate **C1** was analyzed both from malt extract agar (MEA2) and the liquid fermentative medium ME. Growing on the agar medium, C1 produced griseofulvin and 7-dechlorogriseofulvin. Under fermentative conditions, these two substances were no longer part of the metabolite profile. Different compounds were detected and identified as piliformic acid (WH09) and cytochalasin D (WH10) (Figure 3).

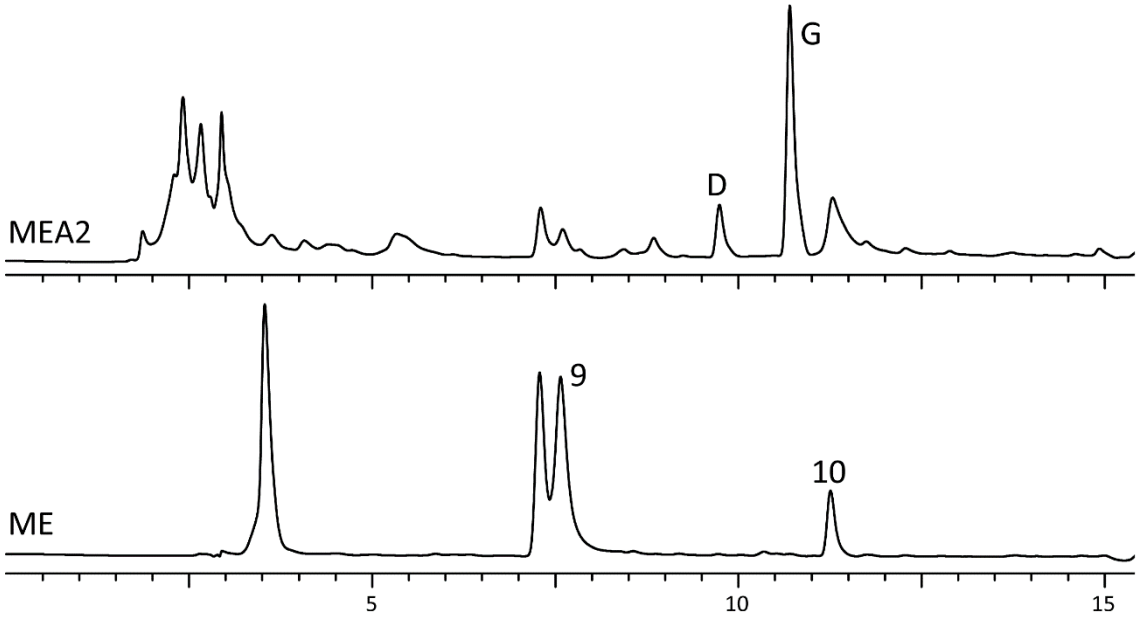


Figure 3. Comparison of HPLC chromatograms at 230 nm of the secondary metabolite profiles of C1 growing on malt extract agar (MEA2) and liquid fermentative medium (ME). Griseofulvin (G) and 7-dechlorogriseofulvin (D) were only produced by the fungus growing on MEA2. Piliformic acid (9) and cytochalasin D (10) were produced by the fungus growing on the liquid medium ME. Pile of peaks on the left represents hydrophilic compounds from the growth medium which were separated by LLE from the $CHCl_3$ phase shown for ME.

## 7.2 Discussion

Fungi for upscale cultivation were chosen by their ability to produce HPLC detectible substances on MEA medium not matching with our labs spectral library. In order to increase the yield of pure isolated compounds for later structure elucidation, fungi were grown under liquid fermentative conditions. Differing only in the absence of agar in the medium, griseofulvin and 7-dechlorogriseofulvin were no longer produced by C1 growing in the liquid medium. A similar change in the metabolite profile was observed for the isolate S1. The change from solid to liquid medium is therefore sufficient to trigger different metabolite production. Liquid media not only lack agar but differ in the availability of oxygen and the amount of available nutrients.

For B17 the difference in the secondary metabolite profile between solid semi-artificial and liquid medium might be caused by the lack of specific nutrients for secondary metabolism in the semi-artificial medium. Paranagama et al. (2007) reported a shift from the production of the polyketides chaetochromin A in liquid medium to radicicol as the main metabolite produced on solid medium by *Chaetomium chiversii*. Furthermore, an addition of six substances to the metabolic profile of *Paraphaeosphaeria quadriseptata* was overserved by adding tap water instead of distilled water to the medium.

Small changes in culture conditions are a suitable method for the discovery of otherwise silent biochemical pathways. The screening for a broad fungal metabolite spectrum by a systematic change of culture conditions is highlighted in the OSMAC approach (one strain many compounds) by Bode et al. (2002). Using this approach, novel substances were described for *Streptomyces* species, sponge associated and endophytic fungi (Rateb et al., 2011, Christian et al., 2005, Hewage et al., 2014). Considering the effects of slight modifications of the medium composition, oxygen availability or temperature to the production of metabolites, the output of novel structures can be increased at small scale for few fungal strains. For screenings of many strains at once, the OSMAC approach would exceed laboratory capacities of labor and material. Standard rice medium was shown to be most efficient for fast screenings regarding amount and diversity of produced secondary metabolites (VanderMolen et al., 2013).

Regarding the high variability of fungal secondary metabolite production *in vitro*, the present results are viewed as preliminary due to limited variation of media applied. The total loss or appearance of single substances in the metabolite profile gives a hint to the vast, unseen biochemical potential of fungi. The optimization of the fermentative process and the use of different media is crucial for the isolation of new secondary metabolites.

# 8. Comparative analysis of fungal and plant crude extracts

## 8.1 Results

For comparative analysis of plant and fungal secondary metabolites, fungal strains and the corresponding host plant organs were analyzed by HPLC. In addition to our already existing HPLC spectral library of *Psychotria* and *Palicourea* substituents, a new database was created. All UV-DAD detectable substances produced by the investigated fungi on the given medium were added to the database containing retention time and UV spectrum. Subsequently, plant organ extracts were cross checked for the production of substances known from fungal cultures. None of the fungal metabolites produced in culture were found in the plant crude extracts.

## 8.2 Discussion

A discrepancy between compositions of plant extracts and fungal endophyte extracts is not uncommon. This may be due to several aspects inherent in fungal cultivation techniques. Firstly, the cultivation of fungi on artificial medium has great influence on the produced secondary metabolite profile as shown in chapter 7. Secondly, many metabolites are not produced constitutively but as a reaction to an environmental change, pathogenic attack or change in the life cycle (Calvo et al., 2002). In addition to a possible influence of culture conditions, also the small proportion of fungal biomass within the analyzed plant samples has to be taken into account.

