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

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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 isolates of fungal endophytes 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 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*.
- 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 of *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 cannot be ignored as it is still capable of destroying entire fields of its hosts, leading to up to 100% yield losses (Nowicki *et al*., 2012). The two major control measures for *Phy. infestans* are resistance breeding and agrochemical applications. While several resistance genes have been identified in screens of wild relatives of *S. lycopersicum* and *S. tuberosum* (Song *et al*., 2003; Van der Vossen *et al*., 2003; Pel *et al*., 2009; Zhang *et al*., 2013), many of them are readily 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

54 approaches, such as biological control, is needed for effective crop protection against this
55 pathogen.

56 One approach that is gaining more and more attention is the use of endophytes for crop
57 protection (Le Cocq *et al*., 2016). Endophytes are microorganisms that grow within plants, and
58 at the time of sampling, do not cause obvious symptoms on their host (Schulz & Boyle, 2005;
59 Le Cocq *et al*., 2016). Many studies have explored the bacterial, fungal and protist endophytic
60 communities associated with different plants (e.g. Bulgarelli *et al*., 2012; Lundberg *et al.*, 2012;
61 Bodenhausen *et al*., 2013; Schlaeppi *et al*., 2013; Bulgarelli *et al*., 2015; Edwards *et al*., 2015;
62 Busby *et al*., 2016a; Coleman-Derr *et al*., 2016; Ploch *et al*., 2016). These studies indicate that
63 the diversity of microbes living inside of plants is largely underestimated and that the
64 distribution of some microorganisms is host and/or environment specific.

65 Furthermore, in some cases such endophytic microorganisms have been evaluated for their
66 potential benefit to their hosts (Busby *et al*., 2016b). Such benefits include growth promotion
67 and protection against parasites and pathogens (e.g. Schulz, 2006; Lahlali & Hijri, 2010;
68 Tellenbach & Sieber, 2012; Panke-Buisse *et al*., 2015; Rolli *et al*., 2015; Busby *et al*., 2016a;
69 Hiruma *et al*., 2016; Martínez-Medina *et al*., 2017). Often these functions are linked to
70 metabolites produced and secreted by the endophytes (Son *et al*., 2008; Puopolo *et al*., 2014;
71 Mousa *et al*., 2016; Suryanarayanan *et al*., 2016), highlighting the endophyte's metabolic
72 versatility (Schulz *et al*., 2002; Strobel & Strobel, 2007; Verma *et al*., 2009; Mousa & Raizada,
73 2013; Brader *et al*., 2014). Endophytes may also directly compete with potential pathogens of
74 their host plants (Albouvette *et al.*, 2009), induce plant defense responses (Shoresh *et al*., 2010)
75 and/or produce bioactive anti-microbial metabolites (Brader *et al*., 2014). An example of an
76 endophyte that can be applied as a direct competitor of a plant pathogenic organism is
77 *Phlebiopsis gigantea* (Adomas *et al*., 2006). *Phl. gigantea* prohibits the infection of stumps of
78 coniferous trees by the pathogen *Heterobasidion annosum sensu lato* and thereby limits the
79 spread of the pathogen (e.g. Annesi *et al*., 2005). Due to its success in limiting the spread of
80 *H. annosum s.l.*, *Phl. gigantea* has been made commercially available. An example for the
81 induction of defense responses by an endophyte is the barley root endophyte *Piriformospora*
82 *indica*, which induces a jasmonic acid-dependent defense response in its host upon co-
83 inoculation with a pathogen (Stein *et al*., 2008). Furthermore, a recent study by Mousa *et al*.
84 (2016) describes an *Enterobacter* sp. strain isolated from an ancient African crop (*Eleusine*
85 *coracana* [finger millet]) with the ability to suppress the grass pathogen *Fusarium*

86 *graminearum*. *Enterobacter* sp. traps *F. graminearum* in the root system of its host and
87 simultaneously produces several antifungal compounds that kill the fungus.

88 Several bacterial and fungal endophytes, with the potential to inhibit *Phy. infestans* growth,
89 have been described (Sturz *et al*., 1999; Kim *et al*., 2007; Miles *et al*., 2012; Puopolo *et al*.,
90 2014). However, these endophytes have only been tested against single isolates of
91 *Phy. infestans;* but alternative approaches, such as biocontrol, can show different outcomes
92 depending on the pathogen isolate (Bahramisharif *et al*., 2013). Therefore, the identification of
93 endophytic species with a broad inhibition spectrum is of critical importance.

94 In this study, we screened the metabolite extracts of 12 fungal endophytes isolated from
95 different plant hosts for their ability to inhibit growth of *Phy. infestans*. Using a plate assay
96 with the four most successful fungal endophytes, we show that they inhibit the growth of a
97 broad spectrum of European *Phy. infestans* isolates in co-culture. According to our
98 phylogenetic analyses, these four endophytes are members of the Ascomycota. The endophyte
99 with the strongest inhibition potential both on plates and *in planta* was *Pho. eupatorii,* isolate
100 8082. This endophyte prohibited proliferation of *Phy. infestans* and in some cases abolished its
101 infection completely. Since we identified *Pho. eupatorii* based on the inhibition potential of its
102 metabolite extract, the active component may be a secreted metabolite or a cocktail of different
103 metabolites. A broad-spectrum activity as observed for *Pho. eupatorii* suggests either a
104 conserved target for such secreted metabolite(s) or several targets that are specific for the
105 pathogen isolate and that are covered by the complexity of the metabolite cocktail. Both can
106 result in slower counter-adaptation of *Phy. infestans* to either the direct application of the
107 endophyte or to the application of its metabolites. Therefore, *Pho. eupatorii* isolate 8082 is a
108 potential novel broad-spectrum biocontrol agent of *Phy. infestans*.

109 **Material and Methods**

110 **Isolation of endophytes**

111 To isolate the endophytes, plant tissues of the respective hosts (Tab. S1) were first thoroughly
112 washed under running water, then immersed for one minute in 70% ethanol, followed by 1-
113 3min in 3% NaOCl and subsequently rinsed three times in sterile water. Sterilized tissues were
114 imprinted on potato-carrot medium (Höller *et al*., 2000) to test for effectiveness of sterilization
115 and to optimize the sterilization procedure. The tissues were then cut with a sterile scalpel into
116 2mm slices and plated on potato-carrot agar medium with antibiotics (Höller *et al*., 2000) and

117 incubated for 3 weeks at 20°C. The emerging mycelia were taken into culture on potato-carrot
118 agar medium and were initially identified according to morphology (Tab. S1).

