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Research Article

RESEARCH ARTICLE

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

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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 overcome 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 Cocq *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 Cocq *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; Bodenhausen, 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; Brader *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 (Shoresh, Harman and Mastouri 2010) and/or produce bioactive anti-microbial metabolites (Brader *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*, *Se. 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 eupatorii*, isolate 8082. This endophyte prohibited proliferation of *Phy. infestans* and in some cases abolished its infection completely. Since we selected *Pho. eupatorii* 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 *Pho. eupatorii* 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 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 biomalt 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 rye 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 $\geq$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, 3928A, 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 experiments depended on the endophytes' growth rates: eight days for all co-cultures that included 9913 and 14–16 days for the remaining co-cultures. A minimum of 10 plates was analyzed per treatment. The Mann–Whitney U test (Mann and Whitney 1947) was used to determine if differences between co-culture and control plates were significant. Average growth inhibition was estimated 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. Pictures were taken with an EOS 70D 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% H_2O-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 endophytes. 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 *Phi. fortinii* killed the seedlings. Hence, only endophyte isolates 8082 and 9913 were used for further inoculation studies. For inoculations with endophyte isolate 8082, 5 or 10 μl of the mycelial suspension or H_2O (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 H_2O was applied to the roots of axenic seedlings at 18 dps. However, the endophyte isolate 9913 did not grow sufficiently, so we performed 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. infestans* zoospore suspension (4°C cold) or with 10 μl H_2O (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 H_2O for 3 min each (treatment 1), (ii) 70% EtOH for 5 min, 0.9% NaOCl for 20 min, followed by washing three times with H_2O (treatment 2, Cao *et al.* 2004) and (iii) 97% EtOH for 30 s, 10% NaOCl for 2 min, followed rinsing four times with H_2O (treatment 3, Terhonen, Sipari and Asiegbu 2016). These sterilization procedures were applied to the roots of the mock controls, as well as the endophyte inoculated 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 camera (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 calculated 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 comparisons using a Tukey-distance approximation (Sachs 1997). Furthermore, 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 Axiophot microscope 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 according to Lindoo and Caldwell (1978). We analyzed three to six biological replicates per treatment. Samples were tested for normality using a Shapiro–Wilk test (Shapiro and Wilk 1965) and for equal variance. Accordingly, significant differences were calculated 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 deionized 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 DNAseI (Thermo Fisher Scientific, Lithuania). For subsequent cDNA synthesis of the DNAseI-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 DNAseI treatment, control reactions were performed. For this, 200 ng of total RNA of each sample was treated with RNAseA (Macherey-Nagel, Germany) and incubated at 37°C for 30 min. These RNAseA 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 *SlElf1α* 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 5.8*S* 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 DNAseI-treated samples.

Molecular identification of endophytes

To determine the phylogenetic placement of the fungal endophytes, we sequenced their *ITS* and 5.8*S* regions. *ITS1* and *ITS4* primers were used. The 20 μl PCR-reaction contained 1x Green GoTaq® Flexi Buffer, 0.1 mM dNTPs, 2 mM $MgCl_2$, 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 the peqGOLD 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 *Phi. 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 Hudspeth, 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 RNA 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′TCGGGGAGAACTTATAGGGGA3′, reverse primer: 5′TGGCTTCACCCTATTCAAGCA3′) 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′TCGACGGCTCTGGTGTCTAC3′, reverse primer: 5′CGCAGTCGGTCTAAGGAAAGT3′). 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*, 5.8*S* 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*, *TIP* and *TIF3H*, were used as tomato (host) reference genes (de Vries, Kloesges 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. eupatorii*. 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 tripolium*, 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 eupatorii* and isolate 9907 showed a global inhibition of all *Phy. infestans* isolates tested (Fig. 2n and o). We further noted that *Pho.*

