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

Sophie de Vries[1,2,5], Janina K. von Dahlen[1], Anika Schnake[1], Sarah Ginschel[1], Barbara Schulz[3] and Laura E. Rose[1,2,4]

1 Institute of Population Genetics, Heinrich-Heine University Duesseldorf, Universitaetsstr. 1, 40225 Duesseldorf, Germany

2 iGRAD-Plant Graduate School, Heinrich-Heine University Duesseldorf, Universitaetsstr. 1, 40225 Duesseldorf, Germany

3 Institute of Microbiology, Technische Universität Braunschweig, Pockelstraße 14, 38106 Braunschweig, Germany

4 Ceplas, Cluster of Excellence in Plant Sciences, Heinrich-Heine University Duesseldorf, Universitaetsstr. 1, 40225 Duesseldorf, Germany

5 Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS B3H 4R2, Canada

*Corresponding author: laura.rose@hhu.de, phone: +49 211 81 13406

Summary

- *Phytophthora infestans* (*Phy. infestans*) is a devastating pathogen of tomato and potato. It readily overcomes resistance genes and applied agrochemicals. Fungal endophytes provide a largely unexplored avenue of control against *Phy. infestans*. Not only do endophytes produce a wide array of bioactive metabolites, they may also directly compete with and defeat pathogens *in planta*.
- Twelve fungal endophyte isolates from different plant species were tested *in vitro* for their production of metabolites with anti-*Phy. infestans* activity. Four well-performing isolates were evaluated for their ability to suppress nine isolates of *Phy. infestans* on plates and *in planta.*
- Two endophytes reliably inhibited all *Phy. infestans* on plates, of which *Phoma eupatorii* isolate 8082 was the most promising. It nearly abolished infection by *Phy. infestans in planta*.
- Here we present a 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 pathogen isolates and may slow the adaptation by *Phy. infestans.*

Keywords: fungal endophytes, antimicrobial metabolites, *Phytophthora infestans,* plant-microbe interaction, biocontrol, late blight

Introduction

Phytophthora infestans is a major pathogen of cultivated tomato (*Solanum lycopersicum*) and cultivated potato (*Solanum tuberosum*). Even today its impact should not to 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 overcome by isolates of *Phy. infestans* (Vleeshouwers et al. 2011). Similarly, agrochemicals 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 et al. 2013, Schlaeppi et al. 2013, Bulgarelli et al. 2015, Edwards et al. 2015, Busby et al. 2016a, Coleman-Derr et al. 2016, Ploch et al. 2016). 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 et al. 2016b). Such benefits include growth promoting effects and protections against parasites and pathogens (e.g. Lahlali and Hijri 2010, Tellenbach and Sieber 2012, Panke-Buisse et al. 2015, Rolli et al. 2015, Busby et al. 2016a, 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, Puopolo et al. 2014, Mousa et al. 2016), highlighting the endophyte's metabolic versatility (Schulz et al. 2002, Strobel and Strobel 2007, Verma et al. 2009, Mousa and Raizada 2013, Brader et al. 2014). Endophytes may also directly compete with potential pathogens of their host plants (Albouvette et al. 2009), induce plant defense responses (Shoresh et al. 2010) and/or produce bioactive anti-microbial metabolites (Brader et al. 2014). An example for an endophyte that can be applied as a direct competitor of a plant pathogenic organism is *Phlebiopsis gigantea* (Adomas et al. 2006). *Phl. gigantea* prohibits the infection of stumps of coniferous trees by the pathogen *Heterobasidion annosum sensu lato* and thereby limits the spread of the pathogen (e.g. Annesi et al. 2005). Due to its success in limiting the spread of *H. annosum s.l.*, *Phl. gigantea* has been made commercially available. An example for the induction of defense responses by an endophyte is the barley root endophyte *Piriformospora indica*, which induces a jasmonic acid-dependent defense response in its host upon co-inoculation with a pathogen (Stein et al. 2008). 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 antimicrobial compounds that killed 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 screened 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 *Pho. eupatorii,* isolate 8082. This endophyte prohibited either proliferation of *Phy. infestans* or abolished its infection completely. Since we identified *Pho. eupatorii* based on the inhibition potential of its metabolite extract, the active component may be a secreted metabolite or a cocktail of different metabolites. A broad-spectrum activity as observed for *Pho. eupatorii* suggests either a conserved target of such secreted metabolite(s) or several pathogen isolate specific targets 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. Therefore, *Pho. eupatorii* isolate 8082 is a potential novel broad-spectrum biocontrol agent of *Phy. infestans*.

Material and Methods

Isolation of endophytes

To isolate the endophytes, plant tissues of the respective hosts (Table S1) were first thoroughly washed under running water, then immersed for one minute 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 and 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).

Screening 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 (Schulz et al. 2011) and/or biomalt agar medium (Höller et al. 2000) at room temperature for 21 days. To isolate the secondary metabolites, the cultures were extracted with ethyl acetate. 25µl of culture extract (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 (Schulz et al. 2011). Only fungal endophytes with a zone of inhibition ≥ 20mm were used for further analyses.