119 **Screening crude metabolite extracts for anti-*Phytophthora infestans* activity**

120 To test the growth inhibition potential of the 12 fungal endophytes, the endophytes were first
121 grown on barley-spelt medium (Schulz *et al*., 2011) and/or biomalt agar medium (Höller *et al*.,
122 2000) at room temperature for 21 days. To isolate the secondary metabolites, the cultures were
123 extracted with ethyl acetate. 25µl of culture extract (40 mg/ml) were then applied to a filter
124 disc and placed onto rye agar medium that had been inoculated with *Phy. infestans* isolate D2*;*
125 subsequent incubation was at 20°C in the dark (Schulz *et al*., 2011). Only fungal endophytes
126 whose culture extracts resulted in a zone of inhibition ≥ 20mm were used for further analyses.

127 **Co-culture on plates**

128 The fungal endophytic isolates 8082, 9907 and 9913, whose culture extracts had inhibited
129 *Phy. infestans* in the agar diffusion assays and *Phialocephala fortinii* isolate 4197 (Schulz,
130 2006) were tested for their bioactivity against nine isolates of the late blight pathogen
131 *Phy. infestans* (NL10001, NL88069, NL90128, IPO-C, IPO428-2, 3928A, D12-2, T15-2 and
132 T20-2). The *Phi. fortinii* isolate was included based on previous experiments (Schulz *et al*.,
133 2002; Schulz, 2006; Schulz, unpublished). The co-culture experiments were performed and
134 evaluated according to Peters *et al*. (1998). Fungal endophytes and *Phy. infestans* isolates were
135 grown on rye-sucrose agar (RSA, Caten & Jinks, 1968) at room temperature. The duration of
136 the experiments was dependent on the endophytes' growth rates: eight days for all co-cultures
137 that included 9913 and 14 to 16 days for the remaining co-cultures. A minimum of ten plates
138 were analyzed per treatment. The Mann-Whitney U test (Mann & Whitney, 1974) was used to
139 determine if differences between co-culture and control plates were significant. Average
140 growth inhibition was estimated as: 1-(average radius in co-culture / average radius in control
141 conditions). All experiments were evaluated again after eight weeks of incubation to assess
142 long-term effects. Pictures were taken with an EOS 70D camera (Canon).

143 **Co-inoculation *in planta***

144 The surfaces of the *S. lycopersicum* seeds were sterilized using 70% ethanol for 3sec, followed
145 by ~5% NaOCl for 30sec. The sterilized seeds were washed three times with sterile water for
146 3min. Seeds were incubated in the dark on 1.2% H_2O-agar with a day-night temperature cycle
147 of 18°C /15°C (16h / 8h). After three days, the seeds were transferred to a day-night cycle with

148 16h light (166 ± 17µmol quanta*m-2*s-1). Temperature conditions were the same as before.
149 Nine to 11 days post sterilization (dps), the germinated seedlings were transferred to 9mm petri
150 dishes containing 0.5% MS-medium (Murashige & Skoog, 1962) with 1% sucrose, poured as
151 a slope.

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

159 For inoculations with endophyte isolate 8082, 5µl or 10µl of the mycelial suspension or H_2O
160 (mock control) was applied to each root at 16dps. After 27dps seedlings were transferred to
161 vessels (10cmx6.5cmx6.5cm) with MS agar medium. For inoculations with endophyte isolate
162 9913, 10µl of dispersed mycelium or H_2O was applied to the roots of axenic seedlings at 18dps.
163 However, the endophyte isolate 9913 did not grow sufficiently, so we performed a second
164 inoculation with undispersed mycelium from the liquid culture at 22dps. These seedlings were
165 transferred to vessels at 28dps. At 34 to 36dps each leaflet of endophyte and mock inoculated
166 plants was inoculated with 10µl of *Phy. infestans* zoospore suspension (4°C cold) or with 10µl
167 H_2O (4°C cold). The zoospore suspension ($5*10^4$ spores/ml) was harvested from a 25 days old
168 culture of *Phy. infestans* isolate D12-2 and was kept on ice during the entire procedure. For the
169 *Phy. infestans* zoospore isolation see de Vries *et al*. (2017). Plants were sampled for
170 microscopic evaluation, to evaluate anthocyanin content and pathogen abundance at three days
171 post inoculation with *Phy. infestans*.
172

173 To confirm endophytic colonization by the fungi, roots from the mock control, endophyte
174 inoculated and co-inoculated samples were surface sterilized using three protocols: i) 70%
175 EtOH for 3sec (isolate 8082) or 30sec (isolate 9913), ~5% NaOCl for 30sec, followed by
176 washing three times with sterile H_2O for 3min each (treatment 1), ii) 70% EtOH for 5min, 0.9%
177 NaOCl for 20min, followed by washing three times with H_2O (treatment 2, Cao *et al*., 2004)
178 and iii) 97% EtOH for 30sec, 10% NaOCl for 2min, followed rinsing four times with H_2O
179 (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 8dps (isolate 8082) and 6dps (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 & Wallis, 1952) combined with a Tukey post-hoc test (Tukey, 1949) and using a Benjamini-Hochberg correction for multiple testing (Benjamini & 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 & 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 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 5min, followed by 5min incubation on ice. This mixture was then visualized on a 2% agarose gel. To ensure that no DNA contamination was present, the

211 RNA was treated with DNAse I (Thermo Scientific). Reactions were adjusted for 200ng of
212 total RNA. cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit
213 (Thermo Fisher Scientific, Lithuania).

214 **Molecular identification of endophytes**

215 To determine the phylogenetic placement of the fungal endophytes, we sequenced their
216 *internal transcribed spacer* (*ITS*) and 5.8S regions. *ITS1* and *ITS4* primers were used (White
217 *et al*., 1990). The 20µl PCR-reaction contained 1x Green GoTaq® Flexi Buffer, 0.1mM
218 dNTPs, 2mM $MgCl_2$, 1U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA),
219 0.2µM of each primer and 40-95ng of template DNA. The PCR protocol included an initial
220 denaturation step of 95°C for 3min, followed by 35 cycles of a denaturation step at 95°C for
221 30sec, an annealing step at 60°C for 30sec and an elongation step at 72°C for 90sec, followed
222 by a final elongation step of 72°C for 7min. All PCR products were purified with the peqGOLD
223 Cycle-Pure Kit (Peqlab, Erlangen, Germany). The products were cloned into the pCR™ 4-
224 TOPO® vector of the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA,
225 USA) and the plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (Qiagen,
226 Hilden, Germany). Sequencing was performed at Eurofins MWG Operon (Ebersberg,
227 Germany). Sequences were blasted using BLASTn (Altschul *et al*., 1990) and the best hits were
228 retrieved. To assemble a dataset of closely related organisms from which to infer the
229 phylogenetic placement of the unknown endophytes, the sequences of species with high
230 similarity to our initial query sequences were downloaded. Taxonomic classification of these
231 sequences was done using mycobank.org (provided by the CBS-KNAW Fungal Biodiversity
232 Center, Utrecht). Additional sequences were retrieved from GenBank (Tab. S2).
233 Taxonomically distant outgroups were chosen based on the systematic classifications in
234 MycoBank (Crous *et al*., 2004). The sequences were aligned using CLUSTAL-W and a
235 Neighbor-Joining phylogeny was inferred using the Kimura-2 model with 5 gamma categories
236 and pairwise deletion of gaps. One hundred bootstrap replicates were evaluated. All analyses
237 were done using MEGA 5.2.2 (Tamura *et al*., 2011).

