Chemical diversity and richness of fungal endophytes from Costa Rican *Palicourea* and *Psychotria* species (Rubiaceae)

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Screening of fungal endophytes from five Costa Rican *Palicourea* and three *Psychotria* species (Rubiaceae) growing in the surroundings of the Tropical Rainforest Station *La Gamba* resulted in the identification of strains belonging to the genera *Xylaria*, *Arthrinium, Fusarium, Clonostachys* and *Colletotrichum*. Metabolic profiles of isolated fungi were analyzed. Several cytochalasin derivatives, piliformic acid as well as the antifungal agents griseofulvin and its derivative 7-dechlorogriseofulvin were identified. Additionally, griseofulvin and its 7-dechloro form were found to be sequestered in the guttation droplets of four strains. Growth inhibiting effects against various microbial test organisms highlight the potential of the isolated fungi to produce powerful antibiotic agents.

Hinterdobler W. & Schinnerl J., 2019: Vielfalt an endophytischen Pilzen und deren chemische Diversität von Costa Ricanischen *Psychotria* und *Palicourea* Arten (Rubiaceae).

Das Ziel dieser Arbeit war die Untersuchung endophytischer Pilze aus acht *Psychotria* und *Palicourea* Arten (Rubiaceae) aus dem der Tropenstation La Gamba angrenzenden Tieflandregenwald in Costa Rica. Insgesamt wurden 102 Pilzkulturen aus Blättern, Sprossen, Früchten und Samen isoliert und dabei konnten Pilze der Gattungen *Xylaria*, *Arthrinium*, *Fusarium*, *Clonostachys* und *Colletotrichum* identifiziert werden. Cytochalasin-Derivate, Piliformic acid sowie Griseofulvin und 7-Dechlorogriseofulvin konnten als Inhaltsstoffe identifiziert werden. Zusätzlich wurde die chemische Zusammensetzung von Guttationstropfen einiger Isolate mit den dazugehörigen Rohextrakten verglichen sowie auch die medium-abhängige Produktion von Sekundärmetaboliten dieser Pilze getestet. Antimikrobielle Eigenschaften der isolierten Endophyten wurden an *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* und *Saccharomyces cerevisiae* getestet. Diese Ergebnisse dokumentieren das Vorkommen endophytischer Pilze in *Psychotria* und *Palicourea* Arten und deren Potential, antimikrobielle Substanzen zu bilden.

Keywords: *Palicourea*, *Psychotria*, fungal endophytes, *Xylaria*, cytochalasin D.

Introduction

Fungi and plants interact in numerous distinct and fascinating ways. Due to the close connection of plants and fungi in the rhizosphere, many fungal species are also capable of growing entirely inside of plant tissues. All plant organs including flowers and seeds are suitable habitats. Besides fungi, also bacteria including actinomycetes are found as colonizers of plants. 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 & Boyle 2005). Presence of endophytic fungi has been reported from higher plants, ferns and mosses, with the highest diversity observed in the tropics (Arnold & Lutzoni 2007, Arnold et al. 2001, Davis et al. 2003). The adaptation to a life within plants lead to a loss of reproduction via spores in some endophytic species. In order to persist 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. Contribution to plant defense mechanisms and increased resistance against pathogens were reported for some prominent and agriculturally important species, such as *Theobroma cacao* L. (cacao tree) (Malvaceae), *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (rubber tree) (Euphorbiaceae), and species of the coffee plant genera *Coffea* sp. (Rubiaceae) (Hanada et al. 2010, Gazis & Chaverri 2010, Vega et al. 2010). Being in constant contact and competition with various other fungi and bacteria sharing the limited habitat within their host, fungal endophytes have been highlighted as 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* Nutt. (Taxaceae) (Stierle et al. 1993), resulted in extensive research on endophytes of pharmaceutically important plants, and their bioactive metabolites. The indole alkaloid vincristine from *Catharanthus roseus* (L.) G.Don (Apocynaceae) and the quinoline alkaloid camptothecin from *Camptotheca acuminata* Decne. (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 & 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 study. 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. 2012, 2016, 2017). To date, 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 near the tropical field station of *La Gamba* 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 of the genus *Psychotria* and the closely related genus *Palicourea*. To the best of our knowledge, this work is the first investigation of endophytic fungal diversity from the selected *Psychotria* and *Palicourea* species.