The influence of fungal endophytes on the biosynthesis of plant secondary metabolites is of outstanding interest. Beside the *de novo* production of metabolites, a shared biosynthesis by the combination of plant and fungal metabolism is possible (Ludwig-Müller, 2015). Yet unclear biosynthetic origins of classical plant secondary metabolites might be uncovered by incorporating enzymatic capacities of endophytic fungi.

## 9. Detection and structure elucidation of fungal secondary metabolites

At the time this thesis was finished, two purified compounds extracted from the fungus C1 were identified (WH09, WH10). The metabolites griseofulvin and 7-dechlorogriseofulvin were identified by comparison to commercially available samples (Sigma Aldrich). Structures of remaining isolated compounds (WH01-WH08 and WH11) will be part of future publications (Hinterdobler et al., in prep.).

### 9.1 Griseofulvin and 7-dechlorogriseofulvin

The fungal metabolite griseofulvin (Figure 4) was identified in 35 of the 102 (34.3 %) fungal isolates growing on MEA2 medium. 7-Dechlorogriseofulvin was present in 30 isolates containing griseofulvin (Appendix 2).

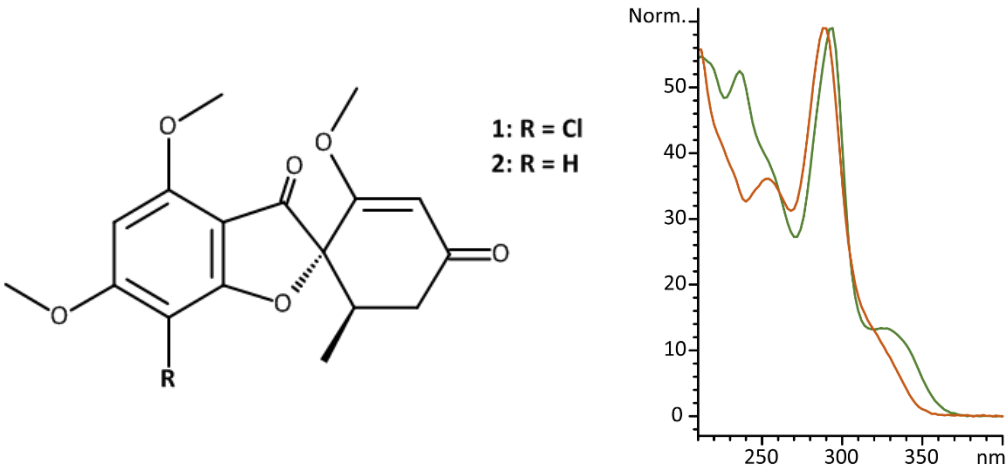


Figure 4. Structure and UV spectra of griseofulvin (**1**, green) and 7-dechlorogriseofulvin (**2**, orange).

Griseofulvin is an antifungal secondary metabolite produced by various fungal genera and was first isolated from *Penicillium griseofulvum* (Petersen et al., 2014, Oxford et al., 1939). The so called *curling factor* produced by *P. janczewskii*, which leads to abnormal growth of hyphae in co-cultured fungi was identified as griseofulvin by Brian et al. (1949).

Griseofulvin was already isolated from an endophytic *Xylaria* species from *Pal. marcgravii* and was shown to be active against *Cladosporium cladosporioides* and *C. sphaerospermum* (Cafêu et al., 2005). Furthermore, griseofulvin is produced *in vitro* by *Xylaria cubensis*, an endophyte of *Asimina triloba* (Annonaceae) und *Silybum marianum* (Asteraceae), and of one *Nigrospora* species isolated from *Moringa oleifera* (Moringaceae) (Sica et al., 2016, Zhao et al., 2012). This antifungal metabolite seems to be produced *in vitro* to defend the limited colonized medium.

## 9.2 Piliformic acid and cytochalasin D

The fungal isolate C1 produced griseofulvin and 7-dechlorogriseofulvin on solid MEA2 medium (see chapter 7). Under fermentative conditions, the compounds piliformic acid (WH09) (Figure 5) and cytochalasin D (WH10) (Figure 6) were produced in sufficient amounts for isolation and NMR structure elucidation.