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

240 To determine whether either the endophyte had overgrown *Phy. infestans* or *Phy. infestans* had
241 overgrown the endophyte on the co-culture plates, we performed PCR reactions on DNA
242 extracted from both sides of eight-week old co-cultures of five to nine *Phy. infestans* isolates

243 with *Phi. fortinii,* isolate 8082 and isolate 9913 as well as their respective controls. We
244 amplified the *ITS* loci (for primers see White *et al*., 1990) and the *cytochrome oxidase subunit2*
245 (*COX2*) using *Phytophthora*-specific primers from Hudspeth *et al*. (2000) with the protocol
246 described above. Between 50-100ng of template DNA was used.

247 **Presence and abundance of *Phytophthora infestans***

248 To quantify the abundance of *Phy. infestans* in the seedlings pre-inoculated with the two
249 endophytes (isolate 8082 and 9913) and the seedlings only inoculated with *Phy. infestans*, we
250 performed a quantitative RT-PCR (qRT-PCR). The two markers, *PiH2a* and *PiElf1α*, were
251 used for the pathogen and the three markers, *SAND*, *TIP* and *TIF3H,* were used as tomato (host)
252 reference genes (de Vries *et al*., 2015; de Vries *et al*., 2017). Two independent qRT-PCR runs
253 were used for the pathogen genes. All qRT-PCRs were performed in a CFX Connect™ Real-
254 Time System (Bio-Rad, Hercules, CA, USA) and included an initial denaturation at 95°C for
255 3min, followed by 40 cycles of a denaturation step at 95°C for 10sec and an annealing and
256 elongation step of 60°C for 45sec. For *PiH2a* the annealing temperature was lower: 59°C in
257 the first run and 55°C in the second run. For the following experiment, each run contained three
258 biological replicates for: i) isolate 8082 (5µl mycelial suspension) with *Phy. infestans*, ii)
259 isolate 9913 with *Phy. infestans* and iii) *Phy. infestans* without endophyte. Two biological
260 replicates were completed for isolate 8082 (10µl mycelial suspension) with *Phy. infestans*. In
261 each run, we analyzed three technical replicates for each biological replicate, resulting in six
262 technical replicates for each biological replicate for both marker genes. To calculate the relative
263 abundance of *Phy. infestans* in these samples, we set the Cq-values of those biological
264 replicates that gave no biomass marker amplicon to 41. As the two independent runs gave the
265 same results, they were combined. *PiH2a* and *PiElf1α* expression was then calculated
266 according to Pfaffl (2001). Data were tested for normal distribution using a Shapiro-Wilk test
267 and the appropriate statistical tests were then applied. For co-inoculations with isolate 8082,
268 significant differences were calculated using a Mann-Whitney U-test. For co-inoculations with
269 isolate 9913, significant differences were calculated using a two-tailed t-test. The statistical
270 analyses were done using R v. 3.2.1.

271 **Results**

272 **Metabolite screening identifies three endophytes with biocontrol potential**

273 To identify fungal endophytes that, on the basis of their secreted metabolites, could be used as
274 biocontrol agents against *Phy. infestans,* we screened culture extracts of 12 fungal endophytes
275 for growth inhibition of *Phy. infestans* isolate D2 using an agar diffusion assay. Inhibition of
276 *Phy. infestans* varied considerably, depending both on the endophyte isolate and on the culture
277 medium. The average growth inhibition was 12.4 ± 8.7mm ranging from 0 and 35mm from the
278 point of extract application (Tab. S3). Culture extracts of three of the 12 isolates inhibited
279 growth of *Phy. infestans* with a radius ≥20mm. These three fungal endophytes (isolates 8082,
280 9907 and 9913) with the greatest *Phy. infestans* growth inhibition were chosen for further
281 studies. An additional fungal strain, *Phi. fortinii* (isolate 4197) was included due to its
282 mutualistic interaction with another host, *Larix decidua* (Schulz *et al*., 2002; Schulz, 2006),
283 growth inhibition of other pathogenic microbes and prior information that it could colonize
284 *S. lycopersicum* asymptomatically (Schulz, unpublished).

285 **Phylogenetic placement of fungal endophytes**

286 To determine the taxonomic identity and phylogenetic placement of the four selected fungal
287 endophytes, we sequenced their *ITS1,* 5.8S and *ITS2* regions. First, we used these sequences in
288 a BLAST search to identify the closest relatives of the fungal endophytes (Tab. S4). All four
289 endophytes belong to the ascomycetes. Our analyses further supported the previous
290 characterization of isolate 4197 as *Phi. fortinii* (99% identity and e-value 0, Grünig *et al.*,
291 2008). For isolate 8082 the best BLAST hit with 100% identity and an e-value of 0 was *Phoma*
292 *eupatorii.* This was additionally supported by the fact that isolate 8082 was isolated from
293 *Eupatorium cannabinum* (Tab. S1). The placement of isolates 4197 and 8082 in our
294 phylogenetic analyses together with the extremely short branch lengths to their best BLAST
295 hits further support these phylogenetic assignments (Fig. 1a,b). The best hit for isolate 9907
296 was *Pyrenochaeta cava* (95% identity and e-value 0) and for isolate 9913 it was
297 *Monosporascus ibericus* (97% identity and e-value 0). This suggests that no completely
298 identical sequence/taxa are currently represented in the database. *Pyrenochaeta* does not form
299 a monophyletic group within the order of Pleosporales (Zhang *et al*., 2009; Aveskamp *et al*.,
300 2010; Fig. 1c), thus based on the phylogenetic analyses isolate 9907 can only be placed within
301 the order Pleosporales. Isolate 9913 was isolated from the roots of *Aster tripolium*, a plant that
302 was growing in the salt marshes of the Mediterranean Sea (Tab. S1). Of note is that
303 *Monosporascus ibericus,* the fungal endophyte clustering most closely with isolate 9913 in the
304 phylogenetic analysis, has been recently described as an endophyte of plants growing in
305 environments with high salinity (Collado *et al*., 2002). Furthermore, the genus *Monosporascus*

306 is monophyletic; isolate 9913 has been placed within this monophyletic group and herewith
307 termed *Monosporascus* sp. (Fig. 1d).

