Broad-spectrum inhibition of *Phytophthora infestans* by fungal endophytes

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**One sentence summary:** Abundance of and symptoms caused by *Phytophthora infestans*, one of the most devastating crop pathogens, can be highly reduced by the fungal endophyte *Phoma eupatorii*.

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**ABSTRACT**

*Phytophthora infestans* is a devastating pathogen of tomato and potato. It readily overcomes resistance genes and applied agrochemicals and hence even today causes large yield losses. Fungal endophytes provide a largely unexplored avenue of control of *Phy. infestans*. Not only do endophytes produce a wide array of bioactive metabolites, they may also directly compete with and defeat pathogens in planta. Here, we tested 12 fungal endophytes isolated from different plant species in vitro for their production of metabolites with anti-*Phytophthora* activity. Four well-performing isolates were evaluated for their ability to suppress nine isolates of *Phy. infestans* on agar medium and in planta. Two endophytes reliably inhibited all *Phy. infestans* isolates on agar medium, of which *Phoma eupatorii* isolate 8082 was the most promising. It nearly abolished infection by *Phy. infestans* in planta. Our data indicate a role for the production of anti-*Phytophthora* compounds by the fungus and/or an enhanced plant defense response, as evident by an enhanced anthocyanin production. Here, we present a potential biocontrol agent, which can inhibit a broad-spectrum of *Phy. infestans* isolates. Such broadly acting inhibition is ideal, because it allows for effective control of genetically diverse isolates and may slow the adaptation of *Phy. infestans*.

**Keywords:** *Phytophthora infestans*; fungal endophytes; *Phoma eupatorii*; plant–microbe interaction; antimicrobial metabolites; biocontrol

**INTRODUCTION**

*Phytophthora infestans* is a major pathogen of cultivated tomato (*Solanum lycopersicum*) and cultivated potato (*Solanum tuberosum*). Even today its impact cannot be ignored as it is still capable of destroying entire fields of its hosts, leading to up to 100% yield losses (Nowicki et al. 2012). The two major control measures for *Phy. infestans* are resistance breeding and agrochemical
applications. While several resistance genes have been identified in screens of wild relatives of S. lycopersicum and S. tuberosum (Song et al. 2003; Van der Vossen et al. 2003; Pel et al. 2009; Zhang et al. 2013), many of them are readily overcame by isolates of Phy. infestans (Vleeshouwers et al. 2011). Similarly, agrochemicals can have a low durability in their protective function against Phy. infestans (Grünwald et al. 2006; Childers et al. 2015). Hence, continual scientific effort in terms of breeding, development of agrochemicals and other approaches, such as biological control, is needed for effective crop protection against this pathogen.

One approach that is gaining more and more attention is the use of endophytes for crop protection (Le Coq et al. 2016). Endophytes are microorganisms that grow within plants, and at the time of sampling, do not cause obvious symptoms on their host (Schulz and Boyle 2005; Le Coq et al. 2016). Many studies have explored the bacterial, fungal and protist endophytic communities associated with different plants (e.g. Bulgarelli et al. 2012; Lundberg et al. 2012; Bodデンhausen, Horton and Bergelson 2013; Schlaeppi et al. 2013; Bulgarelli et al. 2015; Edwards et al. 2015; Busby, Peay and Newcombe 2016; Coleman-Derr et al. 2016; Ploch et al. 2016; U’Ren and Arnold 2016; Sapp et al. 2018). These studies indicate that the diversity of microbes living inside of plants is largely underestimated and that the distribution of some microorganisms is host and/or environment specific.

Furthermore, in some cases such endophytic microorganisms have been evaluated for their potential benefit to their hosts (Busby, Ridout and Newcombe 2016; Fesel and Zuccaro 2016). Such benefits include growth promotion and protection against parasites and pathogens (e.g. Arnold et al. 2003; Schulz 2006; Lahlali and Hijri 2010; Tellenbach and Sieber 2012; Panke-Buisse et al. 2015; Rolli et al. 2015; Busby, Peay and Newcombe 2016; Hiruma et al. 2016; Martínez-Medina et al. 2017). Often these functions are linked to metabolites produced and secreted by the endophytes (Son et al. 2008; Dubey et al. 2013; Puopolo et al. 2014; Mousa et al. 2016; Suryanarayanan, Govinda Rajulu and Vidal 2016), highlighting the endophyte’s metabolic versatility (Schulz et al. 2002; Strobel and Strobel 2007; Verma, Kharwar and Strobel 2009; Mousa and Raizada 2013; Bradler et al. 2014). In addition to secreted compounds, microorganisms produce a spectrum of volatile compounds (Piechulla, Lemfack and Kai 2017), some of which are effective in reducing pathogen growth (Kottb et al. 2015). Endophytes may also directly compete with potential pathogens of their host plants (Alabouvette et al. 2009), induce plant defense responses (Shores, Harm and Mastouri 2010) and/or produce bioactive anti-microbial metabolites (Bradler et al. 2014). Fluorescent Pseudomonas spp. are examples of endophytes able to colonize roots and outcompete other pathogens (O’Sullivan and O’Gara 1992). An example of the induction of defense responses by an endophyte is the root endophyte Serendipita indica (formerly Piriformospora indica). In association with Arabidopsis thaliana, S. indica induces a jasmonic acid-dependent defense response upon co-inoculation with a pathogen (Stein et al. 2008). Furthermore, a recent study by Mousa et al. (2016) describes an Enterobacter sp. strain isolated from an ancient African crop (Eleusine coracana [finger millet]) with the ability to suppress the grass pathogen Fusarium graminearum. Enterobacter sp. traps F. graminearum in the root system of its host and simultaneously produces several antifungal compounds that kill the fungus.

Several bacterial and fungal endophytes with the potential to inhibit Phy. infestans’ growth have been described (Sturz et al. 1999; Kim et al. 2007; Miles et al. 2012; Puopolo et al. 2014). However, these endophytes have only been tested against single isolates of Phy. infestans, but alternative approaches, such as biocontrol, can show different outcomes depending on the pathogen isolate (Bahramisharif et al. 2013). Therefore, the identification of endophytic species with a broad inhibition spectrum is of critical importance.

In this study, we analyzed the metabolite extracts of 12 fungal endophytes isolated from different plant hosts for their ability to inhibit growth of Phy. infestans. Using a plate assay with the four most successful fungal endophytes, we show that they inhibit the growth of a broad spectrum of European Phy. infestans isolates in co-culture. According to our phylogenetic analyses, these four endophytes are members of the Ascomycota. The endophyte with the strongest inhibition potential both on plates and in planta was Phoma eupatoriisolate 8082. This endophyte inhibited proliferation of Phy. infestans and in some cases abolished its infection completely. Since we selected Ph. eupatoriis based on the inhibition potential of its metabolite extract, the active component may be a secreted metabolite or a cocktail of metabolites. A broad-spectrum activity as observed for Ph. eupatoriis suggests either a conserved target for such secreted metabolite(s) or several targets that are specific for the pathogen isolate and that are covered by the complexity of the metabolite cocktail. Both can result in slower counter-adaptation of Phy. infestans to either the direct application of the endophyte or to the application of its metabolites.