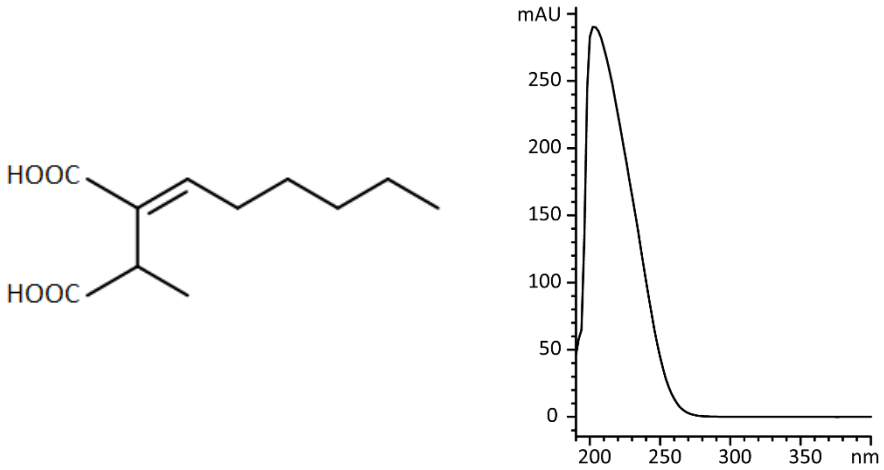


Figure 5. Structure and UV spectrum of piliformic acid isolated from C1

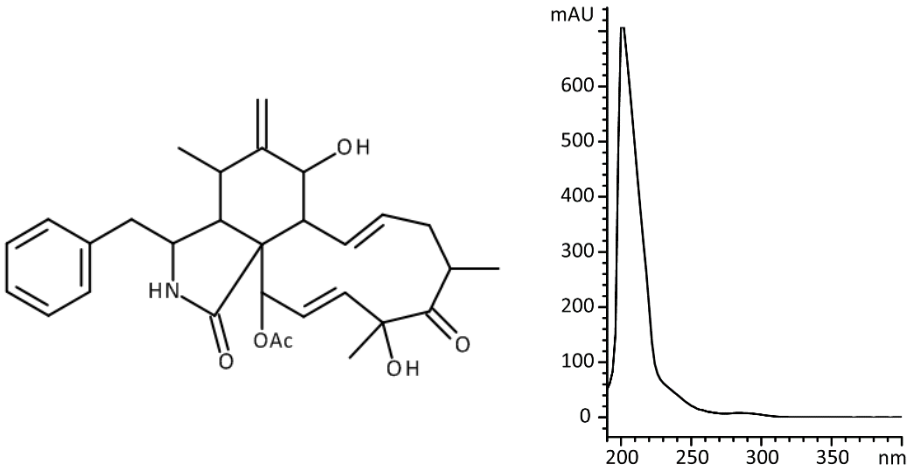


Figure 6. Structure and UV spectrum of cytochalasin D isolated from C1

Piliformic acid (2-hexylidene-3-methylsuccinic acid) is commonly found to be synthesized by xylariaceous fungi and close realatives (Chesters and O'Hagan, 1997). It was further identified to be part of the chemical profile of one *Xylaria* species isolated from mangrove trees in South China and the marine fungus *Halorosellinia oceanica* from Thailand (Liu et al., 2006, Chinworrungsee et al., 2001).

Cytochalasin D is a well-known fungal metabolite with actin polymerization inhibiting properties (Casella et al., 1981). Cytochalasins are produced by a wide range of ascomycetous and basidiomycetous fungal genera (Scherlach et al., 2010). Cytochalasin derivatives have been isolated from various fungal endophytes, e.g. belonging to the genera *Aspergillus*, *Chaetomium* and *Xylaria* (Lin et al., 2009, Ming Ge et al., 2008, Espada et al., 1997). The here described cytochalasin D was found in one *Tubercalaria* species isolated from *Taxus mairei* (Taxaceae) and one *Xylaria* species isolated from *Pal. marcgravii* (Li et al., 2009, Cafêu et al., 2005).

Besides cytochalasin D, also griseofulvin and 7-declorogriseofulvin were reported from the mentioned *Xylaria* species isolated from *Pal. marcgravii*. Additionally, cytochalasin B and a structural isomer from piliformic acid, 2-hexyl-3-methyl-butanodioic acid have been described from this endophyte (Cafêu et al., 2005). Griseofulvin, succinic acid and cytochalasin derivatives produced under culture conditions were further used for chemo-systematic studies within the Xylariaceae (Whalley and Edwards, 1995).

## 10. Analysis of aerial guttation droplets

### 10.1 Results

Guttation droplets from the four fungal isolates C1, D1, L1 and W2 were collected and their secondary metabolite composition compared to the corresponding fungal crude extracts (Figure 7). Compounds were not calibrated, so chromatograms show qualitative differences only. Griseofulvin and 7-dechlorogriseofulvin, produced by all examined species on the applied agar medium (MEA1), were deposited partly in the guttation droplets. In addition, hydrophilic medium ingredients and the antibiotic additive chloramphenicol were also transported and stored in the guttation liquid.

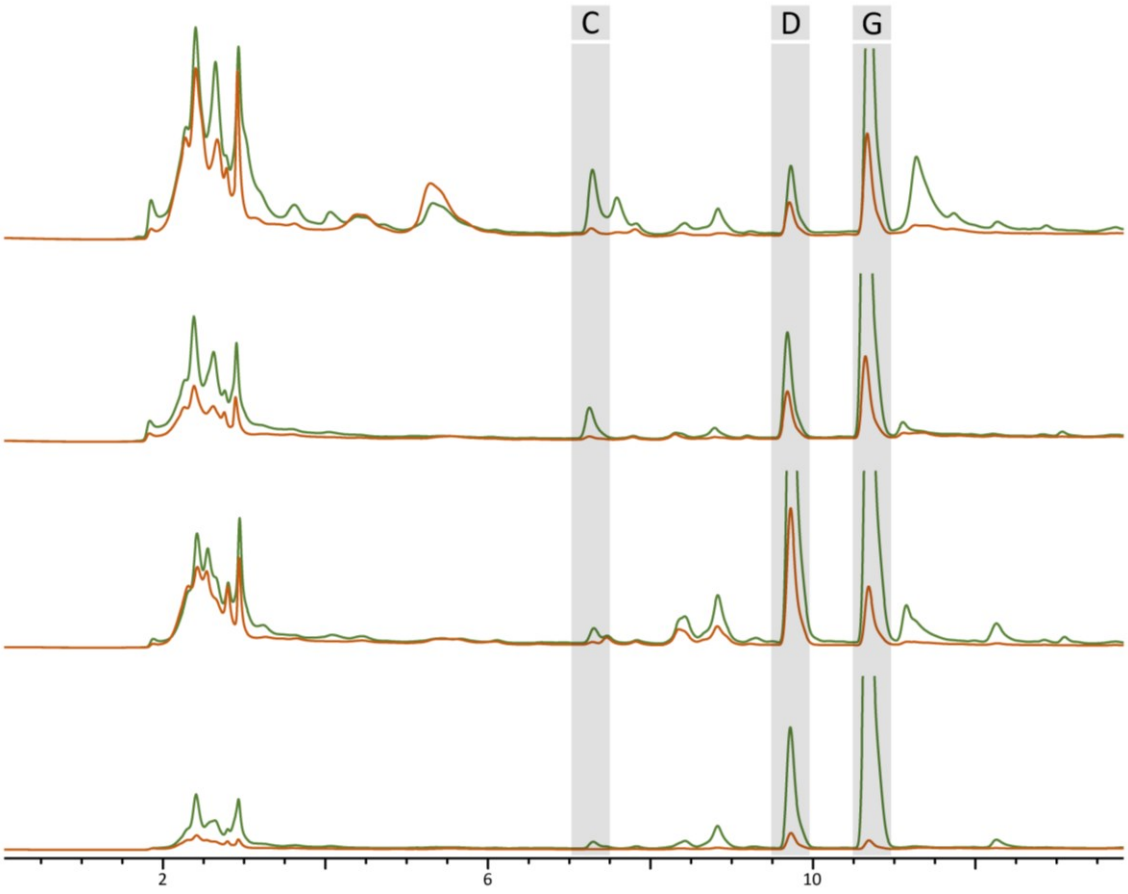


Figure 7. Qualitative comparison of HPLC chromatograms at 230 nm of collected guttation droplets (orange) and the crude extracts of the corresponding fungus (green). From top to bottom: C1, D1, L1, W2. Pile of peaks on the left represents hydrophilic compounds from the growth medium MEA1. Highlighted peaks: Chloramphenicol (C), 7-dechlorogriseofulvin (D) and griseofulvin (G). High peaks were cut for better illustration.