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

309 Our initial screening of the culture extracts identified endophytes with the potential to inhibit
310 the growth of a single *Phy. infestans* isolate. We therefore wondered whether the inhibition
311 could be effective against a wider range of isolates of *Phy. infestans.* To test this, we conducted
312 a co-culture assay on RSA agar medium with the four fungal endophytes against nine European
313 *Phy. infestans* isolates (Fig. 2). In the plate assay all four endophytes were capable of
314 significantly restricting growth of *Phy. infestans* (Fig. 3). *Pho. eupatorii* and isolate 9907
315 showed a global inhibition of all *Phy. infestans* isolates tested (Fig. 3b,c). *Phi. fortinii* inhibited
316 the growth of eight out of nine isolates and *Monosporascus* sp. inhibited the growth of seven
317 of the nine isolates (Fig. 3a,d). *Pho. eupatorii* had the greatest average relative growth
318 inhibition of *Phy. infestans* with 50.6 ± 2.2%, and *Monosporascus* sp. had the lowest with 11.9
319 ± 1.6% (Tab. 1).

320 To exclude a mere reduction based on growth limitations we i) measured the inhibition of the
321 endophyte's growth by *Phy. infestans* after the initial co-cultivation phase and ii) evaluated
322 long-term co-cultures (i.e. eight weeks) to analyze the endophyte and pathogen growth
323 progression. The growth of isolate 9907 was not inhibited by any of the *Phy. infestans* isolates
324 (Fig. S1c). However, some isolates of *Phy. infestans* were able to inhibit the growth of the other
325 three fungal endophytes (Fig. S1a,b,d). In all cases, the average relative inhibition of an
326 endophyte by *Phy. infestans* was, however, less than the average relative inhibition of
327 *Phy. infestans* by an endophyte (Tab. S5). For example, whereas the average relative growth
328 inhibition of *Phy. infestans* by *Pho. eupatorii* was 50.6 ± 2.2%, the average relative inhibition
329 of *Pho. eupatorii* by *Phy. infestans* was 4.7 ± 0.9%.

330 After eight weeks, the endophytes, (except for isolate 9907) visually overgrew the plates,
331 including the regions colonized by *Phy. infestans* (Fig. 4). To substantiate this observation, we
332 extracted DNA from some co-cultures with *Phi. fortinii* (12 co-cultures), *Pho. eupatorii* (18
333 co-cultures) and *Monosporascus* sp. (seven co-cultures) from both sides of the eight-week
334 samples (Tab. S6). In total, we analyzed 37 co-cultures and their respective controls for the
335 presence of endophyte and *Phy. infestans.* We used the marker genes *COX* and *ITS*. Because
336 our *ITS* primers were specific for fungi, we primarily observed amplicons from the fungal
337 endophyte *ITS* loci when both organisms were present. However, presence of *Phy. infestans*

338 could be determined by the presence of a *COX* amplicon. In general, we observed that the
339 endophyte was present on both sides of the plates, whereas *Phy. infestans* was either not
340 detected or only on the side of the plate on which it had been inoculated. Few exceptions
341 occurred in which *Phy. infestans* was observed on the side of original inoculation of the fungal
342 endophyte (2/37 cases). Hence, *Phy. infestans* was usually not able to colonize the side of the
343 plate where the endophyte was growing, while the endophyte was always able to colonize
344 *Phy. infestans'* side of the plate. In addition, the endophytes showed a greater inhibition of
345 *Phy. infestans* than *Phy. infestans* did on the endophytes. Therefore, resource limitation (due
346 to the size of the plates) is unlikely to fully explain the unequal growth differential between
347 *Phy. infestans* and the endophytes during co-cultivation. Instead, we hypothesize that factors
348 actively secreted by the endophytes may also be involved in the growth inhibition of
349 *Phy. infestans.*

350 ***Phoma eupatorii* limits *Phytophthora infestans* infection success**

351 We identified global, non-isolate-specific growth inhibition by all four endophytes in plate
352 assays. To test whether the inhibitory potential of the endophytes holds true *in planta,* we
353 inoculated the fungal endophytes in axenically grown *S. lycopersicum* cv. M82 seedlings. Our
354 preliminary screening showed that *Phi. fortinii* and isolate 9907 were too virulent and killed
355 the *S. lycopersicum* seedlings (Fig. S2a,b,d). In contrast, *S. lycopersicum* seedlings inoculated
356 with *Pho. eupatorii* and *Monosporascus* sp. survived (Fig. S2a,c,e).

357 To confirm the endophytic colonization of the roots, we analyzed fungal outgrowth of surface
358 sterilized roots and their imprints from inoculations with water, endophyte or endophyte and
359 *Phy. infestans* (Tab. 2). Irrespective of the protocol, there was neither fungal growth from the
360 surface sterilized mock control roots nor from their imprints. Generally, imprints of the surface
361 sterilized endophyte inoculated roots did not show fungal growth, except for *Pho. eupatorii*
362 inoculated roots after sterilization treatment 1 (1/16 imprints from the mono-inoculation and
363 5/12 imprints from the co-inoculations). This suggests that surface sterilization was successful
364 in all other cases. *Pho. eupatorii* grew from several roots independently of the sterilization
365 treatment, although the stronger treatments resulted in less outgrowth. Hence, these treatments
366 may partially impact survival of endophytic mycelium. Nevertheless, these results show that
367 *Pho. eupatrorii* is capable of colonizing *S. lycopersicum* roots. *Monosporascus* sp. also showed
368 outgrowth from several of the plated surface sterilized roots, suggesting that, like
369 *Pho. eupatorii, Monosporascus* sp. also grows endophytically in the roots of *S. lycopersicum.*

370 *S. lycopersicum* seedlings colonized by *Pho. eupatorii* are visually smaller than mock control
371 seedlings and seedlings mono-inoculated with *Phy. infestans*. We also observed a reduction in
372 leaflet number (Fig. S3a,c). Since the leaflets appeared sturdier and were darker green than the
373 controls (Fig. 5a-f), we measured chlorophyll levels via chlorophyll fluorescence. However,
374 chlorophyll abundance did not change following any of the treatments (Fig. 5g-m). We also
375 observed that some of the stems of the plants that had been inoculated with *Pho. eupatorii*
376 developed a purple color (Fig. S4c). Therefore, we reasoned that the darker leaflet color may
377 have resulted from anthocyanin accumulation. In fact, we detected a significant increase in
378 anthocyanin content in *Pho. eupatorii* inoculated *versus* mock control plants (p=0.001 without
379 *Phy. infestans*, p=0.04 with *Phy. infestans*, Fig. 5o). In contrast to seedlings colonized by
380 *Pho. eupatorii*, those inoculated with *Monosporascus* sp. did not visibly differ from the mock
381 controls (Fig. S3a,b,S4a,c). In agreement with this, anthocyanin content did not increase
382 significantly in *Monosporascus* sp. inoculated samples compared to the mock control (p=0.08
383 without *Phy. infestans*), but slightly when both endophyte and pathogen were present
384 (p=0.007), suggesting that the increase results from the presence of *Phy. infestans*.

