SHORT COMMUNICATION

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Foliar endophytic fungi as potential protectors from pathogens in myrmecophytic *Acacia* plants

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Abbreviations: PR-proteins, pathogenesis-related proteins; LB media broth, Luria-Bertani media; OD, optical density.

In defensive ant-plant interactions myrmecophytic plants express reduced chemical defense in their leaves to protect themselves from pathogens, and it seems that mutualistic partners are required to make up for this lack of defensive function. Previously, we reported that mutualistic ants confer plants of *Acacia hindsii* protection from pathogens, and that the protection is given by the ant-associated bacteria. Here, we examined whether foliar endophytic fungi may potentially act as a new partner, in addition to mutualistic ants and their bacteria inhabitants, involved in the protection from pathogens in myrmecophytic *Acacia* plants. Fungal endophytes were isolated from the asymptomatic leaves of *A. hindsii* plants for further molecular identification of 18S rRNA gene. Inhibitory effects of fungal endophytes were tested against *Pseudomonas* plant pathogens. Our findings support a potential role of fungal endophytes in pathogen the protection mechanisms against pathogens in myrmecophytic plants and provide the evidence of novel fungal endophytes capable of biosynthesizing bioactive metabolites.

Introduction

Plants have developed sophisticated direct defense mechanisms to deal with pathogen attack. In addition to constitutive barriers against pathogens, such as waxy epidermal cuticles and cell walls,[1] plants can recognize invading pathogens and respond with inducible defenses, such as the production of reactive oxygen species,[2] phytoalexins[3] and pathogenesis-related (PR) protein accumulation.[4] Moreover, plants can also engage the third trophic level as an indirect defense against pathogens. Recently, we found out that mutualistic ants nourished by mesoamerican *Acacia* plants serve to provide an indirect defense against leaf pathogens,[5] and that plants would depend on the mutualistic ant partners to cope with the pathogen colonization. In addition, plants largely rely on their mutualistic interactions with microorganisms to overcome pathogen attack. Plant resistance to the pathogens commonly increases as a result of plant colonization with symbiotic microorganisms, including mycorrhizas,[6,7] plant growth-promoting bacteria or fungi[8] and leaf endophytic fungi.[9] Thus, associations of plants with the multiple mutualistic partners occur recurrently and often provide benefits in plant protection from natural enemies.

In the obligate mutualistic interaction *Acacia-Pseudomyrmex*, *Acacia* myrmecophytic plants produce extrafloral nectar, food bodies and nesting space to house defending ants of *Pseudomyrmex ferrugineus*. In return, ants act as an indirect defense[10] protecting plants from herbivory,[11-13] pruning of neighboring plants[11,13] or pathogenic microorganisms.[5,14,15] Plants of the genera *Acacia* showed a reduced activity of PR proteins in their leaves[15] which render them less able to defend themselves against pathogens. Thus, the questions how and to which extent ant-plants gain protection from microorganisms to compensate for the low abundance of PR-proteins in their leaves still remains open. Recently, we showed that the presence of mutualistic ants of *P. ferrugineus* on the host plant significantly reduced disease symptoms and bacterial abundance in the leaves of *A. hindsii*.[5] Compounds secreted from the ant legs are involved in protection from pathogens in plants of *A. hindsii*. It seems that the ant-associated bacteria isolated from the ant legs contribute, at least partially, to the protective effect provided by mutualistic ants.[5] However, other mechanisms could also be involved in the protection from pathogens in ant-Acacia plants. Chemical secretions produced by exocrine glands of the ants[16] or leaf plant endosymbionts with potential inhibitory effects[8] might also contribute to protection against pathogens.

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Table 1. Identification of fungal endophytes isolated from asymptomatic leaf samples of 5 *Acacia hindsii* plants and their inhibitory effects against the bacterium *Pseudomonas sp*. The inhibition zone (mm) of *Pseudomonas sp* was quantified as the diameter of clear zones of growth inhibition around each endophytic fungus. Values are means ± SE (standard error) of 3 biological replicates. Dash (-) indicates no inhibition

Endophytic fungi	Accession Number	Identity	Abundance	*Pseudomonas sp.* (*Acacia hindsii* isolate)
Cochliobolus geniculatus	JN941621.1	99	3	—
Colletotrichum gloeosporioides	DQ916151.1	98	6	2.4 ± 0.3
Colletotrichum truncatum	AJ301945.1	99	1	—
Eupenicillium javanicum	JN546126.1	100	1	—
Fusarium oxysporum	KC143070.1	99	4	2.2 ± 0.1
Moesziomyces bullatus	DQ831012.1	99	4	7.4 ± 0.4
Paraphaeosphaeria sp	AB665311.1	97	1	—
Phoma sp.	AY646226.1	99	2	—
Pichia anomala	AB126679.1	99	1	4.1 ± 0.1

Here, we complement our research of the protection mechanisms in the ant-Acacia plants and provide information about the potential role of the leaf fungal endophytes as protectors from pathogens in this system.