## 10.2 Discussion

The excretion of liquid exudates on fungal fruit bodies in their natural habitat is known to most mushroom collectors and connoisseurs. Under axenic monoculture conditions some fungal strains also tend to produce exudate droplets on top of their aerial mycelium. These sequestrations are well documented in literature and their chemical constituents have been investigated for a variety of fungal species (Gareis and Gareis, 2007, Gareis and Gottschalk, 2014, Hutwimmer et al., 2010). In analogy to a similar plant phenomenon, these excretions are also called guttation droplets. Regarding their function, external storage of secondary metabolites and water to cope with the unfavorable environment of the growth media were discussed (McPhee and Colotelo, 1977, Jennings, 1991). The presence of griseofulvin in guttation droplets at an even higher concentration than in the mycelium was observed by Sica et al. (2016) for *Xylaria cubensis*. External storage of antifungal substances like griseofulvin might be used as a backup defense system to hold on to the colonized, limited medium. The medium ingredients present in the guttation droplets seem to be a byproduct of the sequestration of water.

The occurrence of small amounts of chloramphenicol in the droplets is a direct evidence that this substance is taken up by the fungus. This raises the question if the use of chloramphenicol in the growth medium has influence on the growth or even on secondary metabolite production. However, the deposition of chloramphenicol indicates that it is not metabolized until it reaches the guttation droplets. Its function as a trigger for substance production or being partly used as substrate cannot be excluded.

# 11. Screening for anti-microbial effects

## 11.1 Results

Endophytic fungi have been reported a promising source for anti-microbial compound isolation (Mousa and Raizada, 2013). Fungal cultures produced and secreted metabolites which diffused into the agar medium. From all isolated endophytes, these substances were tested for their anti-microbial effects on *E. coli* and *B. subtilis*. Additionally, a subset of fungi was tested against *S. cerevisiae* and the remaining on *C. albicans* (Table 4). Due to further diffusion into the bacterial growth medium, a zone of inhibition was visible around the agar plug if an antibiotic substance was present. For *E. coli*, only the fungus C5 showed bacterial growth inhibition around the plated agar plug. The growth of *B. subtilis* was inhibited by seven fungal isolates. Three of them (E3, I3, W8) were cultivated on MYEA and four (I9, R5, S1, T2) on MEA2-C. The isolates I8 and N1 inhibited growth of *S. cerevisiae*. None of the endophytes showed inhibitory effects against *C. albicans*.

Table 4. Fungal isolates with anti-microbial effects on applied test organisms

| | | Microbial test organisms | | | |
|---|---|---|---|---|---|
| **Fungal isolate** | | ***E. coli*** | ***B. subtilis*** | ***S. cerevisiae*** | ***C. albicans*** |
| C5 | *Xylaria* sp. | x | - | - | |
| E3 | *Xylaria* sp. | - | x̶ | | - |
| I3 | | - | x̶ | | - |
| I8 | *Arthrinium arundinis* | - | - | x | |
| I9 | *Fusarium proliferatum* | - | x | - | |
| N1 | | - | - | x | |
| R5 | *Clonostachys* sp. | - | x | - | |
| S1 | *Fusarium proliferatum* | - | x | - | |
| T2 | *Arthrinium arundinis* | - | x | - | |
| W8 | *Colletotrichum* sp. | - | x̶ | | - |

No inhibition (-), inhibition growing on MEA2-C (x) and MYEA (x̶) medium

## 11.2 Discussion

In total, 9.8 % of the tested fungi showed growth inhibiting effects against one of the applied test organism. The two *Arthrinium arundinis* isolates (I8 and T2) showed different effects on the applied test organisms and are therefore regarded as chemotypes. Antibacterial activity against *B. subtilis* was earlier reported for one *Xylaria* species isolated from *Psy. bisculata* (Govinda Rajulu et

al., 2013). Regarding the tight interaction of endophytes with their host and other microorganisms residing within the same limited space, the ability to produce anti-bacterial and anti-fungal substances is essential. The defense of the colonized fungal habitat comes hand in hand with an increased defense of the host plant against pathogens (Arnold et al., 2003).

These results highlight fungal endophytes from *Psychotria* and *Palicourea* species to be promising sources for the discovery of anti-microbial compounds. As it has been described in chapter 7, secondary metabolite production is highly dependent on the composition of the cultivation medium. The agar plug diffusion assay is a basic test for the evaluation of *a priori* produced defense chemicals. As the active principle of the inhibition is not known, this screening underlines the potential for the discovery of anti-microbial substances from the examined endophytes but leaves open further questions on the structure and media dependency of the involved metabolites.

## 12. Evaluation of applied methods

Research on endophytic fungi is highly dependent on the applied methods used for isolation and cultivation. Therefore, the methods chosen for this thesis are evaluated and discussed in view of other techniques not applied in the present study.

Several **surface sterilization** procedures using NaOCl, ethanol or formaldehyde for plant samples have been evaluated and proven to be suitable for endophyte isolation by Schulz et al. (1993). Tropical environments are known for their commonly occurring epiphytic mosses, ferns and algae. Considering the highly colonized surfaces of tropical plants, a rather strong sterilizing protocol was used for the isolation process in Costa Rica. Highly sensitive endophytes might be harmed also inside the plant tissues by the applied disinfectants. This effect has to be taken into account regarding the cultivated endophytic diversity seen after isolation. One inventive method to speed up surface sterilization by bulk processing was published by Greenfield et al. (2015).

The diversity of isolated fungi is strongly affected by the ability of the fungi to grow on the applied **isolation media**. Many fungal strains and species will stay undetected if the given medium does not fit their needs for cultivation (Schulz and Boyle, 2005). Beside the limited isolated diversity, one must reconsider the definition of endophytes. In this thesis, all fungi growing within the sampled, healthy plant organ are considered endophytes. This may also include latent pathogens which do not trigger any visible virulence symptoms in the plant at the moment of harvesting. The used anti-fungal additives in the **selective isolation medium** were chosen by their availability in the laboratory. Besides cycloheximide, benomyl and the anti-bacterial chloramphenicol several other anti-fungal and anti-bacterial additives (e.g. cyclosporine A, natamycin and rose bengal) are commonly used for preselective isolation of fungal endophytes (Stone et al., 2004). In regard of fungal endosymbionts, the use of antibiotics in the isolation and cultivation medium can lead to a loss of endohyphal bacteria in fungal cultures (Hoffman and Arnold, 2010).