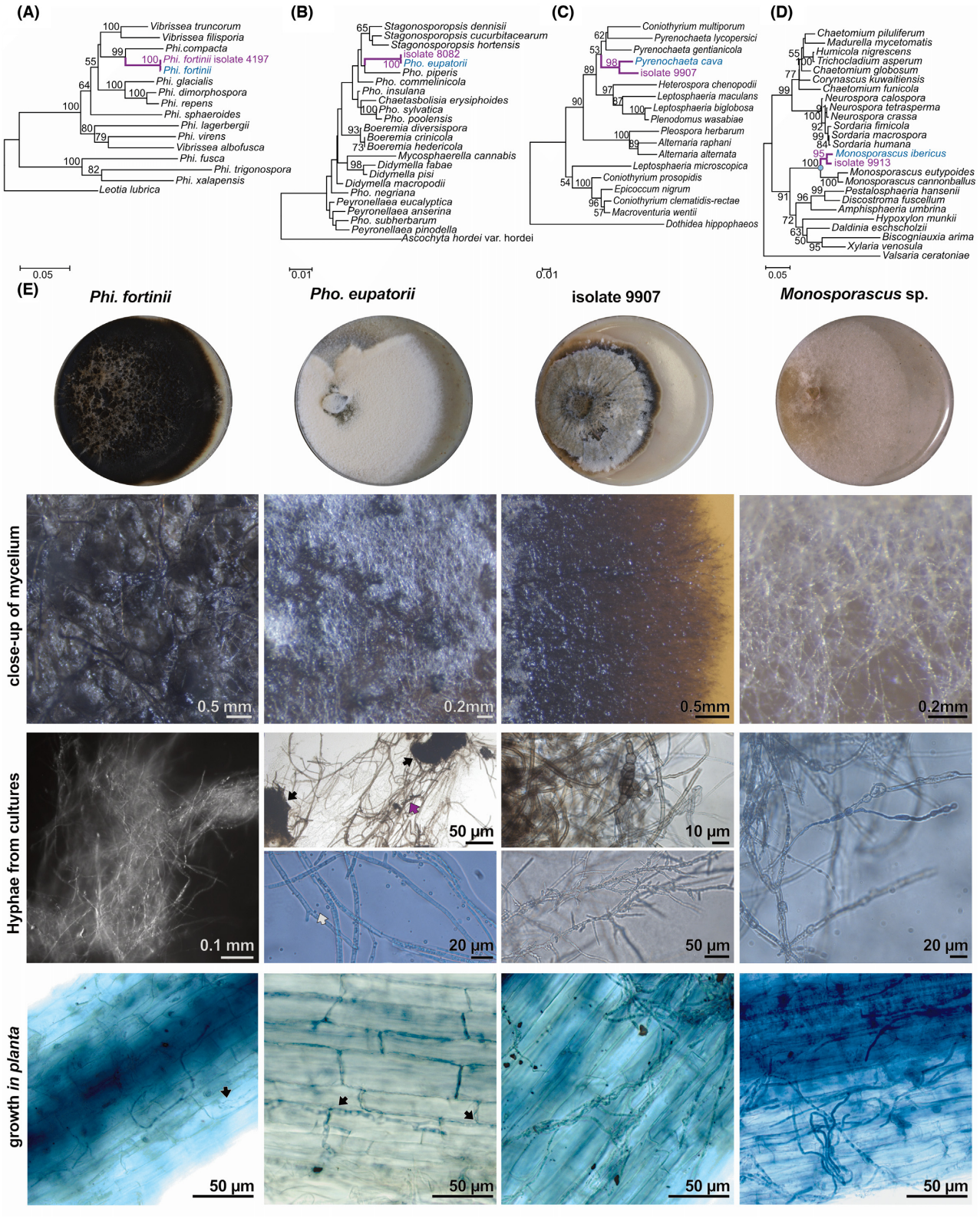

Figure 1. Phylogenetic placement of fungal endophytes. Neighbor-Joining phylogeny of ascomycetes closely related to the four fungal endophytes (**A-D**). Cloned sequences are shown in purple and the best BLAST hit is shown in blue. The monophyletic clade of the genus *Monosporascus* is indicated by the blue dot (**D**). The trees are rooted with *Leotia lubrica* (**A**), *Ascochyta hordei* var. hordei (**B**), *Dothidea hippophaeos* (**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 *Pho. eupatorii* show pycnidia (black arrows) as well as chlamydospores (purple arrow) and pycnidiospores (grey arrow). Note the primarily intercellular growth of *Pho. eupatrii in planta* (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.