Endophytes are microorganisms that live within host plant tissues and do not cause any apparent manifestation of disease, but rather co-exist in mutualistic association with plants for at least part of their life cycle.[17] It is assumed that each plant in natural ecosystems hosts one or more endophytes.[18] Plant leaves appear to be frequently infected by class II fungal endophytes,[19] which comprise species from the phyla Ascomycota and Basidiomycota.[20] In contrast to class I grass-fungal endophytes,[19] class II endophytes are non-systemic, horizontally transmitted and colonize a wide range of plants in the ecosystems. Moreover, different species of class II fungal endophytes can co-occur in the same plant.[20,21] Fungal endophytes may benefit host plants by promoting plant growth,[22] improving tolerance to abiotic stress,[23] or preventing herbivory[24] and pathogen colonization.[9] Endophytes have been recognized to be a novel source of bioactive compounds,[25] because of their ability to produce a number of important bioactive secondary metabolites with antimicrobial activity that is often involved in protection from phytopathogenic microorganisms.[26]

In order to study whether asymptomatic leaves of *A. hindsii* are colonised by fungal endophytes, we isolated fungal endophytes by culture methods from leaflets of 5 plants of *Acacia hindsii* for further molecular identification of the 18S rRNA gene. Then, we tested the potential effects of *A. hindsii* fungal endophytes on the protection against leaf pathogens. A total of 23 isolates of endophytic fungi were obtained from the healthy leaves of *A. hindsii* and were identified as indicated in **Table 1**. The most abundant endophytes were *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and the yeast *Moesziomyces bullatus* (**Table 1**). Several fungal endophytes isolated from the leaves of *A. hindsii* have been already reported to be leaf endophytes, namely, the genus *Phoma*,[27] and the species *Fusarium oxysporum*[28] and *Colletotrichum gloesporioides*.[29] Inhibitory effects of the 9 morphospecies against *Pseudomonas sp*. (previously isolated from symptomatic leaves of *A. hindsii*) showed that the endophytes *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, the yeast *Moesziomyces bullatus* and *Pichia anomala* were able to reduce the growth of *Pseudomonas sp* (**Table 2**). Further inhibitory bioassays showed that crude methanol extracts of the 3 most abundant endophytes were more efficient than ethyl acetate and hexane extracts (**Table 2**). Three methanol extracts of the abovementioned fungal endophytes showed high antibiotic activity against the plant pathogenic bacterium *Pseudomonas syringae* (**Fig. 1**). Endophytes from the genera *Colletotrichum*, *Phoma* and *Fusarium* are known to be rich sources of biologically active secondary metabolites with antimicrobial activity against several pathogens.[27,30] The endophytic yeast *Moesziomyces bullatus* was of particular interest to us as it showed the highest inhibitory effect in all our experiments and it has not been mentioned to be a plant endophyte elsewhere. Our results showed that fungal endophytes isolated from *Acacia* leaves might contribute through metabolites with antimicrobial activity to the protection from phytopathogens. *In vitro,* several studies have shown that endophytic fungi produce substances inhibiting the development of plant pathogens.[31] Nevertheless, since the concentration of endophyte´s substances secreted *in planta* as well as the substantial contribution of the host plant on the *in planta* metabolic processes of the endophytes are unknown, it is necessary to asses *in vivo* the effects of fungal endophytes to their host plants.

Table 2. Inhibitory effects of methanol (MeOH), ethyl acetate (EtAc) and hexane extracts of the most abundant fungal endophytes isolated from *Acacia hindsii* (Fabaceae) tested on 2 bacteria *Pseudomonas syringae* (*P.s.*) and *Escherichia coli* (*E.c.*). The inhibition zone (mm) of the bacteria was quantified as the diameter of clear zones of growth inhibition around each extract drop. Values are means ± SE (standard error) of 3 replicates. Dash (-) indicates no inhibition

	MeOH extract		EtAc extract		Hexane extract	
	P.s.	*E.c.*	*P.s.*	*E.c.*	*P.s.*	*E.c.*
Colletotrichum gloeosporioides	11.6 ± 0.24	12 ± 0.29	8.7 ± 0.21	—	3.5 ± 0.20	—
Fusarium oxysporum	7.1 ± 0.35	—	3 ± 0.20	—	—	—
Moesziomyces bullatus	13.5 ± 0.28	6 ± 0.20	6 ± 0.20	—	—	—

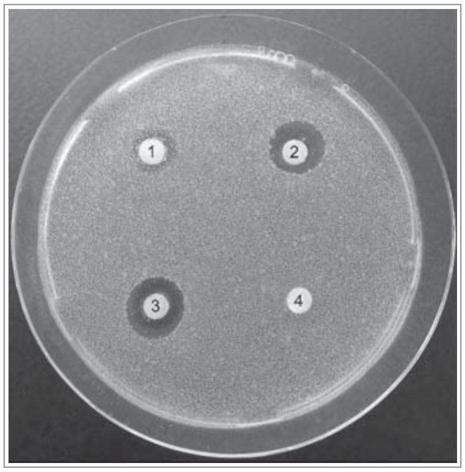

Figure 1. Antibiotic effect of the methanol extracts of 3 fungal endophytes isolated from the leaf samples of *Acacia hindsii* (1: *Fusarium oxysporum*, 2: *Colletotrichum gloeosporioides*, 3: *Moesziomyces bullatus*, 4: methanol as control) on the plant pathogen bacterium *Pseudomonas syringae*.