Results

Richness and organ specificity of endophytic fungi

Fungal endophytes were isolated from 15 *Psychotria* and *Palicourea* individuals comprising eight species. Leaves, basal and central shoots, mature and immature fruits as well as seeds were used for isolation of endophytes. A total of 102 fungal strains were isolated (Tab. 1a). From the standard, malt extract-based isolation medium, 92 fungal strains were recovered from 68 plant samples, leading to an isolation frequency of 1.35 fungal isolates per sample. Selective media supplemented with benomyl or cycloheximide led to the isolation of six and four fungal strains out of 18 and 20 plant samples, respectively. For these selective media the isolation frequency reached 0.33 strains per sample for benomyl and 0.2 for cycloheximide. For *Palicourea solitudinum* (Standl.) Borhidi, *Palicourea elata* (Sw.)

Borhidi, *Palicourea acuminata* (Benth.) Borhidi and *Palicourea pilosa* (Ruiz & Pav.) Borhidi, between 40 to 65 % of the isolated endophytes were collected from leaves followed by 25 to 40 % collected from stems. For *Palicourea tomentosa* (Aubl.) Borhidi, 20 % were isolated from leaves and 60 % from stems. Fruits and seeds account for 15 to 20 % of the total isolates per plant species (Fig. 1). Ten fungal strains were identified at least at the family level by comparison of ITS sequences to the publicly available NCBI database (Tab. 1b).

Tab. 1a: Number of isolated endophytic fungal strains and sampled individuals per plant species. – Tab. 1a: Anzahl an untersuchten Pflanzen je Pflanzenart sowie die Anzahl daraus isolierter Pilze. Tab. 1b: Identified fungal isolates. – Tab. 1b: Identifizierte Pilzgattungen und Arten.

Tab. 1a

Host species	Sampled individuals	Number of fungal isolates						
		Leaf	Shoot B	Shoot C	Fruit	Fruit I	Seed	Total
Pal. 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
Pal. pilosa	2	5	3				2	10
Pal. winkleri Borhidi	1		4			4		8
Psy. cooperi Standl.	1	2						2
Psy. tsakiana Taylor	1		6				2	8
								Total 102

Basal shoots (B), central shoots (C) and immature fruits (I)
Basale Sprossachsen (B), zentrale Sprossachsen (C) und unreife Früchte (I)

Tab. 1b

Isolate	Identification	Host species	Organ	GenBank accession no.
whc1	*Arthrinium* sp.	*Pal. solitudinum*	Seed	MH465392*
C5	*Xylaria* sp.	*Pal. solitudinum*	Leaf	KY192281
whd4	*Fusarium* sp.	*Pal. elata*	Leaf	MH465393*
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*	*Pal. pilosa*	Shoot	KY192278
T2	*Arthrinium arundinis*	*Psy. tsakiana*	Seed	KY192279
W8	*Colletotrichum* sp.	*Psy. solitudinum*	Shoot	KY192280

* Hinterdobler et al. in prep..