**MATERIAL AND METHODS**

**Isolation of endophytes**

To isolate the endophytes, plant tissues of the respective hosts (Table S1, Supporting Information) were first thoroughly washed under running water, and then immersed for 1 min in 70% ethanol, followed by 1–3 min in 3% NaOCl and subsequently rinsed three times in sterile water. Sterilized tissues were imprinted on potato–carrot medium (Höller et al. 2000) to test for effectiveness of sterilization and to optimize the sterilization procedure. The tissues were then cut with a sterile scalpel into 2 mm slices, plated on potato–carrot agar medium with antibiotics (Höller et al. 2000) and incubated for 3 weeks at 20 °C. The emerging mycelia were then taken into culture on potato–carrot agar medium and were initially identified according to morphology (Table S1, Supporting Information).

**Analysis of crude metabolite extracts for anti-Phytophthora infestans activity**

To test the growth inhibition potential of the 12 fungal endophytes, the endophytes were first grown on barley-spelt medium and/or malt agar medium (Schulz et al. 2011) at room temperature for 21 days. To isolate the secondary metabolites, the cultures were extracted with ethyl acetate (Schulz et al. 2011). 25 µl of culture extracts (40 mg/ml) were then applied to a filter disc and placed onto yeast agar medium that had been inoculated with Phy. infestans isolate D2; subsequent incubation was at 20 °C in the dark for 2–3 days (Schulz et al. 2011). Only fungal endophytes whose culture extracts resulted in a zone of inhibition ≥20 mm were used for further analyses.

**Co-culture on plates**

The fungal endophytic isolates 8082 (DSMZ accession: 106 583), 9907 (DSMZ accession: 106 584) and 9913 (DSMZ accession: 106 585), whose culture extracts had inhibited Phy. infestans in the agar diffusion assays and Phialocephala fortinii isolate 4197 (Schulz 2006; DSMZ accession: 106 586) were tested for their
bioactivity against nine isolates of the late blight pathogen Phy.
infestans (NL10001, NL88069, NL90128, IPO-C, IPO428-2, 392BA,
D12-2, T15-2 and T20-2). The Phialocephala fortinii isolate was
included based on previous experiments (Schulz et al. 2002;
Schulz 2006). The co-culture experiments were performed and
evaluated according to Peters et al. (1998). In brief, we estimated
the difference in radial growth of Phy. infestans and endophytes
when grown in co-culture or alone. Fungal endophytes and Phy.
infestans isolates were grown on rye-sucrose agar (RSA, Caten
and Jinks 1968) at room temperature. The duration of the experi-
ments depended on the endophytes’ growth rates: eight days for
all co-cultures that included 9913 and 14–16 days for the remain-
ing co-cultures. A minimum of 10 plates was analyzed per treat-
ment. The Mann–Whitney U test (Mann and Whitney 1947) was
used to determine if differences between co-culture and con-
trol plates were significant. Average growth inhibition was esti-
ated as follows: 1–(average radius in co-culture/average radius
in control conditions). All experiments were evaluated again
after eight weeks of incubation to assess long-term effects. Pic-
tures were taken with an EOS 7D camera (Canon).

Co-inoculation in planta

The surfaces of the S. lycopersicum cv. M82 seeds were sterilized
using 70% ethanol for 3 s, followed by ∼5% NaOCl for 30 s. The
sterilized seeds were washed three times with sterile water for
3 min. Seeds were incubated in the dark on 1.2% H2O-agar with a
day–night temperature cycle of 18°C/15°C (16 h/8 h). After three
days, the seeds were transferred to a day–night cycle with 16 h
light (166 ± 17 μmol quanta·m-2·s-1). Temperature conditions
were the same as before. 9 to 11 days post-sterilization (dps),
the germinated seedlings were transferred to 9 mm Petri dishes
containing 0.5% MS-medium (Murashige and Skoog 1962) with
1% sucrose, poured as a slope.

Preliminary experiments with isolate 8082 inoculated on
different plant tissues showed that root inoculations with a
mycelial suspension resulted in consistent colonization. Hence,
we used this strategy for further co-cultivations with all endo-
phytes. An endophyte mycelial suspension was prepared from a
Two- to four-day old liquid culture for each endophyte (potato–
carrot liquid medium; 100 g potato–carrot mash [prepared
according to Höller et al. 2000] in 1 l medium). Mycelium was
equally dispersed in 25 ml potato–carrot liquid medium using
Tissuelyser II (Qiagen, Hilden, Germany) for a few seconds.

Preliminary inoculations of S. lycopersicum roots with 25–50
μl of mycelial suspensions of all four endophytes were prepared
to assess the effect of the endophytes on the plant. Isolate 9907
and Phy. fortinii killed the seedlings. Hence, only endophyte iso-
lates 8082 and 9913 were used for further inoculation studies.
For inoculations with endophyte isolate 8082, 5 or 10 μl of the
mycelial suspension or H2O (mock control) was applied to each
root at 16 dps. After 27 dps seedlings were transferred to vessels
(10 cm x 6.5 cm x 6.5 cm) with MS agar medium. For inoculations
with endophyte isolate 9913, 10 μl of dispersed mycelium or H2O
was applied to the roots of axenic seedlings at 18 dps. However,
the endophyte isolate 9913 did not grow sufficiently, so we per-
formed a second inoculation with undispersed mycelium from
the liquid culture at 22 dps. These seedlings were transferred to
vessels at 28 dps. At 34–36 dps, each leaflet of endophyte and
mock inoculated plants was inoculated with 10 μl of Phy. infes-
tans zoospore suspension (4°C cold) or 10 μl H2O (4°C cold).
The zoospore suspension (5*10^4 spores/ml) was harvested from
a 25 day old culture of Phy. infestans isolate D12-2 and was kept
on ice during the entire procedure. For the isolation of zoospores
from Phy. infestans, see de Vries et al. (2017). Plants were sampled
for microscopic evaluation, to evaluate anthocyanin content and
pathogen abundance at three days post-inoculation with Phy.
infestans.