The **fungal isolate B17** used for fermentation and isolation of pure compounds was collected from a plant growing for many years in the botanical garden of the University of Vienna. Being in contact with plants and their associated microbes from all over world, the fungal endophyte composition of this *Psy. carthagenensis* individual could be influenced by a loss of endophytes and recolonization from others. Therefore, the endophyte B17 is not considered a typical and naturally occurring endophyte of *Psy. carthagenensis*.

Limited supply of petri dishes during the field work in Costa Rica did not allow fast separation and **axenic cultivation** of the emerging fungi. The result was fully overgrown petri dishes back in the laboratory in Vienna. Nevertheless, morphologically distinct strains could be separated.

The diversity of cultivated fungi was for sure lower due to competition within the petri dish and overgrowing. An improvement of the protocol for the establishment of axenic monocultures in time and technique seems to be necessary for future isolation attempts. The transfer into new petri dishes could be improved by carefully transferring mycelium than whole agar plugs, also in the context of possible co-cultivation of several strains within one apparent axenic culture.

Fungal crude extracts for **analytical screenings** and the comparison to plant extracts were taken from colonies growing on solid malt extract agar. As highlighted in chapter 7, the use of rice medium for large screenings might yield better results regarding the production of fungal secondary metabolites (VanderMolen et al., 2013). Focusing on bioactive metabolites, a bioactivity guided screening (e.g agar plug diffusion assay) *a priori* to analytic measurements should be taken into account in order to decrease working time and material (Strobel, 2003). For following **fermentation** attempts the OSMAC approach with different media can be used to cover a greater possible metabolite output than by the use of one sole medium (Bode et al., 2002). Several media for secondary metabolite production and extraction of fungal cultures were discussed by Frisvad (2012). Furthermore, the addition of plant crude extracts, microbial elicitors or co-cultivation of two fungi in one petri dish can positively influence the production of novel secondary metabolites (Aly et al., 2010, Chagas et al., 2013).

One step towards optimizing the diversity of cultivable endophytic fungi is the **dilution-to-extinction technique**, as used for leaf litter samples (Collado et al., 2007). Surface sterilized leaves are homogenized in sterile water using an electric blender. Particles of 100 – 200 µm are separated and collected after several filtration steps. After washing and dilution of the remaining particles, a small amount of the suspension is pipetted onto multiwell plates containing MEA medium. In theory, if the dilution series fit the examined material, only one endophyte should grow per well without influence from other, possibly faster growing and more competitive strains (Unterseher and Schnittler, 2009).

## 13. Conclusio

*Psychotria* and *Palicourea* species growing in Costa Rica were shown to be a promising source for fungal endophyte research. Also the area of sampling at the Pacific side of Costa Rica with an annual precipitation of 5836 mm and an average temperature of 28.5 °C provides a suitable habitat for fungi of all kinds (Weissenhofer and Huber, 2008, Piepenbring and Ruiz-Boyer, 2008). In the course of this thesis, methods for endophyte isolation and cultivation were established and their limitations discussed. In addition, several results were obtained from comparative analytic, secondary metabolite extraction and bioassay approaches.

The first hypothesis could be confirmed for all sampled plant organs. Endophytic fungi occur in leaves, shoots, fruits and seeds from *Psychotria* and *Palicourea* species. The results indicate that many fungi live side by side within the plant tissue and share this limited habitat. Possible occurrence of fungal endophytes in and on pollen grains needs to be further investigated in future studies.

The limited number of sequenced fungal isolates gives only a hint for answering the second hypothesis. Identified fungi belong to the genus *Xylaria* (Xylariaceae) and related genera. The vast occurrence of cultivable *Xylaria* endophytes in tropical plants seems to also apply for *Psychotria* and *Palicourea* species.

The accumulation of plant like secondary metabolites in fungal cultures, the third hypothesis, could not be confirmed. Due to the high dependency of secondary metabolite production in fungi on the applied media and techniques, this hypothesis might be confirmed in future studies with an enhanced protocol for cultivation and extraction.

The forth hypothesis could be confirmed. Isolated fungal endophytes have the ability to produce anti-microbial defense chemicals against the applied test organisms. Especially these results leave open questions regarding the active principle of the inhibition and its media dependent production.

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## 15. Summary / Zusammenfassung

The aim of this work was to study endophytic fungi associated with eight *Psychotria* and *Palicourea* (Rubiaceae) species growing in a lowland tropical rainforest of Costa Rica. A total of 102 endophytic fungal strains were collected from leaves, shoots, fruits and seeds. Fungi belonging to the genera *Xylaria*, *Arthrinium*, *Fusarium*, *Clonostachys* and *Colletotrichum* were identified by molecular methods. Cultivation medium dependent production of fungal secondary metabolites was monitored on different media. Fungal and plant crude extracts were compared for similarities in secondary metabolite composition. Piliformic acid and cytochalasin D were isolated from fermentative cultures. Griseofulvin and 7-dechlorogriseofulvin were identified by direct comparison with reference compounds. In addition, the chemical composition of fungal guttation droplets was analyzed and compared to corresponding fungal crude extracts. Anti-microbial effects of the fungal isolates were tested by agar-plug diffusion assay on *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae*. In total, 10 isolated endophytes (9.8 %) showed inhibitory effects. The results provide information on the occurrence of fungal endophytes from *Psychotria* and *Palicourea* species and their potential to produce anti-microbial secondary metabolites. Results and applied methods were critically evaluated and experimental adaptations for future studies are suggested.