385 Despite the visible effects of the colonization by *Pho. eupatorii* on the seedlings, we proceeded
386 to investigate the effect of the endophyte on a subsequent infection with *Phy. infestans*. The
387 relative necrotic area caused by the pathogen is significantly higher on plants inoculated only
388 with *Phy. infestans* (in the absence of pre-inoculation by an endophyte) compared to the mock
389 control (Fig. 5n,S4e). To confirm the pathogen infection in the mock/*Phy. infestans* samples,
390 we used the expression of the *Phy. infestans* biomass marker genes *PiH2a* and *PiElf1α*. In
391 agreement with the increase in necrotic area, *Phy. infestans* was present in all biological
392 replicates mono-inoculated with the pathogen, i.e. demonstrating a successful infection.

393 While the relative necrotic area in seedlings that were colonized only by *Pho. eupatorii* was
394 4.7-fold higher compared to the mock control, this was significantly less than the relative
395 necrotic area of seedlings infected with only *Phy. infestans* (Fig. 5n). *S. lycopersicum* seedlings
396 co-inoculated with *Pho. eupatorii* and *Phy. infestans* resulted in a significantly reduced relative
397 necrotic area compared to seedlings mono-inoculated with *Phy. infestans* (Fig. 5n).
398 Importantly, the average relative necrotic area of leaflets colonized by both *Pho. eupatorii* and
399 *Phy. infestans* did not differ from the mono-inoculations with the endophyte (Fig. 5n). Whether
400 5µl or 10µl mycelial suspensions of *Pho. eupatorii* was used had no effect on the outcome of
401 the experiments. The relative necrotic area between the treatment with *Monosporascus* sp. and

402 the mock control did not differ (Fig. S4a,c,e). This endophyte was not able to inhibit
403 *Phy. infestans* infection nor limit development of disease symptoms *in planta* (Fig. S4b,d,e,f).

404 To quantify the biomass of *Phy. infestans in planta* after pre-inoculation with *Pho. eupatorii,*
405 we performed a qRT-PCR with the two biomass marker genes *PiElf1α* and *PiH2A* (Fig. 5o).
406 In total, we tested the three biological replicates from the 5µl *Pho. eupatorii* inoculations and
407 two from the 10µl *Pho. eupatorii* inoculations. In three of those five replicates, we did not
408 detect an amplicon for either *PiH2a* or *PiElf1α.* Yet, *PiH2a* and *PiElf1α* were detected in every
409 biological replicate of the mock/*Phy. infestans* infections. In addition, three plant-specific
410 reference genes were tested; these showed no aberrant expression in any of the samples
411 colonized by the endophyte in which *PiH2a* and *PiElf1α* were not detected. Hence the presence
412 of the fungal endophyte did not affect the efficiency of the qRT-PCR. Also, those samples that
413 were pre-inoculated with *Pho. eupatorii,* but gave an amplicon of the marker genes had reduced
414 Cq-values for both marker genes compared to the mock/*Phy. infestans* samples. This suggests
415 that *Pho. eupatorii* reduced the infection with *Phy. infestans* isolate D12-2 in the sampled
416 leaflets. To estimate the reduction of *Phy. infestans* biomass, we assumed that the Cq-value of
417 those replicates with no amplicon could theoretically have been amplified in later cycles. We
418 therefore set the Cq-values in those samples to 41; i.e. one cycle more than the original runs
419 included. Based on this assumption, we observed a significant reduction of gene expression in
420 both biomass marker genes in the *Pho. eupatorii* pre-treated samples compared to mono-
421 infections of *Phy. infestans* (Fig. 5o). Therefore, *Pho. eupatorii* is capable of significantly
422 inhibiting *Phy. infestans* infection of *S. lycopersicum* leaflets.

423 **Discussion**

424 **Fungal endophytes show a broad-spectrum growth inhibition of European *Phy. infestans***
425 **isolates**

426 Of 12 fungi for which culture extracts were tested for inhibition of *Phy. infestans,* we identified
427 three ascomycetes, *Pho. eupatorii,* isolate 9907 and *Monosporascus* sp., which effectively
428 inhibited growth of the pathogen. While fungal endophytes produce a vast diversity of
429 metabolites (Schulz *et al*., 2002; Strobel & Strobel, 2007; Verma *et al*., 2009; Mousa &
430 Raizada, 2013; Brader *et al*., 2014) and numerous have antimicrobial activity (Son *et al*., 2008;
431 Puopolo *et al*., 2014; Mousa *et al*., 2016), endophytes and their metabolites may have a narrow
432 spectrum of specificity. To avoid narrow spectrum of pathogen inhibition, we screened these
433 three fungal endophytes and the endophyte *Phi. fortinii* for their capacity to inhibit the growth

434 of nine European isolates of *Phy. infestans*. In our co-culture assays, *Pho. eupatorii* and isolate
435 9907 had a broad-spectrum inhibition against all tested isolates, whereas *Monosporascus* sp.
436 and *Phi. fortinii* inhibited most of the isolates. Additionally, after eight-weeks of incubation,
437 the pathogen was not able to grow on sections of the plates, in which the endophytes grew. The
438 consistency of the results from the culture extract experiments and the plate assays of
439 *Pho. eupatorii* and isolate 9907 shows that their inhibition is independent of the growth
440 medium, suggesting an environmentally robust metabolite production of their anti-
441 *Phytophthora* substances. A robust metabolite production would be of great advantage, if these
442 fungal endophytes are to be used as living biocontrol agents in the field.