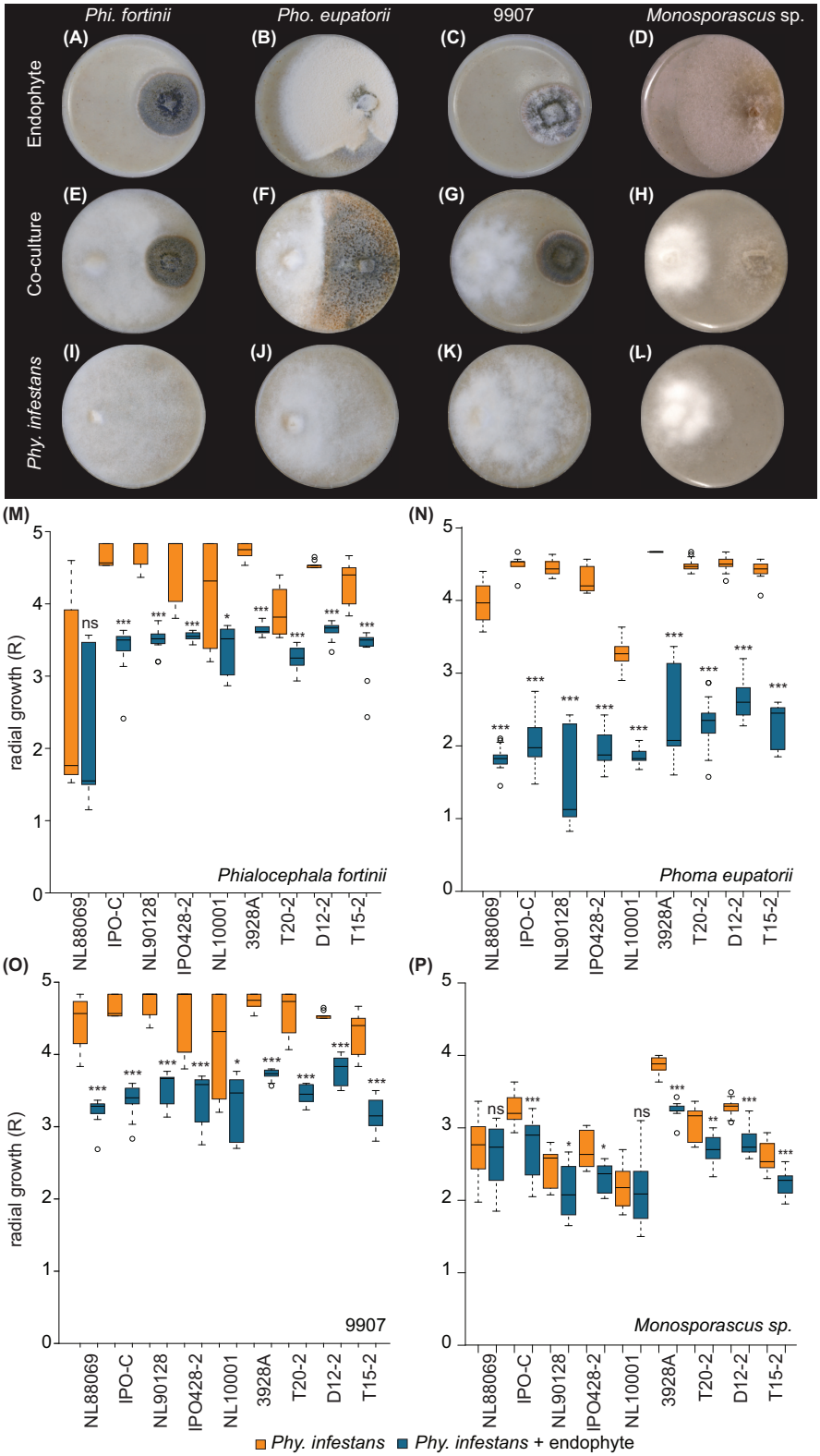

Figure 2. Co-cultivation of fungal endophytes with *Phytophthora infestans* on agar culture medium. Examples of two-week-old single and co-cultures of *Phialocephala fortinii* with *Phy. infestans* isolate 3928A (**A**,**E**,**I**), *Phoma eupatorii* with *Phy. infestans* isolate NL90128 (**B**,**F**,**J**) 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 endophtyes (**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% (Table1).

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 *Phy. infestans* by *Pho. eupatorii* was 50.6 ± 2.2%, the average relative inhibition of *Pho. eupatorii* by *Phy. infestans* was 4.7 ± 0.9%.