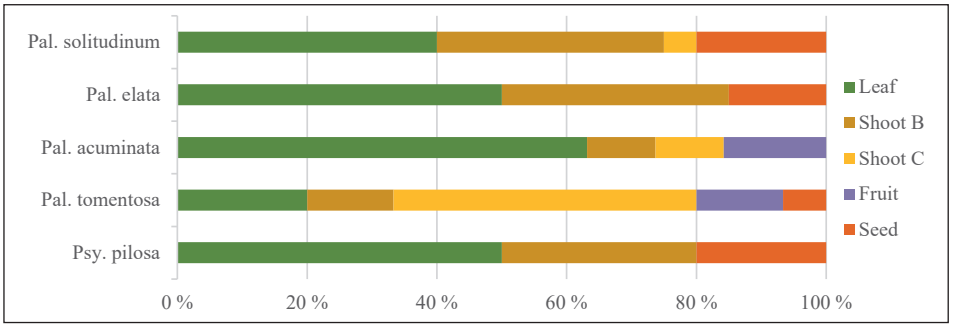

Fig. 1: Proportion of fungal isolates from plant organs (leaves, basal (B) and central (C) shoots, fruits and seeds). Plant species with more than ten fungal isolates are shown. – Abb. 1: Mengenverhältnisse von Pilzisolaten von Blättern, Sprossachsen, Früchte und Samen von Pflanzenarten mit mehr als zehn Isolaten.

Identification and distribution of fungal secondary metabolites

Analysis and extraction of fermentative cultures of a preselected subset of endophytic strains led to the identification of eight compounds (HINTERDOBLER et al. in prep.). The fungal isolate whc1 produced the co-chromatographically identified griseofulvin and 7-dechlorogriseofulvin on solid MEA2 medium (Fig. 2). Under fermentative conditions, piliformic acid and cytochalasin D (Fig. 2) were produced in sufficient amounts for isolation and structure elucidation. Liquid cultivation and extraction of the endophytic strain whd4 led to the isolation of four novel leucin-derived cytochalasins with one containing a rarely described 5/6/5/8-ring system (HINTERDOBLER et al. in prep.). The fungal metabolite griseofulvin was identified in 35 out of 102 (34.3 %) fungal isolates growing on MEA2 medium. 7-dechlorogriseofulvin was present in 30 isolates containing griseofulvin.

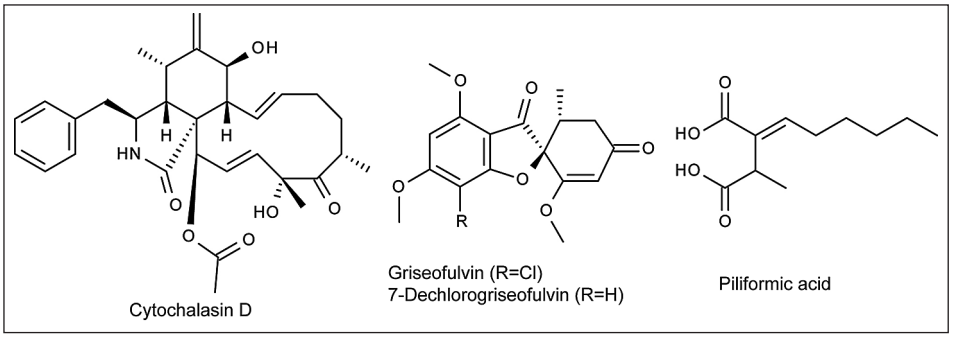

Fig. 2: Structures of cytochalasin D, griseofulvin, 7-dechlorogriseofulvin and piliformic acid. – Abb. 2: Strukturformeln von Cytochalasin D, Griseofulvin, 7-Dechlorogriseofulvin und (2*E*)-2-Hexyliden-3-methylbutandisäure.

Shifts in secondary metabolite production on different growth media was observed in the fungal isolates S1 and whc1. Fungi for upscale cultivation were chosen by their ability to produce HPLC-DAD detectible substances on MEA medium without a match in our spectral library. In order to gain a sufficient amount of the compounds for structure elucidation, fungi were grown under liquid fermentative conditions. Comparative meas-

urements of solid and liquid cultures have shown griseofulvin and 7-dechlorogriseofulvin no longer to be produced by whc1 growing in liquid medium (Hinterdobler et al. in prep.). A similar change in the metabolite profile was observed for the isolate S1. This strain produced three substances with similar UV spectrum but different retention times growing on MEA2. Cultivated under fermentative conditions in liquid medium (ME), these metabolites were no longer detectable and therefore the extract not further fractionated (data not shown).

For comparative analysis of plant and fungal secondary metabolites, fungal cultures and the corresponding host plant organs were analyzed by HPLC. 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.