Co-culture on plates

The fungal endophytic isolates no. 8082, 9907 and 9913, whose culture extracts had inhibited *Phy. infestans* in the agar diffusion assays and *Phialocephala fortinii* isolate 4197 (Schulz 2006) 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 *Phi. fortinii* isolate was included based on preliminary experiments. The co-cultivation experiment was performed and evaluated according to Peters et al. (1998). 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 was dependent on the endophytes' growth rates: eight days for all co-cultivations that included 9913 and 14 to 16 days for the remaining co-cultivations. A minimum of ten plates were analyzed per treatment. The Mann-Whitney U test (Mann and Whitney 1974) was used to determine if differences between co-cultivation and control plates were significant. Average growth inhibition was estimated as 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* seeds were sterilized using 70% ethanol for 3 sec, followed by ~5% NaOCl for 30 sec. 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 ± 17µmol quanta*m-2*s-1). Temperature conditions were the same as before. Nine to 11 days post sterilization (dps), the germinated seedlings were transferred to 9mm petri dishes containing 0.5% MS-medium (Murashige and Skoog 1962) with 1% sucrose, poured as a slope.

An endophyte mycelial suspension was prepared from a two- to four-day old liquid culture for each endophyte (potato-carrot liquid medium; 100g potato-carrot mash [prepared according to Höller et al. 2000] in 1L medium). Mycelium was equally dispersed in 25ml medium using Tissuelyser II (Qiagen, Hilden, Germany) for a few seconds. Preliminary inoculations of *S. lycopersicum* roots with 25 to 50µl of mycelial suspensions of all four endophytes were prepared. Endophyte isolate 9907 and *Phi. fortinii* isolate 4197 killed the seedlings. Hence, only endophyte isolates 8082 and 9913 were used for further inoculation studies.

For inoculations with endophyte isolate 8082, 5µl or 10µl 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 (10cm x 6.5cm x 6.5cm) with MS agar medium. For inoculations with endophyte isolate 9913, 10µl 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 to 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 days old culture of *Phy. infestans* isolate D12-2 and was kept on ice during the entire procedure. For the *Phy. infestans* zoospore isolation see de Vries et al. (2017). Plants were sampled for microscopic evaluation, and to evaluate anthocyanin content and pathogen abundance at three days post inoculation (dpi) with *Phy. infestans*.

To confirm endophytic colonization by the fungi, roots from the mock control, endophyte inoculated and co-inoculated samples were surface sterilized using three protocols: i) 70% EtOH for 3sec (for isolate 8082) or 30sec (isolate 9913), ~5% NaOCl for 30sec, followed by three times washing with sterile H_2O for 3min each (treatment 1), ii) 70% EtOH for 5min, 0.9% NaOCl for 20min, followed by three times washing with H_2O (treatment 2, Cao et al. 2004) and iii) 97% EtOH for 30sec, 10% NaOCl for 2min, followed four times rinsing with H_2O (treatment 3, Terhonen et al. 2016). Roots were imprinted on RSA agar plates to test for efficacy of sterilization and then placed on new RSA agar 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, Tokyo, Japan) using a 600 LP filter (Transmission Filterset F26-010, AHF Analysetechnik, Tübingen, Germany), with an exposure time of 200ms 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, Göttingen, 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, Göttingen, 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 post-hoc test (Tukey 1949) and using a Benjamini-Hochberg correction for multiple testing (Benjamini and Hochberg 1995).

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 whether they showed 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 v 3.2.1.

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, Hilden, 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, 5µl of RNA were treated with 6µl deionized formamide, incubated at 65°C for 5 min, followed by 5 min incubation on ice. This mixture was than visualized on a 2% agarose gel. To ensure that no DNA contamination was present in the original samples, the RNA was treated with DNAse I (Thermo Scientific). Reactions were adjusted for 200 ng of total RNA. cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania).

Molecular identification of endophytes

To determine the phylogenetic placement of the fungal endophytes, we sequenced their *internal transcribed spacer* region (*ITS*). *ITS1* and *ITS4* primers were used (White et al., 1990). The 20µl PCR-reaction contained 1x Green GoTaq® Flexi Buffer, 0.1mM dNTPs, 2mM $MgCl_2$, 1U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), 0.2µM 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 sec, an annealing step at 60°C for 30 sec and an elongation step at 72°C for 90 sec, followed by a final elongation step of 72°C for 7 min. All PCR products were purified with the peqGOLD Cycle-Pure Kit (Peqlab, Erlangen, Germany). The products were cloned into the pCR™ 4-TOPO® vector of the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and the plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Sequencing was performed at Eurofins MWG Operon (Ebersberg, Germany). Sequences were blasted using BLASTn (Altschul et al. 1990) and the best hits were retrieved. To

assemble a large dataset of closely related organisms from which to infer the phylogenetic placement of the unknown endophytes, *ITS* 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, Utrecht). Additional sequences were retrieved from GenBank (Table S2). Taxonomically distant outgroups were always 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 5 gamma categories and pairwise deletion of gaps. One hundered 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 has overgrown *Phy. infestans* or *Phy. infestans* had overgrown the endophyte on the co-cultivation 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* sequences (for primers see White et al. 1990) and the *Phytophthora*-specific *cytochrome oxidase subunit2* (*COX2*) using primers from Hudspeth et al. (2000) with the protocol described above. Between 50-100ng of template DNA was used.