To confirm the endophytic fungal colonization of roots, three
different sterilization procedures were conducted: (i) 70% EtOH
for 3 s (isolate 8082) or 30 s (isolate 9913), ∼5% NaOCl for 30 s,
followed by washing three times with sterile H2O for 3 min each
(treatment 1), (ii) 70% EtOH for 5 min, 0.9% NaOCl for 20 min,
followed by washing three times with H2O (treatment 2, Cao et al.
2004) and (iii) 97% EtOH for 30 s, 10% NaOCl for 2 min, followed
rinsing four times with H2O (treatment 3, Terhonen, Sipari and
Asiegbu 2016). These sterilization procedures were applied to
the roots of the mock controls, as well as the endophyte inocu-
lated and co-inoculated samples. Roots were imprinted on RSA
plates to test for the efficacy of sterilization and then placed on
new RSA plates. The plates were evaluated at 8 dps (isolate 8082)
and 6 dps (isolate 9913).

Microscopy

Two aspects of host physiology were evaluated microscopically
following the co-inoculation: chlorophyll intensity and relative
necrotic area. Pictures to evaluate chlorophyll intensity were
taken with the SMZ18 dissection microscope and a DS-Ri1 cam-
era (Nikon, Japan) using a 600 LP filter (Transmission Filterset
F26-010, AHF Analysetechnik, Germany), with an exposure time
of 200 ms and 100% gain. Intensity was measured using ImageJ2
(Schindelin et al. 2015). Pictures for necrosis measurements were
taken with a SteREO Discovery V8 binocular and an AxioCam
ICC5 camera (Zeiss, Germany). The relative necrotic area was cal-
culated as the necrotic area of a leaflet over the total area of the
leaflet. The necrotic and total leaflet area were estimated using
the ZEN Blue edition (Zeiss, Germany). Differences in relative
necrotic area and chlorophyll content in the treatments were
calculated using a Kruskal–Wallis test (Kruskal and Wallis 1952)
combined with a Tukey and Kramer test for pairwise compar-
isons using a Tukey-distance approximation (Sachs 1997). Fur-
thermore, a Benjamini-Hochberg method was used to correct for
multiple testing (Benjamini and Hochberg 1995).

Photographs of mycelial growth on RSA plates were taken with
the SteREO Discovery V8 binocular and an AxioCam ICC5 camera
(Zeiss, Germany). Additionally, root tissue from co-
inoculations with the endophytes and Phy. infestans as well as
mycelium from RSA plates or potato–carrot liquid medium was
stained with trypan blue (de Vries et al. 2017). The root tissue
was sectioned and the endophytic growth in the root tissue, as
well as trypan blue stained and unstained hyphae from culture-
grown endophytes were visualized using an Axioskop micro-
scope with an AxioCam ICC5 camera with the ZEN blue software
(Zeiss, Germany) and a Zeiss AxioStar Plus and an AxioCam ICC1
with the Axio Vision Release 4.8 (Zeiss, Germany).

Anthocyanin content evaluation

The anthocyanin content was measured and calculated accord-
ing to Lindoo and Caldwell (1978). We analyzed three to six bi-
ological replicates per treatment. Samples were tested for nor-
mality using a Shapiro–Wilk test (Shapiro and Wilk 1965) and
for equal variance. Accordingly, significant differences were cal-
culated using a two-sided t-test with the assumption of equal
or unequal variances depending on the sample combination
tested. All statistical analyses were done in R v3.2.1.
DNA and RNA extraction and cDNA synthesis

DNA was extracted from the mycelium of the fungal endophytes and Phy. infestans isolates grown on RSA medium using the DNeasy® Plant Mini Kit (Qiagen, Germany). RNA was extracted from infected and mock control leaflets of seedlings of S. lycopersicum using the Universal RNA/miRNA Purification Kit (Roboklon, Germany). Three to four leaflets were pooled per replicate. To evaluate RNA quality, 5 μl of RNA were treated with 6 μl denaturated formamide, incubated at 65 °C for 5 min, followed by 5 min incubation on ice. This mixture was then visualized on a 2% agarose gel.

All RNA extractions were treated with DNasel (Thermo Fisher Scientific, Lithuania). For subsequent cDNA synthesis of the DNasel-treated samples, the reactions were adjusted for 200 ng of total RNA and the cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania).

To test whether the RNA samples contained residual DNA even after the DNasel treatment, control reactions were performed. For this, 200 ng of total RNA of each sample was treated with RNAsaE (Macherey-Nagel, Germany) and incubated at 37 °C for 30 min. These RNAsaE-treated samples were then used in a RT reaction using the RevertAid First Strand cDNA Synthesis Kit without the reverse transcriptase and RiboLock.

For the experiments with isolate 9913, we performed a PCR with SIEFlα as described in de Vries et al. 2017. For the samples from the experiments with isolate 8082, we used the ITS1 and ITS4 primers (White et al. 1990). These primers amplify the internal transcribed spacer (ITS) and 5.8S region in S. lycopersicum, the endophytes and Phy. infestans.

For our control experiment to determine if residual DNA was present in the samples, only the positive controls (cDNA from mycelium and untreated leaflets from S. lycopersicum) had an amplicon, showing that there was no remaining DNA contamination in the DNasel-treated samples.

Molecular identification of endophytes

To determine the phylogenetic placement of the fungal endophytes, we sequenced their 5.8S regions. ITS1 and ITS4 primers were used. The 20 μl PCR-reaction contained 1× Green GoTaq® Flexi Buffer, 0.1 mM dNTPs, 2 mM MgCl2, 1U GoTaq® Flexi DNA Polymerase (Promega, WI, USA), 0.2 μM of each primer and 40–95 ng of template DNA. The PCR protocol included an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of a denaturation step at 95 °C for 30 s, an annealing step at 60 °C for 30 s and an elongation step at 72 °C for 90 s, followed by a final elongation step of 72 °C for 7 min. All PCR products were purified with thepeqGOLD Cycle-Pure Kit (Peqlab, Germany). The products were cloned into the PCR 4-TOPO® vector of the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, CA, USA) and the plasmid DNA was extracted with the QiAprep Spin Miniprep Kit (Qiagen, Germany). Sequencing was performed at Eurofins MWG Operon (Germany). Sequences were blasted using BLASTn (Altschul et al. 1990) and the best hits were retrieved. To assemble a dataset of closely related organisms from which to infer the phylogenetic placement of the unknown endophytes, the sequences of species with high similarity to our initial query sequences were downloaded. Taxonomic classification of these sequences was done using mycobank.org (provided by the CBS-KNAW Fungal Biodiversity Center, The Netherlands). Additional sequences were retrieved from GenBank (Table S2, Supporting Information). Taxonomically distant outgroups were chosen based on the systematic classifications in MycoBank (Crous et al. 2004). The sequences were aligned using CLUSTAL-W and a Neighbor-Joining phylogeny was inferred using the Kimura-2 model with five gamma categories and pairwise deletion of gaps. One hundred bootstrap replicates were evaluated. All analyses were done using MEGA 5.2.2 (Tamura et al. 2011).