Das Ziel dieser Arbeit war die Untersuchung von endophytischen Pilzen aus acht *Psychotria* und *Palicourea* Arten (Rubiaceae) aus einem Tieflandregenwald in Costa Rica. Insgesamt wurden 102 Pilzkulturen aus Blättern, Sprossen, Früchten und Samen isoliert. Pilze der Gattungen *Xylaria*, *Arthrinium*, *Fusarium*, *Clonostachys* und *Colletotrichum* wurden mittels Sequenzierung und Datenbankabgleich identifiziert. Die medium-abhängige Produktion von Sekundärmetaboliten in Pilzen wurde auf verschiedenen Medien getestet. Extrakte von Pflanzen und Pilzen wurden auf Ähnlichkeiten ihrer Inhaltsstoffausstattung untersucht. Die Reinstoffe Piliformic acid und Cytochalasin D wurden als Reinstoffe isoliert. Griseofulvin und 7-Dechlorogriseofulvin wurden mittels Vergleich zu Referenzsubstanzen identifiziert. Zusätzlich wurde die chemische Zusammensetzung von Guttationstropfen einiger Pilzisolate mit den dazugehörigen Rohextrakten der Pilze verglichen. Antimikrobielle Eigenschaften der isolierten Endophyten wurden an *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* und *Saccharomyces cerevisiae* getestet. Es zeigten 10 Isolate (9.8 %) einen wachstumshemmenden Effekt. Diese Ergebnisse dokumentieren das Vorkommen von endophytischen Pilzen in *Psychotria* und *Palicourea* Arten und deren Potential, antimikrobielle Substanzen zu bilden. Die Ergebnisse und verwendeten Methoden wurden kritisch bewertet und experimentelle Anpassungen für zukünftige Studien vorgeschlagen.

# 16. Appendix

## 16.1 List of plant specimens

Appendix 1. GPS data, collection and herbarium number of used plant specimens

| Plant species | Collection nr. | Coordinates | | Herbarium nr. |
|---|---|---|---|---|
| *Psy. solitudinum* | WH-181115-1 | 8°41'59.12"N | 83°12'7.68"W | WU 1074827 |
| *Psy. solitudinum* | WH-241115-2 | 8°41'20.23"N | 83°12'29.70"W | WU 1074817 |
| *Psy. solitudinum* | WH-261115-4 | 8°41'45.36"N | 83°12'13.56"W | WU 1014701 |
| *Psy. solitudinum* | WH-301115-4 | 8°42'4.54"N | 83°12'16.91"W | WU 1074812 |
| *Pal. elata* | WH-181115-2 | 8°41'59.11"N | 83°12'7.52"W | WU 1074829 |
| *Pal. elata* | WH-281115-3 | 8°41'58.17"N | 83°12'2.90"W | WU 1074803 |
| *Pal. elata* | WH-301115-3 | 8°42'5.71"N | 83°12'16.88"W | WU 1074810 |
| *Pal. acuminata* | WH-211115-1 | 8°41'20.51"N | 83°12'29.44"W | WU 1074820 |
| *Pal. acuminata* | WH-281115-2 | 8°41'58.22"N | 83°12'3.01"W | WU 1074802 |
| *Pal. tomentosa* | WH-261115-1 | 8°41'27.28"N | 83°12'25.79"W | WU 1014696 |
| *Pal. tomentosa* | WH-261115-2 | 8°41'27.88"N | 83°12'25.28"W | WU 1074813 |
| *Psy. pilosa* | WH-261115-3 | 8°41'38.21"N | 83°12'18.92"W | WU 1014700 |
| *Psy. pilosa* | WH-301115-1 | 8°42'3.43"N | 83°12'15.22"W | WU 1074804 |
| *Pal. winkleri* | WH-241115-1 | 8°41'20.35"N | 83°12'29.10"W | WU 1074818 |
| *Pal. capitata* | WH-241115-3 | 8°41'20.07"N | 83°12'29.35"W | WU 1074814 |
| *Psy. cooperi* | WH-281115-1 | 8°41'59.61"N | 83°12'6.85"W | WU 1014702 |
| *Psy. tsakiana* | WH-301115-2 | 8°42'4.97"N | 83°12'17.01"W | WU 1074809 |

## 16.2 List of fungal isolates

Appendix 2. List of fungal isolates from *Psychotria* and *Palicourea* species with collection number, plant organ they were isolated from and applied isolation media. Isolation medium MEA3 (A), selective isolation medium with benomyl (B) and cycloheximide (C). Production of griseofulvin, 7-dechlorogrisofulvin and no UV-DAD detectible production of secondary metabolites on MEA2 medium. Positive bioassay on *E. coli* (EC), *B. subtilis* (BS), *S. cerevisiae* (SC) and *C. albicans* (CA). * *Psy. carthagenensis* was collected in the botanical garden of the University of Vienna (HBV).

| Fungal isolate | Host species | Collection no. | Organ | Isolation medium | No production | Griseofulvin | 7-Dechlorogriseofulvin | Positive bioassay |
|---|---|---|---|---|---|---|---|---|
| **B17** | *Psy. carthagenensis* | * | Shoot | A | | | | |
| **C1** | *Psy. solitudinum* | WH-181115-1-C1 | Seed | A | | X | X | |
| **C2** | *Psy. solitudinum* | WH-181115-1-C2 | Seed | A | X | | | |
| **C3** | *Psy. solitudinum* | WH-181115-1-C3 | Seed | A | | X | X | |
| **C4** | *Psy. solitudinum* | WH-181115-1-C4 | Seed | A | | | | |
| **C5** | *Psy. solitudinum* | WH-181115-1-C5 | Leaf | A | | | | EC |
| **C6** | *Psy. solitudinum* | WH-181115-1-C6 | Leaf | A | | X | X | |
| **C7** | *Psy. solitudinum* | WH-181115-1-C7 | Leaf | A | | X | X | |
| **C8.1** | *Psy. solitudinum* | WH-181115-1-C8.1 | Leaf | A | | X | X | |
| **C8.2** | *Psy. solitudinum* | WH-181115-1-C8.2 | Leaf | A | | | | |
| **D1** | *Pal. elata* | WH-181115-2-D1 | Leaf | A | | X | X | |
| **D2** | *Pal. elata* | WH-181115-2-D2 | Leaf | A | X | | | |
| **D3** | *Pal. elata* | WH-181115-2-D3 | Leaf | A | | X | X | |
| **D4** | *Pal. elata* | WH-181115-2-D4 | Leaf | **C** | | | | |
| **E1** | *Pal. acuminata* | WH-211115-1-E1 | Shoot C | A | | | | |
| **E10** | *Pal. acuminata* | WH-211115-1-E10 | Leaf | A | | X | X | |
| **E11** | *Pal. acuminata* | WH-211115-1-E11 | Leaf | A | | | | |
| **E12** | *Pal. acuminata* | WH-211115-1-E12 | Leaf | A | X | | | |
| **E2** | *Pal. acuminata* | WH-211115-1-E2 | Shoot C | A | | | | |
| **E3** | *Pal. acuminata* | WH-211115-1-E3 | Fruit | A | | | | BS |
| **E4** | *Pal. acuminata* | WH-211115-1-E4 | Fruit | A | | | | |
| **E5** | *Pal. acuminata* | WH-211115-1-E5 | Fruit | **B** | | | | |
| **E6** | *Pal. acuminata* | WH-211115-1-E6 | Leaf | A | | X | X | |
| **E7** | *Pal. acuminata* | WH-211115-1-E7 | Leaf | A | | | | |
| **E8** | *Pal. acuminata* | WH-211115-1-E8 | Leaf | A | | X | X | |
| **E9** | *Pal. acuminata* | WH-211115-1-E9 | Leaf | A | | X | X | |
| **F1** | *Pal. winleri* | WH-241115-1-F1 | Fruit U | A | | | | |
| **F2** | *Pal. winleri* | WH-241115-1-F2 | Fruit U | A | X | | | |
| **F3** | *Pal. winleri* | WH-241115-1-F3 | Fruit U | **B** | X | | | |
| **F4** | *Pal. winleri* | WH-241115-1-F4 | Fruit U | **C** | X | | | |