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 *PiElf1*α, were used for the pathogen and the three markers, *SAND*, *TIP* and *TIF3H,* were used as tomato (host) reference genes (de Vries et al. 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 sec and an annealing and elongation step of 60°C for 45 sec. For *PiH2a* the annealing temperature was lower: 59°C in the first run and 55°C in the second run. For the following experiment, each run contained three biological replicates: i) isolate 8082 (5µl mycelial suspension) with *Phy. infestans*, ii) isolate 9913 with *Phy. infestans* and iii) *Phy. infestans* without endophyte. Two biological replicates were completed for isolate 8082 (10µl mycelial suspension) with *Phy. infestans*. In each run, we analyzed three technical replicates for each biological replicate, adding up to 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 *PiElf1*α 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 v. 3.2.1.

Results

Metabolite screening identifies three endophytes with biocontrol potential

To identify fungal endophytes that, on the basis of their secreted metabolites, could be used as biocontrol agents against *Phy. infestans,* we screened culture extracts of 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 $\pm$ 8.7mm ranging from 0 and 35mm from the point of extract application (Table S3). Culture extracts of three of the 12 isolates inhibited growth of *Phy. infestans* with a radius ≥20mm (isolates 8082, 9907 and 9913). These three fungal endophyte isolates 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 mutualistic interaction with another host, *Larix decidua* (Schulz 2006), growth inhibition of other pathogenic microbes and prior information that it could colonize *S. lycopersicum* asymptomatically (unpublished).

Phylogenetic placement of fungal endophytes

To determine the taxonomic identity and phylogenetic placement of the four selected fungal endophytes, we sequenced their *ITS1* and *ITS2* regions. First, we used these sequences in a BLAST search to identify the closest relatives to the fungal endophytes (Table S4). All four endophytes belong to the ascomycetes. Our analyses further supported the characterization of isolate 4197 as *Phi. fortinii* (99% identity and e-value 0). For isolate 8082 the best BLAST hit with 100% identity and an e-value of 0 was *Phoma eupatorii.* This was additionally supported by the fact that isolate 8082 was isolated from *Eupatorium cannabinum* (Table S1). 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 (Figure 1a and b). The best hit for isolate 9907 was *Pyrenochaeta cava* (95% identity and e-value 0) and for isolate 9913 it was *Monosporascus ibericus* (97% identity and

e-value 0). This suggests that no completely identical 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, Figure 1c), thus based on the phylogenetic analyses 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). This warrants attention as *Monosporascus ibericus,* the fungal endophyte clustering most closely with isolate 9913 in the phylogenetic anlysis, has been recently 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. (Figure 1d).

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

Our initial screening 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-cultivation assay on RSA plates with the four fungal endophytes against nine European *Phy. infestans* isolates (Figure 2). In the plate assay all four endophytes were capable of significantly restricting growth of *Phy. infestans* (Figure 3). *Pho. eupatorii* and isolate 9907 showed a global inhibition of all *Phy. infestans* isolates tested (Figure 3b, c). *Phi. fortinii* inhibited the growth of eight out of nine isolates and *Monosporascus* sp. inhibited the growth of seven of the nine isolates (Figure 3a, d). *Pho. eupatorii* had the greatest average relative growth inhibition of *Phy. infestans* with 50.6 ± 2.2%, and *Monosporascus* sp. had the weakest with 11.9 ± 1.6% (Table S5).

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 and ii) evaluated long-term co-cultivations (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 (Figure S1c). However, some isolates of *Phy. infestans* were able to inhibit the growth of the other three fungal endophytes (Figure S1a, b, d). 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 S6). 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, all endophytes, (except for isolate 9907), visually overgrew the plates and with that *Phy. infestans* (Figure 4). To substiantiate this observation, we extracted DNA from some co-cultures with *Phi. fortinii*, *Pho. eupatorii* and *Monosporascus* sp. from both sides of

the eight-week samples (Table S7). In total, we analyzed 40 co-cultivations 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 specific for fungi, we primarily observed amplicons from the fungal endophyte *ITS* sequences 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 also observed on the original inoculation side of the fungal endophyte (2/40 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 the *Phy. infestans'* side of the plate. We therefore conclude that only a small proportion of the growth inhibition of *Phy. infestans* by the endophytes can be attributed to a competitive effect on the plate. The differences between growth inhibition of endophytes and that of *Phy. infestans,* as well as *Phy. infestans'* incapability to colonize the endophytic side can only be explained by the endophytes having an independent mechanism other than from direct competition to inhibit growth 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 screening showed that *Phi. fortinii* and isolate 9907 were too virulent and killed the *S. lycopersicum* seedlings (Figure S2a, b, d). In contrast, *S. lycopersicum* seedlings inoculated with *Pho. eupatorii* and *Monosporascus* sp. survived (Figure S2a, c, e).