Assessment of endophyte and Phytophthora infestans growth after eight weeks of co-culture

To determine whether either the endophyte had overgrown Phy. infestans or Phy. infestans had overgrown the endophyte on the co-culture plates, we performed PCR reactions on DNA extracted from both sides of eight-week old co-cultures of five to nine Phy. infestans isolates with Phy. fortinii, isolate 8082 and isolate 9913 as well as their respective controls. We amplified the ITS loci (for primers see White et al. 1990) and the cytochrome oxidase subunit2 (COX2) using Phytophthora-specific primers from Hudspr, Nadler and Hudspeth (2000) with the protocol described above. Between 50 and 100 ng of template DNA was used.

Spread of endophyte isolate 8082 in plant tissue

To evaluate the spread of isolate 8082 in planta over the course of infection, we used molecular analyses (ITS, 28S and beta tubulin sequences) for determining the presence of root-inoculated isolate 8082 (i) in the leaflets from the co-inoculations experiments (ITS and 28S) and (ii) in roots, stems (i.e. between the cotyledons and the first true leaves) following mono-inoculation with 8082 as well as in mock controls (ITS, 28S and beta tubulin). The seedlings were grown and treated as described above, harvested at 34 dps (the time point when Phy. infestans would otherwise be inoculated in a co-inoculation experiment), and DNA was extracted and processed as described above. We amplified the ITS locus to confirm that cDNA synthesis was successful. To determine whether the endophyte was present in the seedling tissues, we used Pe28S primers (forward primer: 5′TCGGGGAAGACTTAAGGGGAA3′, reverse primer: 5′TCTGCTCTACACTTCTAAGGCA3′) designed using NCBI primer BLAST to bind specifically to isolate 8082. Therefore, we first cloned a partial 28S sequence (Accession: MG973066) of isolate 8082 using primers LR0R (Cubeta et al. 1991) and LR5 (Vilgalys and Hester 1990) as described above. We also used the beta tubulin (PeTub) gene (Accession: GU237608.1) of this fungus as a marker (forward primer: 5′TCGACGGCTCTGTTCTCCTAC3′, reverse primer: 5′CGCGTCCGCTTAAAGGAAG3′). The PCR reaction was set up as described above using the GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). For the amplification of the ITS1, 5.8S and ITS2 locus in the leaflet samples from the co-inoculation experiment, the protocol included an initial denaturation at 95 °C for 3 min, 38 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1.30 min, followed by a final elongation step at 72 °C for 5 min. For the root, stem and leaflet samples from seedlings that were root-inoculated with isolate 8082 the number of cycles was reduced to 35. Amplification of the Pe28S and the PeTub genes followed a similar protocol with minor modifications: Pe28S was run with 33 cycles and an annealing temperature of 65 °C and PeTub was run with 40 cycles.
Presence and abundance of Phytophthora infestans

To quantify the abundance of Phy. infestans in the seedlings pre-inoculated with the two endophytes (isolate 8082 and 9913) and the seedlings only inoculated with Phy. infestans, we performed a quantitative RT-PCR (qRT-PCR). The two markers, PiH2a and PiElf1α, were used for the pathogen and the three markers, SAND, TIF and TIF3H, were used as tomato (host) reference genes (de Vries, Kloegees and Rose 2015; de Vries et al. 2017). Two independent qRT-PCR runs were used for the pathogen genes. All qRT-PCRs were performed in a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) and included an initial denaturation at 95 °C for 3 min, followed by 40 cycles of a denaturation step at 95 °C for 10 s and an annealing and elongation step of 60 °C for 45 s. For PiH2a the annealing temperature was lower: 59 °C in the first run and 55 °C in the second run. In general, three biological replicates per treatment were used: (i) isolate 8082 (5 μl mycelial suspension) with Phy. infestans, (ii) isolate 9913 with Phy. infestans and (iii) Phy. infestans without endophyte. The only exception is the treatment with isolate 8082 (10 μl mycelial suspension) with Phy. infestans. In this case, two instead of three biological replicates were used. In each run, we analyzed three technical replicates for each biological replicate, resulting in six technical replicates for each biological replicate for both marker genes. To calculate the relative abundance of Phy. infestans in these samples, we set the Cq-values of those biological replicates that gave no biomass marker amplicon to 41. As the two independent runs gave the same results, they were combined. PiH2a and PiElf1α expression was then calculated according to Pfaffl (2001). Data were tested for normal distribution using a Shapiro–Wilk test and the appropriate statistical tests were then applied. For co-inoculations with isolate 8082, significant differences were calculated using a Mann–Whitney U-test. For co-inoculations with isolate 9913, significant differences were calculated using a two-tailed t-test. The statistical analyses were done using R v3.2.1.

RESULTS

Metabolite assay identifies three endophytes with biocontrol potential

In this study, we analyzed the potential of several endophytes to inhibit the growth of and infection by the plant pathogen Phy. infestans. The endophytes were isolated from eight different plant species from three different European countries (Table S1, Supporting Information) from surfaced sterilized leaves, shoots and roots. Twelve fungal endophytes were selected for further testing of their metabolites for an inhibition potential against Phy. infestans.

To identify fungal endophytes that, on the basis of their secreted metabolites, could be used as biocontrol agents against Phy. infestans, we evaluated culture extracts of the 12 fungal endophytes for growth inhibition of Phy. infestans isolate D2 using an agar diffusion assay. Inhibition of Phy. infestans varied considerably, depending both on the endophyte isolate and on the culture medium. The average growth inhibition was 12.4 ± 8.7 mm ranging from 0 to 35 mm from the point of extract application (Table S3, Supporting Information). Culture extracts of 3 of the 12 isolates inhibited growth of Phy. infestans with a radius ≥20 mm. These three fungal endophytes (isolates 8082, 9907 and 9913) with the greatest Phy. infestans growth inhibition were chosen for further studies. An additional fungal strain, Phi. fortinii (isolate 4197) was included due to its beneficial interaction with another host, Larix decidua (Schulz et al. 2002; Schulz 2006).