| Fungal isolate | Host species | Collection no. | Organ | Isolation medium | No production | Griseofulvin | 7-Dechlorogriseofulvin | Positive bioassay |
|---|---|---|---|---|---|---|---|---|
| **F5** | *Pal. winkleri* | WH-241115-1-F5 | Shoot B | A | | | | |
| **F6** | *Pal. winkleri* | WH-241115-1-F6 | Shoot B | A | | | | |
| **F7** | *Pal. winkleri* | WH-241115-1-F7 | Shoot B | **B** | X | | | |
| **F8** | *Pal. winkleri* | WH-241115-1-F8 | Shoot B | **C** | X | | | |
| **I1** | *Pal. tomentosa* | WH-261115-1-I1 | Shoot C | A | | X | o | |
| **I2** | *Pal. tomentosa* | WH-261115-1-I2 | Shoot C | A | | X | | |
| **I3** | *Pal. tomentosa* | WH-261115-1-I3 | Shoot C | **B** | X | | | BS |
| **I4** | *Pal. tomentosa* | WH-261115-1-I4 | Shoot C | A | X | | | |
| **I5** | *Pal. tomentosa* | WH-261115-1-I5 | Shoot C | A | X | | | |
| **I6** | *Pal. tomentosa* | WH-261115-1-I6 | Leaf | A | | X | X | |
| **I7** | *Pal. tomentosa* | WH-261115-1-I7 | Leaf | A | | | | |
| **I8** | *Pal. tomentosa* | WH-261115-1-I8 | Leaf | A | | X | X | SC |
| **I9** | *Pal. tomentosa* | WH-261115-1-I9 | Shoot C | **B** | X | | | |
| **K1** | *Pal. tomentosa* | WH-261115-2-K1 | Fruit | A | | X | X | |
| **K2** | *Pal. tomentosa* | WH-261115-2-K2 | Shoot C | A | | | | |
| **K3** | *Pal. tomentosa* | WH-261115-2-K3 | Shoot B | A | | | | |
| **K4** | *Pal. tomentosa* | WH-261115-2-K4 | Shoot B | A | X | | | |
| **K5** | *Pal. tomentosa* | WH-261115-2-K5 | Seed | A | | | | |
| **K6** | *Pal. tomentosa* | WH-261115-2-K6 | Fruit | A | | | | |
| **L1** | *Psy. pilosa* | WH-261115-3-L1 | Seed | A | | X | X | |
| **L2** | *Psy. pilosa* | WH-261115-3-L2 | Seed | A | | X | o | |
| **M1** | *Psy. solitudinum* | WH-261115-4-M1 | Shoot C | A | | | | |
| **N1** | *Psy. cooperi* | WH-281115-1-N1 | Leaf | A | | | | SC |
| **N2** | *Psy. cooperi* | WH-281115-1-N2 | Leaf | A | | | | |
| **P1** | *Pal. acuminata* | WH-281115-2-P1 | Leaf | A | | X | X | |
| **P2** | *Pal. acuminata* | WH-281115-2-P2 | Leaf | A | | X | X | |
| **P3** | *Pal. acuminata* | WH-281115-2-P3 | Leaf | A | | | | |
| **P4** | *Pal. acuminata* | WH-281115-2-P4 | Leaf | A | | | | |
| **P5** | *Pal. acuminata* | WH-281115-2-P5 | Leaf | A | | X | X | |
| **P6** | *Pal. acuminata* | WH-281115-2-P6 | Shoot B | A | X | | | |
| **P7** | *Pal. acuminata* | WH-281115-2-P7 | Shoot B | A | X | | | |
| **R1** | *Pal. elata* | WH-281115-3-R1 | Seed | A | | | | |
| **R2** | *Pal. elata* | WH-281115-3-R2 | Seed | A | | X | | |
| **R3** | *Pal. elata* | WH-281115-3-R3 | Seed | A | | | | |
| **R4** | *Pal. elata* | WH-281115-3-R4 | Shoot B | A | X | | | |
| **R5** | *Pal. elata* | WH-281115-3-R5 | Shoot B | A | | | | BS |
| **R6** | *Pal. elata* | WH-281115-3-R6 | Sross B | **B** | | | | |
| **R7** | *Pal. elata* | WH-281115-3-R7 | Shoot B | **C** | X | | | |