After eight weeks, the endophytes (except for isolate 9907) visually overgrew the plates, including the regions colonized by *Phy. infestans* (Fig. 3). To substantiate this observation, we extracted DNA from some co-cultures with *Phi. fortinii* (12 co-cultures), *Pho. 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. eupatrorii* 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 microcospy 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 Informaton). 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 $\text{content}_{\text{mock}} = 0.5 \pm 0.1$ vs relative anthocyanin $\text{content}_{9913/\text{mock}.} = 2.3 \pm 1.3$; $P = 0.08$ without *Phy. infestans*). However, when both endophyte and pathogen were present, the anthocyanin content was elevated (relative anthocyanin $\text{content}_{\text{mock}} = 0.5 \pm 0.1$ vs relative anthocyanin $\text{content}_{9913/\text{Phy}.} = 1.2 \pm 0.1$; $P = 0.007$), 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 *PiElf1α*. In agreement with the increase

Table 1. Average relative growth inhibition of *Phy. infestans* (upper row) by endophytes (first column).

	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.

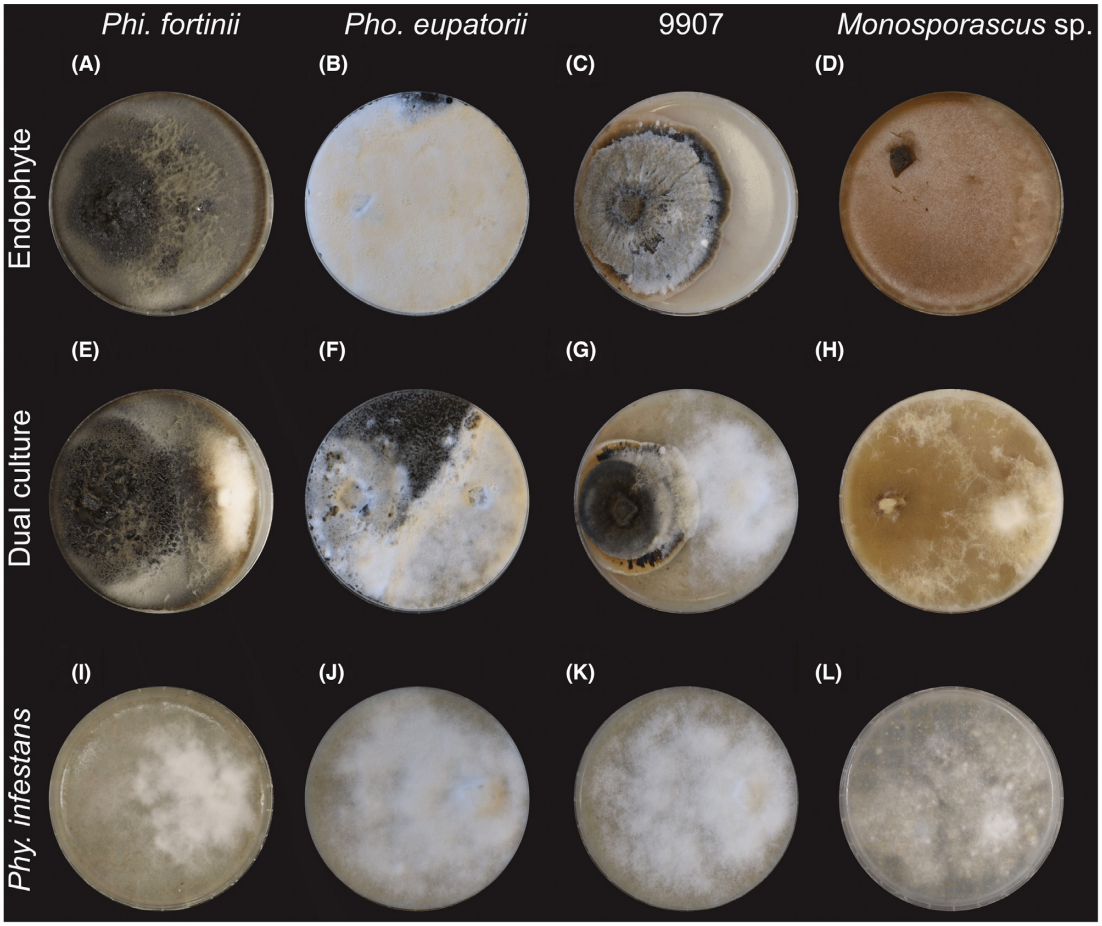

Figure 3. Long-term co-cultivation of fungal endophytes with *Phytophthora infestans* on agar growth medium. Examples of eight-week-old co-cultures and their respective controls. *Phi. fortinii* with *Phy. infestans* isolate NL88069 (**A**,**E**,**I**), *Pho. eupatorii* with *Phy. infestans* isolate 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).