Phylogenetic placement of fungal endophytes

To determine the taxonomic identity and phylogenetic placement of the four selected fungal endophytes, we sequenced their ITS1, 5.8S and ITS2 regions. To support their phylogenetic placement, we further used morphological and ecological information on the endophytes (Table S1, Supporting Information). First, we used the cloned sequences in a BLASTn search to identify the closest relatives of the fungal endophytes (Table S4, Supporting Information). All four endophytes belong to the ascomycetes. Our analyses further supported the previous characterization of isolate 4197 as Phi. fortinii (99% identity, Grünig et al. 2008). For isolate 8082, the best BLAST hit with 100% identity was Pho. eupatori. This is in agreement with its morphological description as Phoma sp. (Table S1, Supporting Information). Additionally, it was supported by the fact that isolate 8082 was isolated from Eupatorium cannabinum (Table S1, Supporting Information). The placement of isolates 4197 and 8082 in our phylogenetic analyses together with the extremely short branch lengths to their best BLAST hits further support these phylogenetic assignments (Fig. 1a and b). The best hit for isolate 9907 was Pyrenochaeta cava (95% identity) and for isolate 9913 it was Monosporascus ibericus (97% identity). This suggests that no completely identical sequence/taxa are currently represented in the database. Pyrenochaeta does not form a monophyletic group within the order of Pleosporales (Zhang et al. 2009; Aveskamp et al. 2010; Fig. 1c), thus based on the phylogenetic analysis, isolate 9907 can only be placed within the order Pleosporales. Isolate 9913 was isolated from the roots of Aster triplolum, a plant that was growing in the salt marshes of the Mediterranean Sea (Table S1, Supporting Information). Of note is that Monosporascus ibericus, the fungal endophyte clustering most closely with isolate 9913 in the phylogenetic analysis, has been described as an endophyte of plants growing in environments with high salinity (Collado et al. 2002). Furthermore, the genus Monosporascus is monophyletic; isolate 9913 has been placed within this monophyletic group and herewith termed Monosporascus sp. (Fig. 1d).

Fungal endophytes show broad-spectrum inhibition of Phytophthora infestans growth

Our initial analysis of the culture extracts identified endophytes with the potential to inhibit the growth of a single Phy. infestans isolate. We therefore wondered whether the inhibition could be effective against a wider range of isolates of Phy. infestans. To test this, we conducted a co-culture assay on RSA medium with the four fungal endophytes against nine European Phy. infestans isolates (Fig. 2). For this analysis, the fungal isolates were co-cultured with Phy. infestans isolates for 14–16 days, with the exception of the co-cultivations with Monosporascus sp., which were evaluated after eight days of co-culture due to its fast growth rate. We then compared the radial growth of the Phy. infestans isolates and the endophytes with their respective controls. In the plate assay, all four endophytes were capable of significantly restricting growth of Phy. infestans (Fig. 2m–p). Phoma eupatori and isolate 9907 showed a global inhibition of all Phy. infestans isolates tested (Fig. 2n and o). We further noted that Pho.
Figure 1. Phylogenetic placement of fungal endophytes. Neighbor-Joining phylogeny of ascomycetes closely related to the four fungal endophytes (A–D). Cloned sequences are shown in purple and the best BLAST hit is shown in blue. The monophyletic clade of the genus Monosporascus is indicated by the blue dot (D). The trees are rooted with Leotia lubrica (A), Ascochyta hordei var. hordei (B), Dothidea hippophaeae (C) and Valsaria ceratoniae (D). Only bootstrap values > 50 are shown. The bar below the phylogeny indicates the distance measure for the branches. (E) Pictures of the four fungal endophytes on plates, as well as close-ups of the mycelial growth on plates, microscopic pictures of hyphal growth in culture and in roots of S. lycopersicum. The cultures of Phi. fortinii show pycnidia (black arrows) as well as chlamydospores (purple arrow) and pycnidiospores (grey arrow). Note the primarily intercellular growth of Phi. eupatori on plants (black arrows), which may contribute to its asymptomatic root colonization of S. lycopersicum. Phi. fortinii, in contrast, colonized both inter- and intracellularly, as was observed for this isolate in other hosts (black arrow). Scale bars are given in the corner of each photograph.
Figure 2. Co-cultivation of fungal endophytes with *Phytophthora infestans* on agar culture medium. Examples of two-week-old single and co-cultures of *Phialocephala fortinii* with *Phy. infestans* isolate 3928A (A,E,I), *Phoma eupatorii* with *Phy. infestans* isolate NL00128 (B,F,J) and 9907 with *Phy. infestans* isolate T15-2 (C,G,K) and eight-day old single and co-cultures of *Monosporascus* sp. with *Phy. infestans* isolate D12-2 (D,H,L). The diameter of each Petri dish is 9 cm. Radial growth inhibition of *Phy. infestans* isolates by fungal endophytes (M–P). Radial growth (R) of the different *Phy. infestans* isolates denoted on the x-axis when grown alone (orange) or in co-culture with the four fungal endophytes (blue): *Phi. fortinii* (M), *Pho. eupatorii* (N), isolate 9907 (O) and *Monosporascus* sp. (P). At least 10 biological replicates per control or co-cultivation were measured. The box indicates the upper and lower 50% quartile (interquartile range, IQR), the horizontal line in each box shows the median, the whiskers indicate the upper and lower bounds of the 1.5x IQR and the circles show data points, which are outliers. Significant differences are noted as *P* < 0.05, **P** < 0.01, ***P*** < 0.001 and ns = not significant.
eupatorii showed a color change, which is due to enhanced spore production. Spore production occurred infrequently but did not alter the inhibition rate in co-culture. Phialocephala fortinii inhibited the growth of eight out of nine isolates and Monosporascus sp. inhibited the growth of seven of the nine isolates (Fig. 2m and p). Phoma eupatorii caused the greatest average relative growth inhibition of Phy. infestans with 50.6 ± 2.2%, and Monosporascus sp. the lowest with 11.9 ± 1.6% (Table 1).