To confirm 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 1). Irrespective of the protocol, there was no fungal growth from the mock control nor from their imprints. Generally, imprints of the endophyte inoculated roots did not show fungal growth, except for *Pho. eupatorii* inoculated roots after treatment 1 (1/16 imprints mono-inoculation and 5/12 imprints co-cultivation). This suggests that surface sterilization was successful in all other cases. *Pho. eupatorii* grew from several roots independent of the treatment, although the stronger treatments showed 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 roots, suggesting that,

like *Pho. eupatorii, Monosporascus* sp. also grows endophytically in the roots of *S. lycopersicum.*

S. 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 (Figure S3a, c). Since the leaflets appeared sturdier and were darker green than the controls (Figure 5a-f), we measured chlorophyll levels via chlorophyll fluorescence. However, chlorophyll abundance did not change following any of the treatments (Figure 5g-m). We also observed that some of the stems of the plants that had been inoculated with *Pho. eupatorii* developed a purple color (Figure S4c). Therefore, we reasoned that the darker leaflet color may have resulted from anthocyanin accumulation. 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*, Figure 5o). In contrast to seedlings colonized by *Pho. eupatorii*, those inoculated with *Monosporascus* sp. did not visibly differ from the mock controls (Figure S3a, b, S4a, c). In agreement with that, anthocyanin content did not increase significantly in *Monosporascus* sp. inoculated samples compared to the mock control ($p=0.08$ without *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 (Figure 5n, S4e). To confirm the pathogen infection in the mock/*Phy. infestans* samples, we used the expression of the *Phy. infestans* biomass marker genes *PiH2a* and *PiElf1*α. In agreement with the increase in necrotic area, *Phy. infestans* was present in all biological replicates mono- inoculated with the pathogen, i.e. showing a successful infection.

While the relative necrotic area in seedlings that were only colonized 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* (Figure 5n). *S. lycopersicum* seedlings inoculated with *Pho. eupatorii* followed by inoculation with *Phy. infestans* resulted in a significantly reduced relative necrotic area compared to seedlings mono-inoculated with *Phy. infestans* (Figure 5n). 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 (Figure 5n). The use of a 5µl or 10µl mycelial suspensions of *Pho. eupatorii* did not change the result. In contrast, while the relative necrotic area between the treatment with *Monosporascus* sp. and the mock control did not differ (Figure S4a, c, e), this endophyt was

not able to inhibit *Phy. infestans* infection nor limit its disease symptoms *in planta* (Figure S4b, d, e, f).

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 *PiElf1*α and *PiH2A* (Figure 5o). In total, we tested the three biological replicates for the 5µl *Pho. eupatorii* inoculations and two for the 10µl *Pho. eupatorii* inoculations. In three of those five replicates we did not detect an amplicon for either *PiH2a* or *PiElf1*α. Yet, *PiH2a* and *PiElf1*α 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 *PiElf1*α 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* at least 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* (Figure 5o). Therefore, *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 *Phy. 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 et al. 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), endophytes and their metabolites may have a narrow spectrum of specificity. To avoid narrow spectrum of pathogen inhibition, we screened 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, while *Monosporascus* sp. and *Phi. fortinii* covered nearly all isolates. Additionally, after an eight-week incubation experiment,

the pathogen was not able to grow on areas of the plates, where the endophytes grew. The consistency of the results from the culture extract experiments and the plate assays of *Pho. eupatorii* and isolate 9907 shows that their inhibition is independent of the growth medium, suggesting a potentiallyu 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.

For application in the field, it needs to be clarified i) whether infection by the endophyte causes is damaging to the host in the absence of a pathogen and ii) whether the endophyte can successfully inhibit the pathogen in the host. In our study, the former is of extreme importance, because the fungal endophytes in question were not originally isolated from Solanaceae plants (i.e. plants of the same family). Furthermore, whether an endophyte remains benign and asymptomatic is likely to be affected by a number of different circumstances and in some cases the host endophyte relationship may shift to a pathogenic outcome from an initially protective interaction (Schulz and Boyle 2005, Junker et al. 2012, Schulz et al. 2015, Busby et al. 2016b). 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 vary depending on their environments, i.e. *in vitro* and *in planta* (Barder 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 inoculate with *Phy. infestans.* Nevertheless, the outcome of the *in planta* co-inoculations do 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. 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. It is hence of utmost importance to also test the metabolite extracts for their protective capabilities and a lack of cytotoxicity *in planta.*

Phoma eupatorii isolate 8082 may inhibit _Phytophthora infestans_ via a combination of secreted toxic metabolites and the induction of host defense mechanisms

Pho. 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, while *Phy. infestans* was inoculated on the leaves, niche competition is an unlikely mechanism by which *Pho. eupatorii* protects the *S. lycopersicum* seedlings. Therefore, the two other possible mechanisms by which the plant is defended against the pathogen include endophyte-dependent induction of defense responses or the production of anti-*Phytophthora* metabolites. The induction of plant defense responses by endophytes, including *Pir. 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, among other factors, positively regulated by jasmonic acid (Franceschi and Grimes 1991, Feys et al. 1994, Shan et al. 2009, Li et al. 2006). Hence, it is possible that jasmonic acid dependent defense responses are induced upon colonization of *Pho. eupatorii*. This may be a response to *Pho. eupatorii* itself, however such accumulation of jasmonic acid may have contributed to the inhibition of the *Phy. infestans* infection we observed. However, the role of jasmonic acid in defense against *Phy. infestans* is not clear: In one study, application of jasmonic acid to leaves of tomato and potato plants resulted in reduced infection of the pathogen (Cohen et al. 1993). In another study, it is 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, RNA interference lines downregulating jasmonic acid biosynthesis and signaling components, did not alter the infection success 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 hits host plant detrimental to the host plant's 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 the co-cultivations on plate. This makes it likely that *Pho. eupatorii* also produces such metabolites during *in planta* co-inoculations with *Phy. infestans.* A combination of this two mechanism is, however, also possible.