Analyses of aerial guttation droplets

Guttation droplets were collected from four fungal isolates (whc1, D1, L1, W2) and their secondary metabolite composition was qualitatively compared to the corresponding fungal crude extracts (Fig. 3). Griseofulvin and 7-dechlorogriseofulvin, produced by these strains growing on the agar medium MEA1, were deposited in the guttation droplets. In addition, hydrophilic medium ingredients and the antibiotic additive chloramphenicol were also identified as part of the guttation liquid.

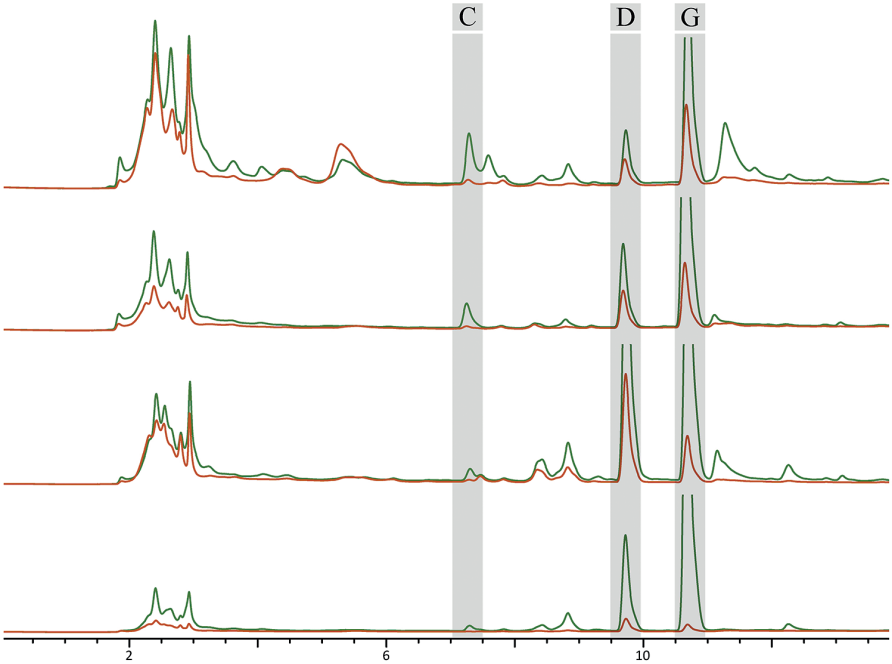

Fig. 3: Qualitative comparison of HPLC chromatograms at 230 nm of collected guttation droplets (orange) and the crude extracts of the corresponding fungus (green). From top to bottom: whc1, D1, L1, W2. Pile of peaks on the left represents hydrophilic components from the growth medium MEA1. Highlighted peaks: Chloramphenicol (C), 7-dechlorogriseofulvin (D) and griseofulvin (G). – Abb. 3: Vergleichende HPLC Chromatogramme von Guttationstropfen mit den mit den Rohextrakten der korrespondierenden Pilze. Von oben nach unten: whc1, D1, L1, W2. Markierte Peaks entsprechen Chloramphenicol (C), 7-Dechlorogriseofulvin (D) und Griseofulvin (G).

Bioassays

Metabolites that are constitutively sequestered into the growth medium were tested for their anti-microbial effects on *Escherichia coli* and *Bacillus subtilis*. Additionally, a subset of fungi was tested against *Saccharomyces cerevisiae* and on *Candida albicans* (Tab. 2). 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. The isolates I8 and N1 inhibited growth of *S. cerevisiae* whereas none of the tested strains showed inhibitory effects against *C. albicans*.

Tab. 2: Fungal isolates with anti-microbial effects on applied test organisms. – Tab. 2: Pilzisolate mit antimikrobieller Wirkung gegen die verwendeten Testorganismen.