To exclude a mere reduction based on growth limitations we (i) measured the inhibition of the endophyte’s growth by Phy. infestans after the initial co-cultivation phase (14–16 days, or eight days in case of co-cultures including Monosporascus sp.) and (ii) evaluated long-term co-cultures (i.e. eight weeks) to analyze the endophyte and pathogen growth progression. The growth of isolate 9907 was not inhibited by any of the Phy. infestans isolates (Fig. S1c, Supporting Information). However, some isolates of Phy. infestans were able to inhibit the growth of the other three fungal endophytes (Fig. S1a,b, and d, Supporting Information). In all cases, the average relative inhibition of an endophyte by Phy. infestans was, however, less than the average relative inhibition of Phy. infestans by an endophyte (Table 1, Table S5, Supporting Information). For example, whereas the average relative growth inhibition of Pho. eupatorii extracted DNA from some co-cultures with all cases, the average relative inhibition of an endophyte by Phy. infestans (Fig. S1a,b, and d, Supporting Information). In total, we analyzed 37 co-cultures), Pho. eupatorii cultures), Phy. fortinii (12 co-cultures), Phy. eupatorii (18 co-cultures) and Monosporascus sp. (seven co-cultures) from both sides of the eight-week samples (Table S6, Supporting Information). In total, we analyzed 37 co-cultures and their respective controls for the presence of endophyte and Phy. infestans. We used the marker genes COX and ITS. Because our ITS primers were designed for fungi, we primarily observed amplicons from the fungal endophyte ITS loci when both organisms were present. However, presence of Phy. infestans could be determined by the presence of a COX amplicon. In general, we observed that the endophyte was present on both sides of the plates, whereas Phy. infestans was either not detected or only on the side of the plate on which it had been inoculated. Few exceptions occurred in which Phy. infestans was observed on the side of the original inoculation of the fungal endophyte (2/37 cases). Hence, Phy. infestans was usually not able to colonize the side of the plate where the endophyte was growing, while the endophyte was always able to colonize Phy. infestans’ side of the plate. In addition, the endophytes showed a greater inhibition of Phy. infestans than Phy. infestans did of the endophytes. Therefore, resource limitation (due to the size of the plates) is unlikely to fully explain the unequal growth differential between Phy. infestans and the endophytes during co-cultivation. Instead, we hypothesize that factors actively secreted by the endophytes may also be involved in the growth inhibition of Phy. infestans.

Phoma eupatorii limits Phytophthora infestans infection success

We identified global, non-isolate-specific growth inhibition by all four endophytes in plate assays. To test whether the inhibitory potential of the endophytes holds true in planta, we inoculated the fungal endophytes in axenically grown S. lycopersicum cv. M82 seedlings. Our preliminary analysis showed that Phi. fortinii and isolate 9907 were too virulent and killed the S. lycopersicum seedlings (Fig. S2 a, b, and d, Supporting Information). In contrast, S. lycopersicum seedlings inoculated with Pho. eupatorii or Monosporascus sp. survived (Fig. S2 a, c, and e, Supporting Information).

To confirm the endophytic colonization of the roots, we analyzed fungal outgrowth of surface sterilized roots and their imprints from inoculations with water, endophyte or endophyte and Phy. infestans (Table 2). Irrespective of the protocol, there was no fungal growth from the surface sterilized mock control roots or from their imprints. Generally, imprints of the surface sterilized endophyte inoculated roots did not show fungal growth, except for Pho. eupatorii inoculated roots after sterilization procedure 1 (1/16 imprints from the mono-inoculation and 5/12 imprints from the co-inoculations). This suggests that surface sterilization was successful in all other cases. Phoma eupatorii grew from several roots independently of the sterilization procedure, although the stronger treatments resulted in less outgrowth. Hence, these treatments may partially impact survival of endophytic mycelium. Nevertheless, these results show that Pho. eupatorii is capable of colonizing S. lycopersicum roots. Monosporascus sp. also showed outgrowth from several of the plated surface sterilized roots, suggesting that, like Pho. eupatorii, Monosporascus sp. also grows endophytically in the roots of S. lycopersicum. This was further confirmed by microscopy of the roots inoculated with the endophytes (Fig. 1e).

Solanum lycopersicum seedlings colonized by Pho. eupatorii are visually smaller than mock control seedlings and seedlings mono-inoculated with Phy. infestans. We also observed a reduction in leaflet number (Fig. S3 a and c, Supporting Information). Since the leaflets appeared sturdier and were darker green than the controls (Fig. 4a-f), we measured chlorophyll levels via chlorophyll fluorescence. However, chlorophyll abundance did not change following any of the treatments (Fig. 4g-m). We also observed that some of the stems and leaflets of the plants that had been inoculated with Pho. eupatorii developed a purple color (Fig. S3c, Supporting Information). Therefore, we reasoned that the darker leaflet color may have resulted from anthocyanin accumulation. Anthocyanin is a plant stress compound and hence we evaluated if Pho. eupatorii may stress the seedlings. In fact, we detected a significant increase in anthocyanin content in Pho. eupatorii inoculated versus mock control plants (P = 0.001 without Phy. infestans, P = 0.04 with Phy. infestans, Fig. 4n). In contrast to seedlings colonized by Pho. eupatorii, those inoculated with Monosporascus sp. did not visibly differ from the mock controls (Figs S3a and b, and S4a and c, Supporting Information). In agreement with this, anthocyanin content did not differ in Monosporascus sp. inoculated and mock control samples (relative anthocyanin contentmock = 0.5 ± 0.1 vs relative anthocyanin contentPiH2a = 2.3 ± 1.3; P = 0.08 without Phy. infestans). However, when both endophyte and pathogen were present, the anthocyanin content was elevated (relative anthocyanin contentmock = 0.5 ± 0.1 vs relative anthocyanin contentPiElf1a = 1.2 ± 0.1; P = 0.007), suggesting that the increase results from the presence of Phy. infestans.

Despite the visible effects of the colonization by Pho. eupatorii on the seedlings, we proceeded to investigate the effect of the endophyte on a subsequent infection with Phy. infestans. The relative necrotic area caused by the pathogen is significantly higher on plants inoculated only with Phy. infestans (in the absence of pre-inoculation by an endophyte) compared to the mock control (Fig. 4o; Fig. S4e, Supporting Information). To confirm the pathogen infection in the mock/Phy. infestans samples, we used the expression of the Phy. infestans biomass marker genes PiH2a and PiElf1a. In agreement with the increase
Table 1. Average relative growth inhibition of Phy. infestans (upper row) by endophytes (first column).

| Endophyte       | NL88069 | IPO-C | NL90128 | IPO428-2 | NL10001 | 3928A | T20-2 | D12-2 | T15-2 | Average +/- SEM |
|-----------------|---------|-------|---------|----------|---------|-------|-------|-------|-------|-----------------|
| Phi. fortinii   | 0.100   | 0.273 | 0.254   | 0.211    | 0.190   | 0.232 | 0.166 | 0.200 | 0.216 | 0.205 +/- 0.016 |
| Pho. eupatorii  | 0.537   | 0.546 | 0.661   | 0.539    | 0.429   | 0.471 | 0.480 | 0.411 | 0.482 | 0.506 +/- 0.022 |
| 9907            | 0.276   | 0.276 | 0.251   | 0.250    | 0.210   | 0.215 | 0.244 | 0.166 | 0.262 | 0.239 +/- 0.011 |
| Monosporascus sp.| 0.038   | 0.157 | 0.138   | 0.137    | 0.020   | 0.161 | 0.129 | 0.144 | 0.147 | 0.119 +/- 0.016 |

The relative inhibition is calculated from the average radii estimated for co-cultivations and control plates. A minimum of ten biological replicates per control or co-cultivation were analyzed.