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

	Pho. eupatorii imprint	8 dps roots	*Monosporascus* sp. imprint	6 dps roots
Procedure1				
mock/mock	0/10	0/10	0/13	0/13
endophyte/mock	1/16	13/16	0/12	3/12
endophyte/*Phy. infestans*	5/12	10/12	0/12	3/12
Procedure 2				
mock/mock	0/10	0/10	0/12	0/12
endophyte/mock	0/13	2/13	0/12	3/12
endophyte/*Phy. infestans*	0/12	3/12	0/12	0/12
Procedure 3				
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

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.

To quantify the biomass of *Phy. infestans in planta* after pre-inoculation with *Pho. eupatorii,* we performed a qRT-PCR with the two biomass marker genes *PiElf1α* and *PiH2A* (Fig. 4p). In total, we tested the three biological replicates from the 5 μl *Pho. eupatorii* inoculations and two from the 10 μl *Pho. eupatorii* inoculations. In three of those five replicates, we did not detect an amplicon for either *PiH2a* or *PiElf1α*. Yet, *PiH2a* and *PiElf1α* 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 *PiElf1α* 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. eupatorii,* 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. eupatorii* 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 to 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. eupatorii* pre-treated samples compared to mono-infections of *Phy. infestans* (Fig. 4p).

We further explored whether *Pho. eupatorii* 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. eupatorii,* despite being root-inoculated, was able to colonize the leaflets in the co-inoculation experiment and (ii) how far *Pho. eupatorii* could spread from the time point of root inoculation with the endophyte to the day of leaflet-inoculation with *Phy. infestans*. Using two *Pho. eupatorii* 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. eupatorii* 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. eupatorii,* 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. eupatorii* was detected, it suggests that even the presence of *Pho. eupatorii* 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. eupatorii* 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. eupatorii,* 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 *Phi. fortinii* for their capacity to inhibit the growth of nine European isolates of *Phy. infestans*. In our co-culture assays, *Pho. eupatorii* and isolate 9907 had a broad-spectrum inhibition against all tested isolates, whereas *Monosporascus* sp. and *Phi. 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. eupatorii* and