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

Out of a screen of 12 fungal endophytes, we discovered four ascomycetes that inhibited 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 towards nine European *Phy. infestans* isolates, the other two showing a near-global inhibition. This indicates that there is a highly conserved target within *Phy. infestans* for a particular metabolite produced by these four endophytes. Alternatively, a complex metabolite mixture is involved. In either case, the result could slow the counter-adaptation of *Phy. infestans* to the new anti-*Phytophthora* compound(s). Hence, all four fungal endophytes can be considered good candidates for the production of such new and urgently needed compounds. Additionally, out of the four fungal endophytes, *Pho. eupatorii* functioned as an effective biocontrol agent *in planta.* Therefore, *Pho. eupatorii* may not only synthesize a reservoir of highly useful antimicrobial metabolites, but could serve as a novel biocontrol agent providing an alternative to resistance gene breeding and application of agrochemicals.

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Author contribution

SdV, BS and LER wrote the manuscript. SdV, JKvD, AS and SG performed the experimental work and data analyses. BS provided the fungal isolates and the metabolite screening. All authors read and approved the manuscript.

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Tables and Figures

Table 1. Endophytic outgrowth from surface sterilized roots after inoculation with the endophyte. 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). Treatment 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.

	Pho. eupatorii **8dps**		***Monosporascus* sp.** **6dps**	
	imprint	roots	imprint	roots
Treatment 1				
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
Treatment 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
Treatment 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

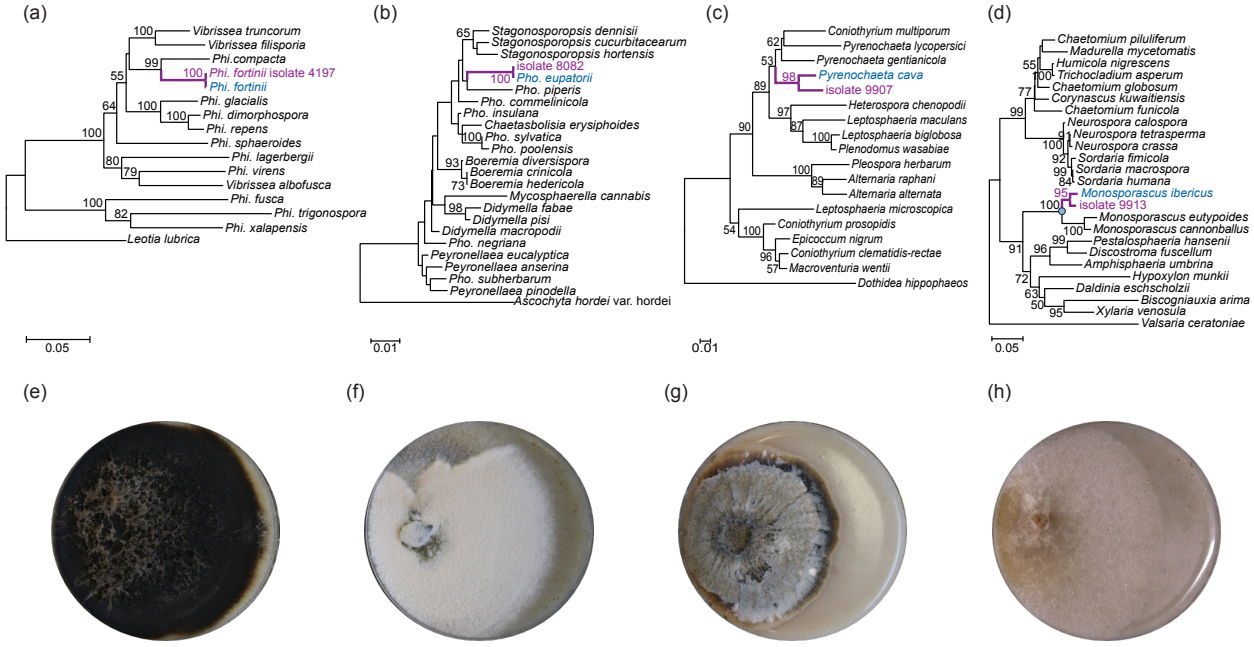

Figure 1. Phylogenetic placement of fungal endophytes.

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. The corresponding fungal endophyte in culture is shown below each tree: *Phialocephala fortinii* (e), *Phoma eupatorii* (f), isolate 9907 (g) and *Monosporascus* sp. (h).