		Microbial test organisms			
Fungal isolates		***E. coli***	***B. subtilis***	***S. cerevisiae***	***C. albicans***
C5	*Xylaria* sp.	x	-	-	
E3	*Xylaria* sp.	-	X		-
I3	-	-	X		-
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.	-	X		-

No inhibition (-), inhibition growing on MEA2-C (x) and MYEA (X) medium
Keine Aktivität (-), Aktivität, bei Wachstum auf MEA2-C (x) und MYEA(X) Medium

Discussion

Richness and organ specificity of endophytic fungi

Multiple fungal species exist side by side inside the plant tissue. In order to increase the chance of harvesting a high diversity of cultivable strains, repetition of the isolation process is necessary. 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 well-known technique, the plant fragment size for isolation also influences recovered fungal diversity. The reduction of fragment size comes with an increased cutting margin where the hyphae have access to the provided medium. It is estimated that one leaf fragment of 4 cm² 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 & BAYMAN (2001) described an isolation frequency of 1.4 fungal species per sample for *Guarea guidonia* (L.) Sleumer (Meliaceae) with 20 mm² fragments and increased the frequency to 2.9 by the use of 4 mm² pieces. The time between collection work in Costa

Rica and axenic cultivation of single strains is also considered a major factor influencing the isolation frequency. Growing together in one petri dish, 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 fungal species emerging on the standard medium. For isolation work in Costa Rica, benomyl and cycloheximide were 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, is 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. The latter is argued by Bills & Polishook (1992) 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* Walter (Betulaceae) bark disks using cycloheximide medium, the isolation using cycloheximide and benomyl for *Chamaecyparis thyoides* (L.) Britton, Sterns & Poggenb. (Cupressaceae) leaves hindered fungal growth and isolation (Bills & Polishook 1991, Bills & Polishook 1992). An optimization of the isolation process regarding the plants' properties and different organs might increase the output of cultivable endophytes.

The identified fungal genera and species fit well in the expected diversity of a tropical endophyte community. Fungal species belonging to the Xylariaceae family are regularly encountered endophytes and saprobes in the tropics (Rogers 2000). The genus *Arthrinium* harbors typical endophytic species as well, but also human- and phytopathogens. *Arthrinium* species have further been proven a good source for novel anti-microbial metabolites (Crous & Groenewald 2013, Ramos et al. 2010). *Fusarium proliferatum* on the other hand is a typical crop pathogen but is also found frequently as an endophyte in healthy leaves of various plant species (Stępień et al. 2011). Like *Fusarium*, also *Colletotrichum* species were described as endophytes in *Theobroma cacao* and *Taxus* × *media* (Taxaceae) (Rubini et al. 2005, Xiong et al. 2013). One *Colletotrichum* species similar to the strain W8 was isolated previously from *Trichilia tuberculata* (Triana & Planch.) C. DC. (Meliaceae) from Costa Rica (GenBank accession number KU204655). One isolate could be assigned to the genus *Clonostachys*. This genus harbors also entomopathogenic species that colonize leafhoppers and nematodes (Toledo et al. 2006, Zhang et al. 2008). The relatively small sample size in this study already shows the huge diversity of species and ecotypes that exist side by side in such an extraordinary niche as the inside of the plant body. From the plant's perspective, the genera and species identified here range from real mutualists providing an additional chemical reservoir against pathogens to temporarily silent saprobes and plant pathogens. The endophytic lifestyle provides an advantage for saprobes in early colonization and degradation of dying plant parts. The entomopathogenic fungi use their endophytic state as an effective way to colonize insects feeding on these plants and therefore can also be seen as mutualists against herbivores.

The ubiquity of fungal endophytes encountered 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 distribution via the plant (Steiner et al. 2006, Steiner & Leistner 2012). Bacterial endophytes collected from *Psychotria* were also found to be partly transferred vertically (Lemaire et al. 2012a). 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* (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 into early fungal colonization tendencies and potential protection mechanisms for the seedling.