443 For application in the field, two issues must be examined: i) Does infection by the endophyte
444 damage the host in the absence of a pathogen? and ii) Does the endophyte successfully inhibit
445 the pathogen in the host? In our study, the former is of extreme importance, because the fungal
446 endophytes in question were not originally isolated from the Solanaceae, to which tomato
447 belongs. Furthermore, whether an endophyte remains benign and asymptomatic is likely to be
448 affected by a number of different circumstances and in some cases the host endophyte
449 relationship may shift to a pathogenic outcome from an initially protective interaction (Schulz
450 & Boyle, 2005; Junker *et al*., 2012; Schulz *et al*., 2015; Busby *et al*., 2016b). Along these lines
451 we excluded two isolates, *Phi. fortinii* and isolate 9907, for direct applications as biocontrol
452 agents: Seedlings of *S. lycopersicum* infected with either of these two isolates quickly died
453 after inoculation. A third isolate, *Monosporascus* sp., neither inhibited *Phy. infestans* infection
454 nor hindered its infection progress. This may not be surprising, because *Monosporascus* sp.
455 had the lowest inhibition potential in our co-culture assays. It should, however, be noted that
456 the metabolite composition of fungal endophytes varies depending on their environments, i.e.
457 *in vitro* and *in planta* (Barder *et al*., 2014). It is therefore possible that the metabolite
458 composition *Monosporascus* sp. produces *in planta* does not include the active anti-
459 *Phytophthora* compound. Alternatively, the active compound may be only produced in specific
460 stages of the infection. In the latter scenario, the infection of *Monosporascus* sp. may not have
461 progressed far enough by the time we inoculated with *Phy. infestans.* Nevertheless, the
462 outcome of the *in planta* co-inoculations does not exclude the possibility that the *in vitro*
463 produced metabolites could be effective in field applications, especially since they showed a
464 broad-spectrum reduction in *Phy. infestans* growth in our co-culture experiments. The broad-
465 spectrum effectiveness of inhibition suggests that the metabolite composition either includes a
466 metabolite with a conserved target in *Phy. infestans* or a mixture of anti-*Phytophthora*

467 metabolites. Both would slow the counter-adaptation of the pathogen to the metabolites if used
468 in field application. As a next step, the metabolite extracts with protective capabilities should
469 be tested for their cytotoxicity *in planta*.

470 ***Phoma eupatorii* isolate 8082 may inhibit *Phytophthora infestans* via secreted toxic**
471 **metabolite(s) and/or the induction of host defense mechanisms**

472 *Pho. eupatorii* was the most effective fungal endophyte in our experiments, excelling both in
473 co-culture as well as *in planta*. The presence of *Pho. eupatorii* not only reduced or inhibited
474 the pathogen's growth, but perhaps entirely prevented infection. Here we used root inoculations
475 of *Pho. eupatorii* combined with leaflet inoculations of *Phy. infestans* isolate D12-2. Because
476 *Pho. eupatorii* was applied to roots, while *Phy. infestans* was inoculated on the leaves, niche
477 competition is an unlikely mechanism by which *Pho. eupatorii* protects the *S. lycopersicum*
478 seedlings. Therefore, two other possible mechanisms by which the plant is defended against
479 the pathogen include endophyte-dependent induction of defense responses or the production of
480 anti-*Phytophthora* metabolites. The induction of plant defense responses by endophytes, such
481 as *Pir. indica* and non-pathogenic *Fusarium oxysporum*, has been previously shown (Stein *et*
482 *al*., 2008; Aimé *et al*., 2013). Here, we observed an elevation of anthocyanin levels in leaf
483 tissue of *S. lycopersicum* after root colonization of *Pho. eupatorii.* Accumulation of
484 anthocyanins is, among other factors, positively regulated by jasmonic acid (Franceschi &
485 Grimes, 1991; Feys *et al*., 1994; Shan *et al*., 2009; Li *et al*., 2006). Hence, it is possible that
486 jasmonic acid dependent defense responses are induced upon colonization with *Pho. eupatorii*.
487 This may be a response to *Pho. eupatorii* and elevated levels of jasmonic acid may have
488 contributed to the inhibition of the *Phy. infestans* infection we observed. The role of jasmonic
489 acid in defense against *Phy. infestans* is not clear: In one study, application of jasmonic acid to
490 leaves of tomato and potato plants resulted in reduced infection of the pathogen (Cohen *et al*.,
491 1993). In another study, it is reported that jasmonic acid is required for the initiation of defense
492 responses triggered by a peptide secreted by *Phy. infestans* (Halim *et al*., 2009). Yet, potato
493 RNA interference lines that downregulated jasmonic acid biosynthesis and signaling
494 components, showed no alterations in the infection rates of *Phy. infestans* (Halim *et al*., 2009).
495 Hence, the production of anti-*Phytophthora* metabolites may be a more likely explanation for
496 the observed reduction of *Phy. infestans* infection. A recently published example of a
497 metabolite based endophyte-mediated pathogen protection is that of *Enterobacter* sp. This
498 endophyte produces many different antimicrobial compounds in its host plant and these are
499 detrimental to the host plant's pathogen *F. graminearum* (Mousa *et al*., 2016). In our study,

500 each of the four fungal endophytes undoubtedly produces anti-*Phytophthora* metabolites in the
501 crude extract tests and in the co-cultures on agar media. This makes it likely that *Pho. eupatorii*
502 also produces such metabolites during *in planta* co-inoculations with *Phy. infestans.* A
503 combination of these two mechanisms is, however, also possible.

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

506 Out of a screen of 12 fungal endophytes, we discovered four ascomycetes that inhibited the
507 growth of *Phy. infestans* in co-culture, presumably through the secretion of secondary
508 metabolites, particularly since their culture extracts were also active. Most importantly, two of
509 the endophytes exhibited global inhibition of nine European *Phy. infestans* isolates, the other
510 two showing a near-global inhibition. This indicates that a conserved target within
511 *Phy. infestans* for a particular metabolite may be produced by these four endophytes.
512 Alternatively, a complex metabolite mixture could be involved. In either case, the use of these
513 fungi for biocontrol could slow the counter-adaptation of *Phy. infestans*. Hence, all four fungal
514 endophytes can be considered good candidates for the production of such new and urgently
515 needed compounds. Additionally, of the four fungal endophytes, *Pho. eupatorii* functioned as
516 an effective biocontrol agent *in planta.* Therefore, *Pho. eupatorii* may not only synthesize a
517 reservoir of highly useful antimicrobial metabolites, but could serve as a novel biocontrol agent
518 providing an alternative to resistance gene breeding and application of agrochemicals.

519 **Acknowledgements**

520 We thank Tuba Altinmakas, Melissa Mantz and Klaudia Maas-Kantel for technical support,
521 Siegfried Draeger for isolation of the endophytes and initial identification based on
522 morphology. We thank the TGRC Institute for providing the seeds of *S. lycopersicum* cv. M82
523 and Francine Govers (Wageningen University) for the *Phy. infestans* isolates NL10001,
524 NL88069, NL90128, IPO-C, IPO428-2, 3928A, D12-2, T15-2 and T20-2. We thank Dr. Bärbel
525 Schöber-Butin for providing the German isolate *Phy. infestans* D2. This work was supported
526 by the Deutsche Forschungsgemeinschaft (Ro 2491/5-2, Ro 2491/6-1, Research Training
527 Group GRK1525).