| Fungal isolate | Host species | Collection no. | Organ | Isolation medium | No production | Griseofulvin | 7-Dechlorogriseofulvin | Positive bioassay |
|---|---|---|---|---|---|---|---|---|
| **S1** | *Psy. pilosa* | WH-301115-1-S1 | Shoot B | A | | | | BS |
| **S2** | *Psy. pilosa* | WH-301115-1-S2 | Shoot B | A | | | | |
| **S3** | *Psy. pilosa* | WH-301115-1-S3 | Shoot B | A | X | | | |
| **S4** | *Psy. pilosa* | WH-301115-1-S4 | Leaf | A | X | | | |
| **S5.1** | *Psy. pilosa* | WH-301115-1-S5.1 | Leaf | A | | o | o | |
| **S5.2** | *Psy. pilosa* | WH-301115-1-S5.2 | Leaf | A | X | | | |
| **S6** | *Psy. pilosa* | WH-301115-1-S6 | Leaf | A | | X | X | |
| **S7** | *Psy. pilosa* | WH-301115-1-S7 | Leaf | A | | X | | |
| **T1** | *Psy. tsakiana* | WH-301115-2-T1 | Seed | A | X | | | |
| **T2** | *Psy. tsakiana* | WH-301115-2-T2 | Seed | A | X | | | BS |
| **T3** | *Psy. tsakiana* | WH-301115-2-T3 | Shoot B | A | | X | X | |
| **T4** | *Psy. tsakiana* | WH-301115-2-T4 | Shoot B | A | | | | |
| **T5** | *Psy. tsakiana* | WH-301115-2-T5 | Shoot B | A | | | | |
| **T6** | *Psy. tsakiana* | WH-301115-2-T6 | Shoot B | A | | X | X | |
| **T7** | *Psy. tsakiana* | WH-301115-2-T7 | Shoot B | A | | | | |
| **T8** | *Psy. tsakiana* | WH-301115-2-T8 | Shoot B | A | | | | |
| **U1** | *Pal. elata* | WH-301115-3-U1 | Shoot B | A | X | | | |
| **U2** | *Pal. elata* | WH-301115-3-U2 | Shoot B | A | | | | |
| **U3** | *Pal. elata* | WH-301115-3-U3 | Shoot B | A | X | | | |
| **U4.1** | *Pal. elata* | WH-301115-3-U4.1 | Leaf | A | X | | | |
| **U4.2** | *Pal. elata* | WH-301115-3-U4.2 | Leaf | A | | X | X | |
| **U5** | *Pal. elata* | WH-301115-3-U5 | Leaf | A | | X | X | |
| **U6** | *Pal. elata* | WH-301115-3-U6 | Leaf | A | X | | | |
| **U7** | *Pal. elata* | WH-301115-3-U7 | Leaf | A | | X | X | |
| **U8** | *Pal. elata* | WH-301115-3-U8 | Leaf | A | X | | | |
| **W1** | *Psy. solitudinum* | WH-301115-4-W1 | Shoot B | A | | X | | |
| **W10** | *Psy. solitudinum* | WH-301115-4-W10 | Leaf | A | | X | | |
| **W11** | *Psy. solitudinum* | WH-301115-4-W11 | Leaf | A | X | | | |
| **W2** | *Psy. solitudinum* | WH-301115-4-W2 | Shoot B | A | | X | X | |
| **W3** | *Psy. solitudinum* | WH-301115-4-W3 | Shoot B | A | X | | | |
| **W5.1** | *Psy. solitudinum* | WH-301115-4-W5.1 | Shoot B | A | | o | o | |
| **W5.2** | *Psy. solitudinum* | WH-301115-4-W5.2 | Shoot B | A | | X | X | |
| **W6** | *Psy. solitudinum* | WH-301115-4-W6 | Shoot B | A | X | | | |
| **W8** | *Psy. solitudinum* | WH-301115-4-W8 | Shoot B | A | X | | | BS |
| **W9** | *Psy. solitudinum* | WH-301115-4-W9 | Leaf | A | | | | |

## 16.3 BLAST search results

Appendix 3. BLAST search results of sequenced fungal isolates

| | | BLAST results | | | |
|---|---|---|---|---|---|
| **Isolate** | **Accession nr.** | **BLAST match** | **Accession nr.** | **Similarity** | **Reference** |
| C5 | KY192281 | Fungal sp. | FJ613084 | 96 % | U'Ren et al., 2009 |
| | | *Xylaria* sp. | KU683903 | 95 % | U'Ren et al., 2016 |
| | | *Xylaria tuberoides* | KP133545 | 90 % | Thomas et al., 2016 |
| | | *Xylaria tuberoides* | GU300074 | 90 % | Hsieh et al., 2010 |
| D4 | KY192282 | *Xylaria* sp. | KC771489 | 98 % | CE-QCA Ecuador |
| | | Fungal sp. | KX271333 | 94 % | Christian et al., 2016 |
| | | *Xylaria* cf. *heliscus* | JQ761642 | 93 % | U'Ren et al., 2012 |
| | | *Xylaria castorea* | JN225908 | 93 % | Johnston et al., 2012 |
| E3 | KY192283 | Fungal sp. | FJ613075 | 99 % | U'Ren et al., 2009 |
| | | *Nemania* sp. | KP133235 | 99 % | Thomas et al., 2016 |
| | | *Entonaema* sp. | KF746156 | 99 % | Higginbotham et al., 2014 |
| | | *Xylaria* sp. | FJ799948 | 99 % | Van Bael et al., 2009 |
| I8<br>T2 | KY192275<br>KY192279 | *Arthrinium arundinis* | KU935651 | 99 % | Bovio et al., 2017 |
| | | *Arthrinium* sp. | KX378907 | 99 % | Xie et al., 2016 |
| | | *Arthrinium arundinis* | KX533933 | 99 % | Unpublished |
| | | *Arthrinium arundinis* | KU214522 | 99 % | Unpublished |
| I9<br>S1 | KY192276<br>KY192278 | *Fusarium proliferatum* | KU377463 | 99 % | Sosa et al., 2016 |
| | | *Fusarium proliferatum* | KR350650 | 99 % | Unpublished |
| | | *Fusarium proliferatum* | KU184608 | 99 % | CSUFT China |
| | | *Fusarium proliferatum* | LC101942 | 99 % | Hafizi et al., 2013 |
| R5 | KY192277 | *Clonostachys* sp. | LC133895 | 100 % | Unpublished |
| | | *Clonostachys* sp. | KP006353 | 100 % | Unpublished |
| | | *Clonostachys* sp. | KP006352 | 100 % | Unpublished |
| | | *Clonostachys rosea* | HQ607798 | 100 % | Rodrigues et al., 2011 |
| W8 | KY192280 | *Colletotrichum* sp. | KU204655 | 99 % | Unpublished |
| | | *Colletotrichum fructicola* | KX129709 | 99 % | Unpublished |
| | | Fungal endophyte | KF436343 | 99 % | Higginbotham et al., 2013 |
| | | *Colletotrichum gloeosporioides* | JQ814328 | 99 % | Unpublished |