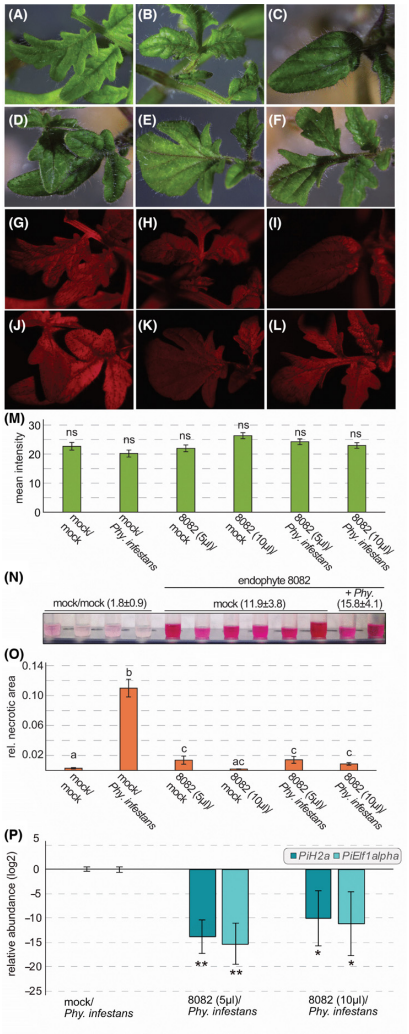

Figure 4. *In planta* co-inoculations of *Phoma eupatorii* isolate 8082 and *Phytophthora infestans*. *Solanum lycopersicum* cv. M82 seedlings were mock treated (**A**) or inoculated with *Phy. infestans* isolate D12-2 (**B**), 5 μl of *Pho. eupatorii* mycelium suspension (**C**), 10 μl of *Pho. eupatorii* mycelium suspension (**D**), 5 μl of *Pho. eupatorii* mycelium suspension and *Phy. infestans* isolate D12-2 (**E**) and 10 μl of *Pho. eupatorii* mycelium suspension and *Phy. infestans* isolate D12-2 (**F**). Chlorophyll fluorescence is depicted in red false coloring for all combinations (**G**–**L**) and was measured as mean fluorescence intensity using ImageJ2 (**M**). Bars give the average mean fluorescence ($n_{leaflets}$ = 17–37). Error bars give the standard error of the mean (SEM); ns = not significant. Differences in anthocyanin content (**N**). A darker pink in the examples shown indicates a higher concentration of anthocyanins in the sample. Average relative anthocyanin content with standard deviation is given in brackets following each treatment. In total, three to six biological replicates per treatment were analyzed. The average relative necrotic area of the leaflets was calculated for each treatment ($n_{leaflets}$ = 38–156). Bars give the average necrotic area per treatment and error bars indicate the SEM. Significant differences between the treatments are indicated by different letters above the bars with a cutoff of $P < 0.05$; same letter = not significant. The relative abundance of *Phy. infestans* isolate D12-2 was measured with a qRT-PCR of the two biomass marker genes *PiH2a* and *PiElf1α* (**P**). Bars show average relative expression of the two biomass markers normalized against the three plant reference genes *SAND*, *TIP* and *TIF3H*. It compares relative abundance of *Phy. infestans* in *Pho. eupatorii*–*Phy. infestans* co-inoculations with that in control treatments (*Phy. infestans* only). Three biological replicates per treatment were used in all cases except for *Pho. eupatorii* (10 μl mycelial suspension) with *Phy. infestans*, in which only two biological replicates were used. The error bars indicate the SEM. Significant differences between relative *Phy. infestans* abundance in samples pre-inoculated with the endophyte and the control are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$. In all bar graphs, treatments with *Pho. eupatorii* are indicated by its isolate number 8082.

isolate 9907 shows that their inhibition is independent of the growth medium, suggesting an environmentally robust metabolite production of their anti-*Phytophthora* substances. A robust metabolite production would be of great advantage if these fungal endophytes are to be used as living biocontrol agents in the field.

As a first step towards identifying a potential biocontrol agent, we examined two essential questions: (i) Does infection by the endophyte damage the host in the absence of a pathogen? (ii) Does the endophyte successfully inhibit the pathogen in the host? In this study, the first question is especially relevant, because the fungal endophytes in question were not originally isolated from Solanaceae, to which tomato belongs. Furthermore, whether an endophyte remains benign and asymptomatic is likely to be affected by a number of different factors and in some cases the host endophyte relationship may shift to a pathogenic outcome from an initially protective interaction (Schulz and Boyle 2005; Junker, Draeger and Schulz 2012; Schulz *et al.* 2015; Busby, Ridout and Newcombe 2016). Along these lines, we excluded two isolates, *Phi. fortinii* and isolate 9907, for direct applications as biocontrol agents: seedlings of *S. lycopersicum* infected with either of these two isolates quickly died after inoculation. A third isolate, *Monosporascus* sp., neither inhibited *Phy. infestans* infection nor hindered its infection progress. This may not be surprising, because *Monosporascus* sp. had the lowest inhibition potential in our co-culture assays. It should, however, be noted that the metabolite composition of fungal endophytes varies depending on their environments, i.e. *in vitro* and *in planta* (Brader *et al.* 2014). It is therefore possible that the metabolite composition *Monosporascus* sp. produces *in planta* does not include the active anti-*Phytophthora* compound. Alternatively, the active compound may be only produced in specific stages of the infection. In the latter scenario, the infection of *Monosporascus* sp. may not have progressed far enough by the time we inoculated with *Phy. infestans*. Nevertheless, the outcome of the *in planta* co-inoculations does not exclude the possibility that the *in vitro* produced metabolites could be effective in field applications, especially since they showed a broad-spectrum reduction in *Phy. infestans* growth in our co-culture experiments. The broad-spectrum effectiveness of inhibition suggests that the metabolite composition either includes a metabolite with a conserved target in *Phy. infestans* or a mixture of anti-*Phytophthora* metabolites. Both would slow the counter-adaptation of the pathogen to the metabolites if used in field application. As a next step, the metabolite extracts with protective capabilities should be tested for their cytotoxicity *in planta*.

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 *Se. 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; Feys *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 pathogen 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-inoculations 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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