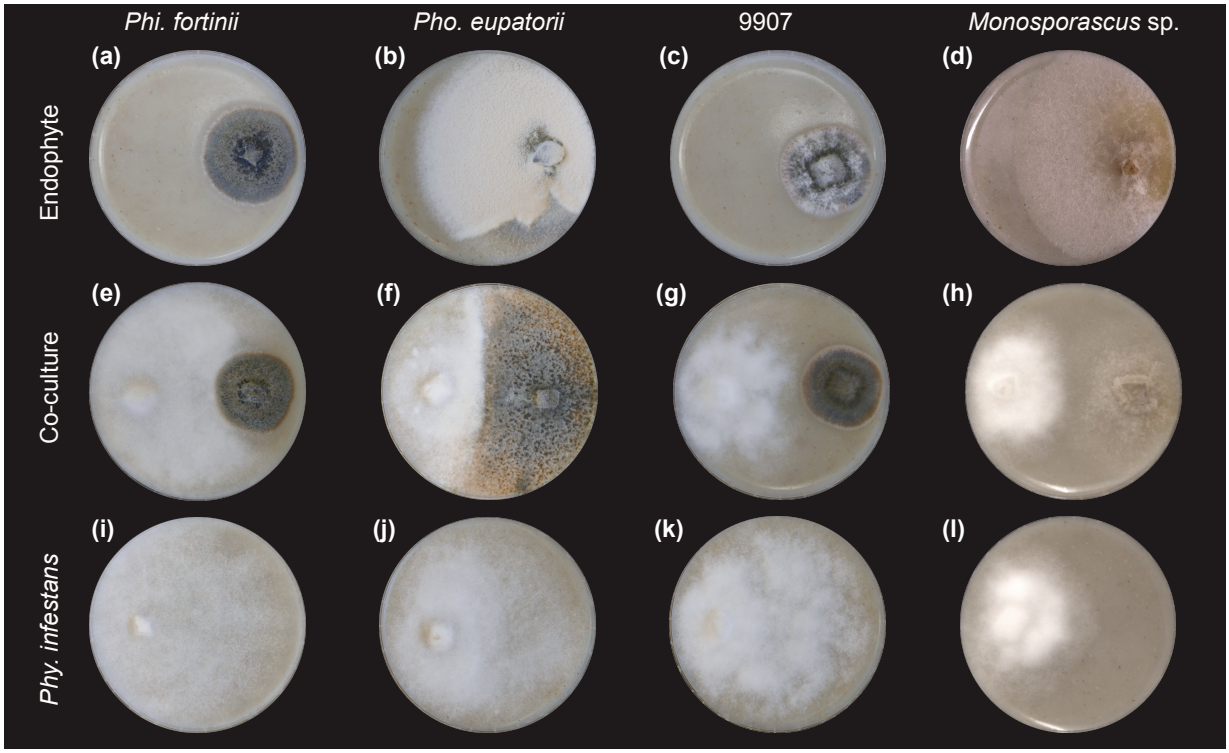

Figure 2. Co-cultivation of fungal endophytes with *Phytophthora infestans* on plate.

Examples of two-week-old single and co-cultivations 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-cultivations of *Monosporascus* sp. with *Phy. infestans* isolate D12-2 (d, h, l). The diameter of each plate is nine cm.