Identification and distribution of fungal secondary metabolites

The identified 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 isolated from an endophytic *Xylaria* species from *Palicourea marcgravii* A.St.-Hil. and was shown to be active against *Cladosporium cladosporioides* and *C. sphaerospermum* (Cafêu et al. 2005). Furthermore, this compound is produced *in vitro* by *Xylaria cubensis*, an endophyte of *Asimina triloba* (L.) Dunal (Annonaceae) and *Silybum marianum* (L.) Gaertn. (Asteraceae), and by a *Nigrospora* species isolated from *Moringa oleifera* Lam. (Moringaceae) (Sica et al. 2016, Zhao et al. 2012). Piliformic acid is frequently found in xylariaceous fungi and close relatives (Chesters & O'Hagan 1997). It was further identified to be part of the chemical profile of one *Xylaria* strain 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 another 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 belonging to the genera *Aspergillus*, *Chaetomium* and *Xylaria* (Lin et al. 2009, Ming Ge et al. 2008, Espada et al. 1997). The cytochalasin D described here was identified in one *Tubercularia* isolate from *Taxus mairei* (Lemée & H.Lév.) S.Y.Hu (Taxaceae) and one *Xylaria* species found in *Palicourea marcgravii* (Li et al. 2009, Cafêu et al. 2005).

Severe media-dependent changes in the metabolite pattern were observed in the fungal strains whc1 and S1. Paranagama et al. (2007) reported a shift from the production of the polyketide 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 observed by adding tap water instead of distilled water to the medium. 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 a small scale. Regarding the high variability of fungal secondary metabolite production *in vitro*, the presented 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 these fungi.

Analysis of aerial guttation droplets

The excretion of liquid exudates of basidiomycetous fruiting bodies in their natural habitat is known to most mushroom collectors and connoisseurs. Under axenic *in vitro* conditions some ascomycetous fungal species 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 & Gareis 2007, Gareis & Gottschalk 2014, Hutwimmer et al. 2010). In analogy to a similar plant phenomenon, these excretions are 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 & 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 droplets seem to be a byproduct of the sequestration of water. The occurrence of small amounts of chloramphenicol in the droplets is direct evidence that this substance is taken up by the fungus. This raises the question of whether the use of chloramphenicol in the growth medium influences growth or even secondary metabolite production. However, the deposition of chloramphenicol indicates that it is not entirely metabolized until it reaches the guttation droplets. Its function as a trigger for substance production or being partly used as substrate cannot be excluded.

Screening for anti-microbial effects

Endophytic fungi have been reported as a promising source for anti-microbial compound isolation (Mousa & Raizada 2013). In total, 9.8 % of the tested fungal isolates showed growth-inhibiting effects against one of the applied test organisms. Antibacterial activity against *B. subtilis* was earlier reported for one *Xylaria* strain isolated from *Psychotria bisulcata* Wight & Arn. (Govinda Rajulu et al. 2013). Regarding the close interaction of endophytes with their host and other microorganisms residing within the same limited space, the ability to produce antibacterial and antifungal 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 reveal fungal endophytes from *Psychotria* and *Palicourea* species as a promising source for the discovery of antimicrobial compounds. As mentioned before, secondary metabolite production is highly dependent on the applied cultivation medium. The agar plug diffusion assay is a basic assay 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 antimicrobial substances from the examined endophytes but leaves open further questions on the structure and media-dependency of the involved metabolites.

Evaluation of applied methods

Research on endophytic fungi is highly dependent on the methods used for isolation and cultivation. Several surface sterilization procedures using sodium hypochlorite (NaOCl), ethanol or formaldehyde for plant samples have been evaluated and proven to be suit-

able 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 observed after isolation. The diversity is also strongly affected by the ability of the present fungi to grow on the isolation media thus leading to many fungal strains and species staying undetected if the given medium does not fit their needs (Schulz & Boyle 2005). Beside these limitations of the isolation process, one must reconsider the definition of endophytes. In this study, 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 antifungal additives in the selective isolation media were chosen based on availability and literature search. Besides cycloheximide, benomyl and the antibacterial chloramphenicol, several other antifungal and antibacterial additives (e.g. cyclosporine A, natamycin and rose bengal) are commonly used for pre-selective isolation of fungal endophytes (Stone et al. 2004). Regarding fungal endosymbionts, the use of antibiotics in the isolation and cultivation medium can lead to a loss of endohyphal bacteria in fungal cultures (Hoffman & Arnold 2010) and thereby further influence the production of secondary metabolites *in vitro*.