528 **Author contribution**

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

532 **References**

533 **Adomas A, Eklund M, Johansson M, Asiegbu FO. 2006.** Identification and analysis of
534 differentially expressed cDNAs during nonself-competitive interaction between *Phlebiopsis*
535 *gigantea* and *Heterobasidion parviporum. FEMS Microbiology Ecology* **57:** 26 – 39.

536 **Aimé S, Alabouvette C, Steinerg C, Olivain C. 2013.** The endophytic strain *Fusarium*
537 *oxysporum* Fo47: A good candidate for priming the defense responses in tomato roots.
538 *Molecular Plant-Microbe Interactions* **26**: 918 – 926.

539 **Alabouvette C, Olivain C, Migheli Q, Steinberg C. 2009.** Microbiological control of soil-
540 borne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum*.
541 *New Phytologist* **184**: 529 – 544.

542 **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990.** Basic local alignment search
543 tool. *Journal of Molecular Biology* **215**: 403 – 410.

544 **Annesi T, Curcio G, D'Amico L, Motta E. 2005.** Biological control of *Heterobasidion*
545 *annosum* on *Pinus pinea* by *Phlebiopsis gigantea. Forest Pathology* **35**: 127 – 134.

546 **Aveskamp MM, de Gruyter J, Woudenberg JHC, Verkley GJM, Crous PW. 2010.**
547 Highlights of the Didymellaceae: A polyphasic approach to characterise *Phoma* and related
548 pleosporalean genera. *Studies in Mycology* **65**: 1 – 60.

549 **Bahramisharif A, Lamprecht SC, Calitz F, McLeod A. 2013.** Suppression of *Pythium* and
550 *Phytophthora* damping-off of rooibos by compost and a combination of compost and non-
551 pathogenic *Pythium*-taxa. *Plant disease* **97**: 1605 – 1610.

552 **Benjamini Y, Hochberg Y. 1995.** Controlling the false discovery rate: a practical and
553 powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* **57**: 289
554 – 300.

555 **Brader G, Compant S, Mitter B, Trognitz F, Sessitsch A. 2014.** Metabolic potential of
556 endophytic bacteria. *Current Opinion in Biotechnology* **27**: 30 – 37.

557 **Bodenhausen N, Horton MW, Bergelson J. 2013.** Bacterial communities associated with
558 leaves and the roots of *Arabidopsis thaliana. Plos One* **8**: e56329.

559 **Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza**
560 **F, Rauf P, Huettel B, Reinhardt R, Schmelzer E, Peplies J, et al. 2012.** Revealing structure
561 and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**: 91 – 95.

562 **Bulgarelli D, Garrido-Oter R, Münch PC, Weiman A, Dröge J, Pan Y, McHardy AC,**
563 **Schulze-Lefert P. 2015.** Structure and function of the bacterial root microbiota in wild and
564 domesticated barley. *Cell Host and Microbe* **17**: 392 – 403.

565 **Busby PE, Peay KG, Newcombe G. 2016a.** Common foliar fungi of *Populus trichocarpa*
566 modify *Melampsora* rust disease severity. *New Phytologist* **209**: 1681 – 1692.

567 **Busby PE, Ridout M, Newcombe G. 2016b.** Fungal endophytes: modifiers of plant disease.
568 *Plant Molecular Biology* **90**: 645 – 655.

569 **Cao L, Qiu Z, You J, Tan H, Zhou S. 2004.** Isolation and characterization of endophytic
570 *Streptomyces* strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. *Letter in*
571 *Applied Microbiology* **39**: 425 – 430.

572 **Caten CE, Jinks JL. 1968.** Spontanous variability of single isolates of *Phytophthora infestans.*
573 I. Culture variation. *Canadian Journal of Botany* **46**: 329 – 348.

574 **Childers R, Danies G, Myers K, Fei Z, Small IM, Fry WE. 2015.** Acquired resistance to
575 mefenoxam in sensitive isolates of *Phytophthora infestans. Phytopathology* **105**: 342 – 349.

576 **Collado J, Gonzalez A, Platas G, Stchigel AM, Guarro J, Pelaez F. 2002.** *Monosporascus*
577 *ibericus* sp. nov. an endophytic ascomycete from plants on saline soils, with observations on
578 the position of the genus based on sequence analysis of the 18S rDNA. *Mycological Research*
579 **106**: 118 – 127.

580 **Cohen Y, Gisi U, Niderman T. 1993.** Local and systemic protection against *Phytophthora*
581 *infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester.
582 *Phytopathology* **83**: 1054 – 1062.

583 **Coleman-Derr D, Desgarennes D, Fonseca-Garcia C, Gross S, Clingenpeel S, Woyke T,**
584 **North G, Visel A, Partida-Martinez LP, Tringe SG. 2016.** Plant compartment and
585 biogeography affect microbiome composition in cultivated and native *Agave* species. *New*
586 *Phytologist* **209**: 798 – 811.

587 **Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G. 2004.** MycoBank: an online
588 initiative to launch mycology into the 21st century. *Studies in Mycology* **50**: 19 – 22.

589 **Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen**
590 **JA, Sundaresan V. 2015.** Structure, variation, and assembly of the root-associated
591 microbiomes of rice. *Proceedings of the National Academy of Sciences of the United States of*
592 *America* **112:** E911 – E920.

593 **Feys BJ, Benedetti CE, Penfold CN, Turner JG. 1994.** *Arabidopsis* mutants selected for
594 resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate and
595 resistant to a bacterial pathogen. *The Plant Cell* **6**: 751 – 759.

596 **Franceschi VR, Grimes HD. 1991.** Induction of soybean vegetative storage proteins and
597 anthocyanins by low-level atmospheric methyl jasmonate. *Proceedings of the National*
598 *Academy of Sciences of the United States of America* **88**: 6745 – 6749.

599 **Grünig CR, Queloz V, Sieber TN, Holdenrieder O. 2008.** Dark septate endophytes (DSE)
600 of the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex in tree roots:
601 classification, population biology, and ecology. *Botany* **86**: 1355 – 1369.

602 **Grünwald NJ, Sturbaum AK, Montes GR, Serrano EG, Lozoya-Saldaña H, Fry WE.**
603 **2006.** Selection for fungicide resistance within a growing season in field populations of
604 *Phytophthora infestans* at the center of origin. *Phytopathology* **96**: 1397 – 1403.