Figure 3. Long-term co-cultivation of fungal endophytes with Phytophthora infestans on agar growth medium. Examples of eight-week-old co-cultures and their respective controls. Phi. fortinii with Phy. infestans isolate NL88069 (A,E,I), Pho. eupatorii with Phy. infestans isolate NL88069 (B,F,J), isolate 9907 with Phy. infestans isolate T15-2 (C,G,K) and Monosporascus sp. with Phy. infestans isolate NL10001 (D,H,L). The diameter of each Petri dish is 9 cm.

in necrotic area, Phy. infestans was present in all biological replicates mono-inoculated with the pathogen, i.e. demonstrating a successful infection.

While the relative necrotic area in seedlings that were colonized only by Pho. eupatorii was 4.7-fold higher compared to the mock control, this was significantly less than the relative necrotic area of seedlings infected with only Phy. infestans (Fig. 4o). Solanum lycopersicum seedlings co-inoculated with Pho. eupatorii and Phy. infestans resulted in a significantly reduced relative necrotic area compared to seedlings mono-inoculated with Phy. infestans (Fig. 4o). Importantly, the average relative necrotic area of leaflets colonized by both Pho. eupatorii and Phy. infestans did not differ from the mono-inoculations with the endophyte (Fig. 4o). Whether 5 or 10 μl mycelial suspensions of Pho. eupatorii were used had no effect on the outcome of the experiments. The relative necrotic area between the treatment with Monosporascus sp. and the mock control did not differ (Fig. S4a, c and e, Supporting Information). This endophyte was neither able to inhibit Phy. infestans infection nor limit development of disease symptoms in planta (Fig. S4b, d, e and f, Supporting Information).
To quantify the biomass of *Phy. infestans* in planta after pre-inoculation with *Pho. eupatori*, we performed a qRT-PCR with the two biomass marker genes *PiElf1a* and *PiH2A* (Fig. 4p). In total, we tested the three biological replicates from the 5 µl *Pho. eupatori* inoculations and two from the 10 µl *Pho. eupatori* inoculations. In three of those five replicates, we did not detect an amplicon for either *PiH2A* or *PiElf1a*. Yet, *PiH2A* and *PiElf1a* were detected in every biological replicate of the mock/*Phy. infestans* infections. In addition, three plant-specific reference genes were tested; these showed no aberrant expression in any of the samples colonized by the endophyte in which *PiH2A* and *PiElf1a* were not detected. Hence, the presence of the fungal endophyte did not affect the efficiency of the qRT-PCR. Also, those samples that were pre-inoculated with *Pho. eupatori*, but gave an amplicon of the marker genes had reduced Cq-values for both marker genes compared to the mock/*Phy. infestans* samples. This suggests that *Pho. eupatori* reduced the infection with *Phy. infestans* isolate D12-2 in the sampled leaflets. To estimate the reduction of *Phy. infestans* biomass, we assumed that the Cq-value of those replicates with no amplicon could theoretically have been amplified in later cycles. We therefore set the Cq-values in those samples that were pre-inoculated with *Pho. eupatori*, but gave an amplicon for either *PiH2A* or *PiElf1a* to be 41; i.e. one cycle more than the original runs included. Based on this assumption, we observed a significant reduction of gene expression in both biomass marker genes in the *Pho. eupatori* pre-treated samples compared to mono-infections of *Phy. infestans* (Fig. 4p).

We further explored whether *Pho. eupatori* inhibits *Phy. infestans* due to direct competition in the leaflets or indirectly via some form of long-distance signal. To do this, we analyzed (i) whether *Pho. eupatori*, despite being root-inoculated, was able to colonize the leaflets in the co-inoculation experiment and (ii) how far *Pho. eupatori* could spread from the time point of root inoculation with the endophyte to the day of leaflet-inoculation with *Phy. infestans*. Using two *Pho. eupatori* marker genes (Fig. S5a, Supporting Information), we found that some of the co-inoculated leaflets, but not all, were colonized by the endophyte at the time of harvest. Additionally, in assays using the Pe28S marker, the endophyte was detected in all roots, many stems and some leaflets (Fig. S5b, Supporting Information). PeTub was detected in all root samples, but in contrast to Pe28S, only in two of the stems and none of the leaflet samples (Fig. S5b, Supporting Information). In agreement with PeTub, the ITS1, 5.8S and ITS2 band specific to *Pho. eupatori* was also only found in two stem samples and no leaflet samples. Differences between endophyte detection across tissues by these three markers is likely related to differences in their sensitivity, with the greatest sensitivity provided by Pe28S, due to its high species specificity and substantial genomic copy number. All in all, these data show that in leaflet samples where we detected *Pho. eupatori*, the endophyte was potentially in the leaflet tissue at the time of inoculation with *Phy. infestans*. However, since all co-inoculated samples showed a significant reduction in *Phy. infestans* infection, whether or not leaflet colonization with *Pho. eupatori* was detected, it suggests that even the presence of *Pho. eupatori* in the roots and stems brought about substantial pathogen suppression. In summary, despite—or perhaps because of—an increased stress response of the infected seedlings, *Pho. eupatori* is capable of significantly inhibiting *Phy. infestans* infection of *S. lycopersicum* leaflets.

### DISCUSSION

**Fungal endophytes show a broad-spectrum growth inhibition of European Phytophthora infestans isolates**

Of 12 fungi for which culture extracts were tested for inhibition of *Phy. infestans*, we identified three ascomycetes, *Pho. eupatori*, isolate 9907 and *Monosporascus* sp., which effectively inhibited growth of the pathogen. While fungal endophytes produce a vast diversity of metabolites (Schulz et al. 2002; Strobel and Strobel 2007; Verma, Kharwar and Strobel 2009; Mousa and Raizada 2013; Brader et al. 2014) and numerous have antimicrobial activity (Son et al. 2008; Puopolo et al. 2014; Mousa et al. 2016), their metabolites may have a narrow spectrum of specificity. To avoid narrow spectrum of pathogen inhibition, we studied these three fungal endophytes and the endophyte *Ph. fortinii* for their capacity to inhibit the growth of nine European isolates of *Phy. infestans*. In our co-culture assays, *Pho. eupatori* and isolate 9907 had a broad-spectrum inhibition against all tested isolates, whereas *Monosporascus* sp. and *Ph. fortinii* inhibited most of the isolates. Furthermore, after eight weeks of incubation, the pathogen was not able to grow on sections of the plates, in which the endophytes grew. The consistency of the results from the culture extract experiments and the plate assays of *Pho. eupatori* and