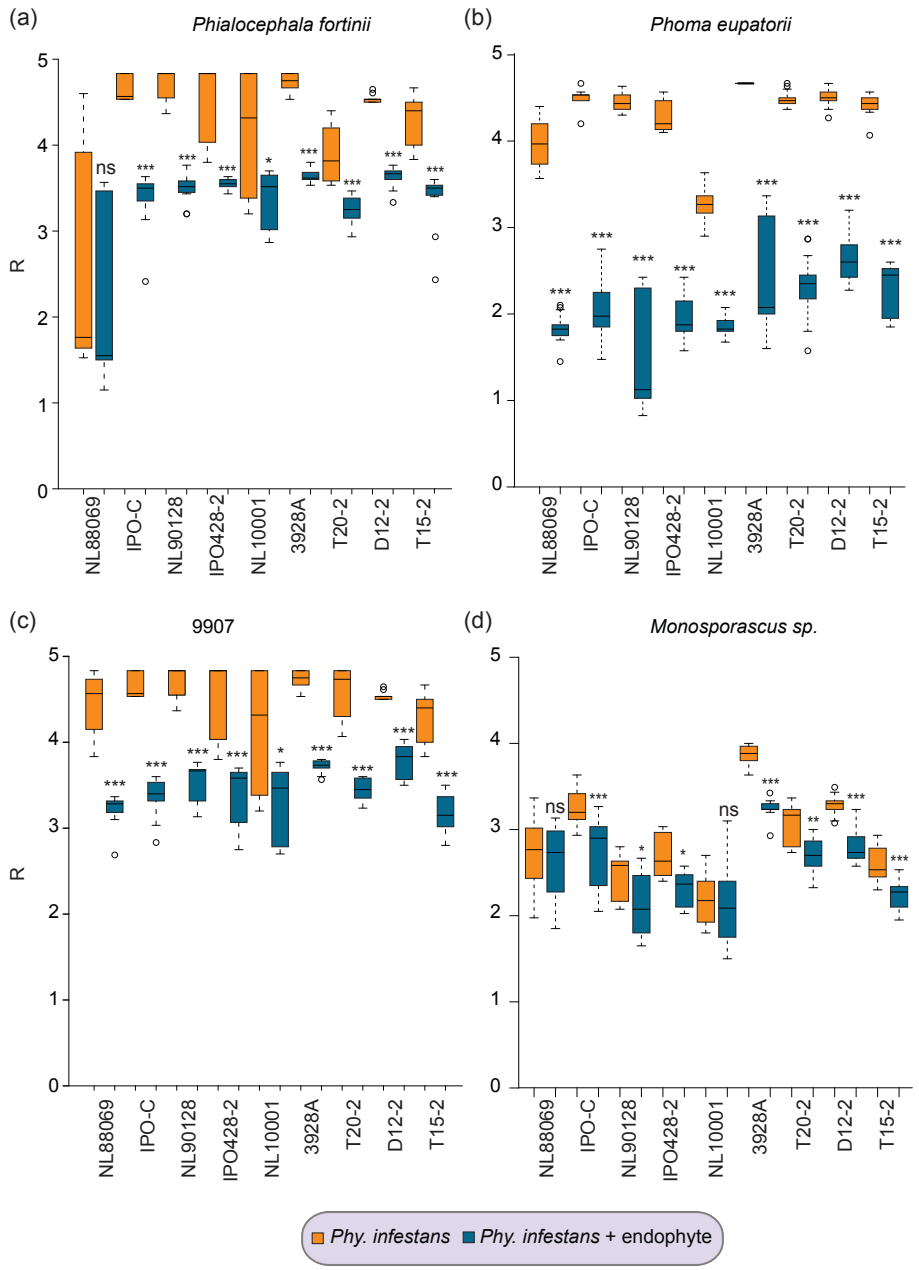

Figure 3. Radial growth inhibition of *Phytophthora infestans* isolates by fungal endophtyes.

Radial growth (R) of the different *Phy. infestans* isolates denoted on the x-axis when grown alone (orange) or in dual culture with the four fungal endophytes (blue): *Phi. fortinii* (a), *Pho. eupatorii* (b), isolate 9907 (c) and *Monosporascus* sp. (d). 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.