Although, it is still required to identify the substances produced by fungal endophytes in order to understand their contribution to *Acacia* plant protection, there is a potential for the selected endophytic fungi to produce novel bioactive compounds.

In summary, we showed that in addition to the protection mechanisms provided by the mutualistic ants and their bacteria inhabitants, foliar endophytic fungi might potentially act as a third partner involved in protection from pathogens in myrmecophytic *Acacia hindsii* plants. Plant-ant-fungus symbiosis has been already described in ant-plant mutualisms[32,33] and it seems that mutualistic ants are responsible for the establishment and persistence of the fungus.[33] The way how mutualistic ants shape the occurrence of endophytic fungi in *Acacia* leaves still requires further elucidation.

Material and Methods

Fungal endophytes were isolated from the healthy leaflets of 5 plants of *A. hindsii* according to the method described by Arnold et al.[34] Small pieces of leaflets (2-3 mm) were placed on potato-dextrose-agar (Sigma-Aldrich). Petri plates were incubated at room temperature for several days, and the emerging colonies were subcultured to obtain pure isolates. Pure isolates were grown on potato-dextrose-broth (Sigma Aldrich, Germany) at room temperature for 2 weeks for the molecular identification. Genomic DNA was extracted from the mycelial mat using a modified method described by Nicholson et al., 2001. Species identification of endophytic fungi was performed using the primers NS1 and FR1. Amplification of the partial 18S rDNA (1.65 kbp) was conducted with 50 μL of PCR reaction mixtures, each containing 4 μL of total fungal genomic DNA, 4 μL of 10 μM forward primer, 4 μL of 10 μM reverse primer, l μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany), 1 μL deoxynucleoside triphosphate (at a concentration of 10 mM for each nucleotide; Bioline GmbH, Luckenwalde, Germany), 3.75 μL 50 mM $MgCl_2$, 5 μL of 10 × PCR buffer, 0.4 μL of Taq polymerase (5 U μL^{-1}) and distilled water to complete the total volume. PCR was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) with the following program: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 45 sec and primer extension at 72°C for 3 min, completed with a final extension at 72°C for 10 min. PCR products were purified with the kit Invisorb Fragment Clean Up (Invitek GmbH, Berlin, Germany) and then bidirectionally sequenced. Sequencing was carried out at the Max Planck Institute for Chemical Ecology, Jena, Germany. DNA sequences were cleaned and assembled with the DNASTAR Lasergene software package (DNASTAR Inc. Madison, WI, USA). The initial assembly of the sequences was performed with a 99% threshold. Consensus sequences were used for BLAST searches at the NCBI (http://www.ncbi.nlm.nih.gov). Fungal 18S rRNA gene sequences have been deposited at the NCBI with accession numbers KP027006-KP027014 for fungal endophyte OTUs.

Inhibitory effects of the 9 morphospecies of endophytic fungi isolated from *A. hindsii* were evaluated on the growth of *Pseudomonas sp*. (previously isolated from the symptomatic *A. hindsii* leaves, KF623094) and *Escherichia coli*. Pathogenic test bacteria were cultivated in LB media broth for 16 h at 37°C and then inoculated into LB media agar plates (100 μL of each culture bacteria with an OD = 0.6 – 0.7 was used for 20 mL of agar plate). An agar slice (2 cm × 2 cm) of each fungal endophyte was placed in the center of Petri dishes containing the test bacteria. Inhibitory effects of endophytic fungi were quantified by the diameter (mm) of clear zones of growth inhibition around each endophytic fungal. The experiment was repeated with 3 independent endophyte samples for all test bacteria.

The most abundant endophytes (*Fusarium oxysporum*, *Colletotrichum gloeosporioides* and the yeast *Moesziomyces bullatus*), which showed a good activity against the *Pseudomonas sp* isolate, have been extracted with 100 mL of methanol (MeOH), 100 mL of ethyl acetate (EtAc) and 100 mL of n-hexane for 6–8 hours in a shaker and used for further tests in diffusion assays. Extracted volumes were concentrated under the reduced pressure to the final volume of approximately 2 mL, and the crude extracts were tested separately in bioassays. The antibacterial activity of each fungal endophyte extract was assessed against *Pseudomonas syringae* var. *glycinea* in the disk diffusion assay. The size of the inhibition zone was determined by the diameter of the clear zone around each extract drop. The assay was performed for 3 biological replicates of each endophyte fungal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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