Experimental

Plant and fungal material

Plant and fungal material was collected during 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 & Baumgartner 2001). The nearby laboratory facilities were used for fast processing of the collected material. In the course of three weeks, 15 individuals of eight *Psychotria* and *Palicourea* species were successfully sampled. Altogether 102 fungal strains were isolated and further cultivated. For each plant individual collected, 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. Plant specimens were deposited in the herbarium of the University of Vienna (WU) and in the national herbarium of Costa Rica (CR) – one copy in each.

Isolation and cultivation of endophytic fungi

Fungal endophytes were isolated the same day of collection of plant material. 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 cm² of leaves including the midrib were prepared for surface sterilization. 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 min, followed by 5 min 3 % NaOCl solution and again 70 % ethanol

for one min to remove excess hypochlorite solution. To guarantee 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 incubated at room temperature. If no fungal colonies were visible after one week, sterilization was considered successful. After surface sterilization, margins of leaf and shoot samples damaged by NaOCl were removed using sterile razor blades. Shoots, fruits and seeds were divided lengthwise and plated with the cut surface onto the isolation medium. For each collected plant organ, several pieces were used for isolation (one per petri dish). Petri dishes were stored in darkness at room temperature. All isolates linked to a positive imprint test were disposed.

The standard 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 for MEA1 and 5 g/L malt extract for MEA2. 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 selective isolation media, 4 mg/L benomyl and 200 mg/L cycloheximide were added to the MEA3 recipe. Benomyl was 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. Excess acetone was evaporated under lamina flow. Emerging fungi from plated plant samples were separated by transferring small agar plugs to new petri dishes. Morphologically distinct strains were separated again until isolates were morphologically homogenous. Five fungal isolates were assigned to fermentation after first analytical screenings. Agar plugs with mycelium were cut from cultures on MEA1 and used to inoculate the liquid (ME) medium. S1 and whd4 were cultivated in 280 mL ME medium (2 × 140 mL) in darkness at 27° C for 49 days and additionally in 250 mL medium for 24 days (Hinterdobler et al. in prep.). Whc1 and C4 were cultivated in 280 mL ME medium (2 × 140 mL) in darkness at 27° C for 68 days and additionally in 250 mL medium for 43 days (ibid.).

Species identification

A subset of fungal isolates was identified by Sanger sequencing and comparison to the NCBI database by BLAST search (see also Hinterdobler et al. in prep.). Internal transcribed spacer (ITS) regions of fungal ribosomal DNA were amplified using the standard ITS5 forward primer for fungal isolates C5 and 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 with the help of Dr. Alexander Urban and sequenced at the Division of Systematic and Evolutionary Botany, University of Vienna.

Secondary metabolite analysis and extraction

For isolation procedure of whc1 and whd4 crude extracts see Hinterdobler et al. (in prep).

Agar plug diffusion assay

Fungi were grown on MEA2-C or MYEA medium until full colonization. Agar plugs of 5×5 mm were transferred to petri dishes covered with test organisms. Results of the screenings were evaluated the next day. Endophytic fungi were tested against *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.

Conclusion

Psychotria and *Palicourea* species growing in Costa Rica were shown to be a rich source for fungal endophyte research. The area of sampling at the Pacific side of Costa Rica provides a suitable habitat for fungi of all kinds (Weissenhofer & Huber 2008, Piepenbring & Ruiz-Boyer 2008). The results showed that endophytic fungi occur in leaves, shoots, fruits and seeds of the studied plant species. The identified endophytic fungi belong to the frequently isolated genus *Xylaria* (Xylariaceae) and related genera. Production of plant-like secondary metabolites in fungal cultures could not be observed during this study but the isolated fungal endophytes have the ability to produce anti-microbial defense chemicals against applied test organisms. These defense chemicals could not be detected in previous studied plant material (Berger et al. 2012, 2016, 2017). Further studies are required to illuminate the symbiotic interactions between plants and their endophytes.

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