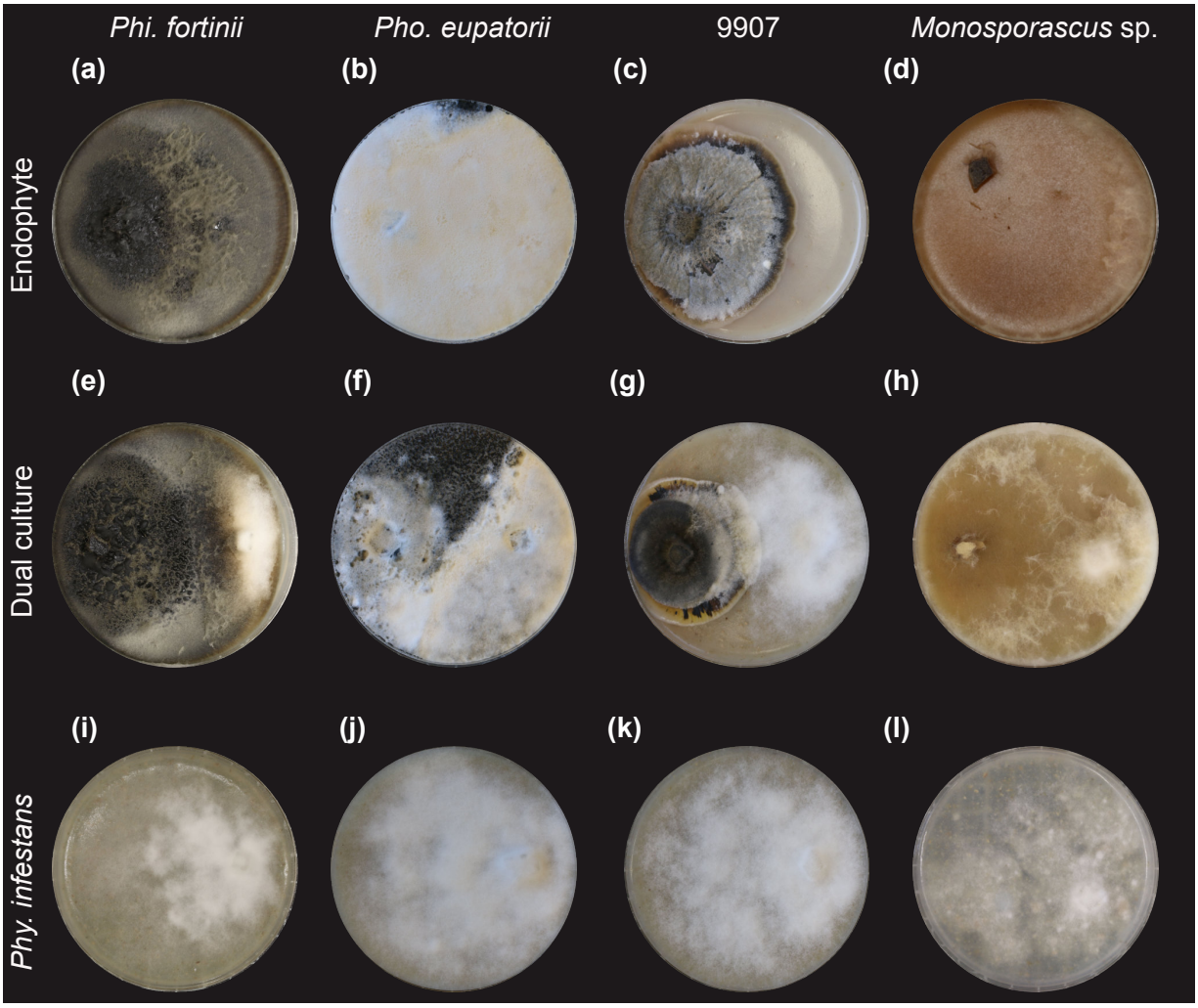

Figure 4. Long-term co-cultivation of fungal endophytes with *Phytophthora infestans* on plate

Examples of eight-week-old co-cultivations 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 plate is nine cm.

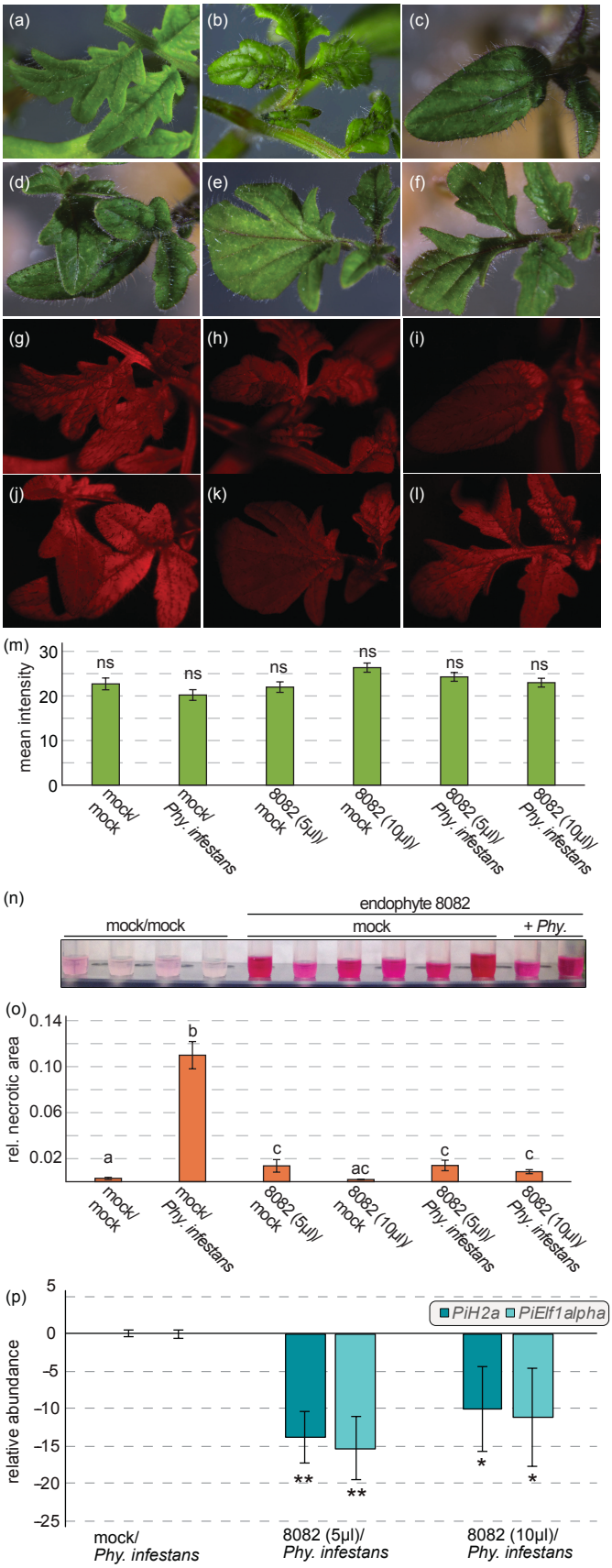

Figure 5. *In planta* co-inoculations of *Phoma eupatorii* isolate 8082 and *Phytophthora infestans*

S. lycopersicum cv. M82 seedlings were mock treated (a) or inoculated with *Phy. infestans* isolate D12-2 (b), 5µl of *Pho. eupatorii* mycelium suspension (c), 10µl of *Pho. eupatorii* mycelium suspension (d), 5µl of *Pho. eupatorii* mycelium suspension and *Phy. infestans* isolate D12-2 (e) and 10µl 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 ImageJ (m). Bars give the average mean fluorescence ($n_{leaflets}$=17-37). Error bars give the standard error (SEM); ns = not significant. Differences in anthocyanin content (n). A darker pink in the examples shown indicates a higher amount of anthocyanins in the sample. The average relative necrotic area of the leaflets was calculated for each treatment ($n_{leaflets}$=38-156, o). 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* and compared between the *Pho. eupatorii* – *Phy. infestans* co-inoculations and the control treatment (*Phy. infestans* only). 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.