### Table 2. Endophytic outgrowth from surface sterilized roots after inoculation with the endophyte.

| Procedure | 8 dps root | 6 dps root |
|-----------|------------|------------|
| mock/mock | 0/10       | 0/13       | 0/13       |
| endophyte/mock | 1/6        | 13/16      | 0/12       | 3/12 |
| endophyte/Phy. infestans | 5/12 | 10/12 | 0/12 | 3/12 |

**Procedure 1**

**mock/mock** | 0/10 | 0/10 | 0/12 | 3/12 |

**endophyte/mock** | 0/17 | 2/13 | 0/12 | 0/12 |

**endophyte/Phy. infestans** | 0/12 | 0/12 | 0/12 | 0/12 |

**Procedure 2**

**mock/mock** | 0/11 | 0/11 | 0/12 | 0/12 |

**endophyte/mock** | 0/15 | 4/15 | 0/12 | 0/12 |

**endophyte/Phy. infestans** | 0/12 | 2/12 | 0/8  | 2/8  |

**Procedure 3**

**mock/mock** | 0/10 | 0/10 | 0/12 | 0/12 |

**endophyte/mock** | 0/10 | 0/10 | 0/12 | 0/12 |

**endophyte/Phy. infestans** | 0/12 | 0/12 | 0/12 | 0/12 |

Roots were surfaces sterilized and an imprint of each root was prepared to test for efficiency of the treatment. The days after which the roots were surveyed is given as days post sterilization (dps). Procedure 1, 2 and 3 indicate the type of surface sterilization as described in the Material and Method section. The number of imprints and roots with fungal growth and the total number of analyzed roots is given for each sample type.
Phoma eupatorii isolate 8082 may inhibit Phytophthora infestans via secreted toxic metabolite(s)

Phoma eupatorii was the most effective fungal endophyte in our experiments, excelling both in co-culture as well as in planta. The presence of Pho. eupatorii not only reduced or inhibited the pathogen’s growth, but perhaps entirely prevented infection. Here we used root inoculations of Pho. eupatorii combined with leaflet inoculations of Phy. infestans isolate D12-2. Because Pho. eupatorii was applied to roots and did not consistently spread to the leaflets by the time the plants were inoculated with Phy. infestans, niche competition is less likely to be the only mechanism by which Pho. eupatorii protects the seedlings of S. lycopersicum. Therefore, two other possible mechanisms by which the plant is defended against the pathogen include endophyte-dependent...
induction of defense responses or the production of mobile anti-Phytophthora metabolites. The induction of plant defense responses by endophytes, such as S. indica and non-pathogenic Fusarium oxysporum, has been previously shown (Stein et al. 2008; Aimé et al. 2013). Here, we observed an elevation of anthocyanin levels in leaf tissue of S. lycopersicum after root colonization of Pho. eupatorii. Accumulation of anthocyanins is a stress response and, among other factors, positively regulated by jasmonic acid (Franceschi and Grimes 1991; Feyes et al. 1994; Li et al. 2006; Shan et al. 2009). Hence, it is possible that jasmonic acid dependent defense responses are induced upon colonization with Pho. eupatorii and may contribute to the inhibition of the Phy. infestans infection that we observed. Yet, the role of jasmonic acid in defense against Phy. infestans is not clear. The application of jasmonic acid to leaves of tomato and potato plants resulted in reduced infection of the pathogen (Cohen, Gisi and Niderman 1993). It is further reported that jasmonic acid is required for the initiation of defense responses triggered by a peptide secreted by Phy. infestans (Halim et al. 2009). Yet, potato RNA interference lines that downregulated jasmonic acid biosynthesis and signaling components showed no alterations in the infection rates of Phy. infestans (Halim et al. 2009). Hence, the production of anti-Phytophthora metabolites may be a more likely explanation for the observed reduction of Phy. infestans infection. A recently published example of a metabolite-based endophyte-mediated protection is that of Enterobacter sp. This endophyte produces many different antimicrobial compounds in its host plant and these are detrimental to the plant pathogen F. graminearum (Mousa et al. 2016). In our study, each of the four fungal endophytes undoubtedly produces anti-Phytophthora metabolites in the crude extract tests and in the co-cultures on agar media. This makes it likely that Pho. eupatorii also produces such metabolites during in planta co-infections with Phy. infestans. A combination of an elevated stress response (jasmonic acid mediated or not) and inhibition of Phy. infestans by antimicrobial compounds is, however, also possible.

Development of Phoma eupatorii as a biocontrol agent

Further questions should be addressed to determine if Pho. eupatorii is fit to become a biocontrol. For example: (i) How long do endophytes survive in the soil? (ii) Could the endophyte become an invasive species and/or pathogenic on other plants? (iii) Is a practical and efficient mode of application of the potential biocontrol available, i.e. could spores be used as a source of inoculum as shown for other biocontrol agents (Annesi et al. 2005)? We have shown that Pho. eupatorii is able to produce spores on plate. However, which plant organ would be suitable for reliable infection and how the potential biocontrol agent could be formulated would need to be determined and (iv) are the metabolites mycotoxins? If so registration would be problematic.

The longevity of the endophyte in soil is important, especially considering that the relationship between host and endophyte is environment dependent and that some endophytes may become pathogenic under certain conditions (Schulz and Boyle 2005). Moreover, Pho. eupatorii seems to have a broad host range, given that it was isolated from E. cannabinum and is also able to infect S. lycopersicum. A broad host range may become problematic for other plants in the environment, for example, if Pho. eupatorii is pathogenic on them. Hence, its ability to infect several common weeds, as well as other crop plants should be assessed.

Conclusion: Phoma eupatorii isolate 8082 is a potential novel Phytophthora infestans biocontrol agent

Out of an analysis of 12 fungal endophytes, we discovered four ascomycetes that inhibited the growth of Phy. infestans in co-culture, presumably through the secretion of secondary metabolites, particularly since their culture extracts were also active. Most importantly, two of the endophytes exhibited global inhibition of nine European Phy. infestans isolates, the other two showing a near-global inhibition. This indicates that a conserved target within Phy. infestans for a particular metabolite may be produced by these four endophytes. Alternatively, complex metabolite mixtures could be involved. In either case, the use of these fungi for biocontrol could slow the counter-adaptation of Phy. infestans. Hence, all four fungal endophytes can be considered good candidates for the production of such new and urgently needed compounds. Additionally, of the four fungal endophytes, Pho. eupatorii functioned as an effective biocontrol agent in planta. Phoma eupatorii may not only synthesize a reservoir of highly useful antimicrobial metabolites but may additionally induce resistance in the plant. Phoma eupatorii is hence a potential candidate to be tested as a novel biocontrol agent in the field providing an alternative to resistance gene breeding and application of agrochemicals.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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