605 **Halim VA, Altmann S, Ellinger D, Eschen-Lippold L, Miersch O. 2009.** PAMP-induced
606 defense responses in potato require both salicylic acid and jasmonic acid. *The Plant Journal*
607 **57**: 230 – 242.

608 **Hiruma K, Gerlach N, Sacristán S, Nakano RT, Hacquard S, Kracher B, Neumann U,**
609 **Ramírez D, Bucher M, O'Connell RJ, et al. 2016.** Root endophyte *Colletrichum tofieldiae*
610 confers plant fitness benefits that are phosphate status dependent. *Cell* **165**: 464 – 474.

611 **Höller U, Wright AD, Matthée GF, König GM, Draeger S, Aust H-J, Schulz B. 2000.**
612 Fungi from marine sponges: diversity, biological activity and secondary metabolites.
613 *Mycological Research* **104**: 1354 - 1365.

614 **Hudspeth DSS, Nadler SA, Hudspeth MES. 2000.** A COX2 molecular phylogeny of the
615 Peronosporomycetes. *Mycologia* **92**: 674 – 684.

616 **Junker C, Draeger S, Schulz B. 2012.** A fine line – endophytes or pathogens in *Arabidopsis*
617 *thaliana. Fungal Ecology* **5**: 657 - 662.

618 **Kim H-Y, Choi GJ, Lee HB, Lee S-W, Lim HK, Jang KS, Son SW, Lee SO, Cho KY,**
619 **Sung ND, et al. 2007.** Some fungal endophytes from vegetable crops and their anti-oomycete
620 activities against tomato late blight. *Letters in Applied Microbiology.* **44**: 332 – 337.

621 **Kruskal WH, Wallis WA. 1952.** Use of ranks in one-criterion variance analysis. *Journal of*
622 *the American Statistical Association* **47**: 583 – 621.

623 **Lahlali R, Hijri M. 2010.** Screening, identification and evaluation of potential biocontrol
624 fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. *FEMS Microbiology*
625 *Letters.* **311**: 152 – 159.

626 **Le Cocq K, Gurr SJ, Hirsch PR, Mauchline TH. 2016.** Exploiting of endophytes for
627 sustainable agriculture intensification. *Molecular Plant Pathology* doi: 10.1111/mpp.12483.

628 **Li J, Brader G, Kariola T, Palva ET. 2006.** WRKY70 modulates the selection of signaling
629 pathways in plant defense. *The Plant Journal* **46**: 477 – 491.

630 **Lindoo SJ, Caldwell MM. 1978.** Ultraviolet-B radiation-induced inhibition of leaf expansion
631 and promotion of anthocyanin production. *Plant Physiology* **61**: 178 – 282.

632 **Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J,**
633 **Engelbrekston A, Kunin V, Glavina del Rio T, et al. 2012.** Defining the core *Arabidopsis*
634 *thaliana* root microbiome. *Nature* **488**: 86 – 90.

635 **Mann HB, Whitney DR. 1947.** On a test of whether one of two random variables is
636 stochastically larger than the other. *Annals of Mathematical Statistics* **1**: 50 – 60.

637 **Martínez-Medina A, Fernandez I, Lok GB, Pozo MJ, Pieterse CMJ, Van Wees SCM.**
638 **2017.** Shifting from priming of salicyliuc acid- to jasmonate acid-regulated defences by
639 *Trichoderma*protects tomato against the root knot nematode *Meloidogyne incognita. New*
640 *Phytologist* **213**: 1363 – 1377.

641 **Miles LA, Lopera CA, González S, Cepero de García MC, Franco AE, Restrepo S. 2012.**
642 Exploring the biocontrol potential of fungal endophytes from an Andean Colombian paramo
643 ecosystem. *BioControl* **57**: 697 – 710.

644 **Mousa WK, Raizada MN. 2013.** The diversity of anti-microbial secondary metabolites
645 produced by fungal endophytes: an interdisciplinary approach. *Frontiers in Microbiology* **4**:
646 65.

647 **Mousa WK, Shearer C, Limay-Rios V, Ettinger CL, Eisen JA, Raizada MN. 2016.** Root-
648 hair endophyte stacking in finger millet creates a physiochemical barrier to trap the fungal
649 pathogen *Fusarium graminearum. Nature Microbiology* **1**: 16167.

650 **Murashige T, Skoog F. 1962.** A revised medium for rapid growth and bio assays wity tobacco
651 tissue cultures. *Physologia Plantarum* **15**: 473 – 497.

652 **Nowicki M, Foolad MR, Nowakowska M, Kozik EU. 2012.** Potato and tomato late blight
653 caused by *Phytophthora infestans*: an overview of pathology and resistance breeding. *Plant*
654 *disease* **96**: 4 – 17.

655 **Panke-Buisse K, Poole AC, Goodrich JK, Ley RE, Kao-Kniffin J. 2015.** Selection on soil
656 microbiomes reveals reproducible impacts on plant function. *The ISME Journal* **9**: 980 – 989.

657 **Pel MA, Foster SJ Park T-H, Rietman H, van Arkel G, Jones JDG, van Eck HJ, Jacobsen**
658 **E, Visser RGF, van der Vossen E. 2009.** Mapping and cloning of late blight resistance genes
659 from *Solanum venturii* using an interspecific candidate gene approach. *Molecular Plant-*
660 *Microbe Interactions* **22**: 601 – 615.

661 **Peters S, Aust H-J, Draeger S, Schulz B. 1998.** Interactions in dual cultures of endophytic
662 fungi with host and nonhost plant calli. *Mycologia* **90**: 360 – 367.

663 **Pfaffl MW. 2001.** A new mathematical model for relative quantification in real-time RT-PCR.
664 *Nucleic Acids Research* **29**: e45.

665 **Ploch S, Rose LE, Bass D, Bonkowski M. 2016.** High diversity revealed in leaf-associated
666 protists (Rhizaria: Cercozoa) of Brassicaceae. *Journal of Eukaryotic Microbiology* **63**: 635 –
667 641.

668 **Puopolo G, Cimmino A, Palmieri MC, Giovannini O, Evidente A, Pertot I. 2014.**
669 *Lysobacter capsici* AZ78 produces cyclo(L-Pro-L-Tyr), a 2,5-diketopiperazine with toxic
670 activity against sporangia of *Phytophthora infestans* and *Plasmopara viticola. Journal of*
671 *Applied Microbiology* **117**: 1168 – 1180.

672 **Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Gandolfi C, Casati**
673 **E, Previtali F, Gerbino R. 2015.** Improved plant resistance to drought is promoted by the

674 root-associated microbiome as a water stress-dependent trait. *Environmental Microbiology* **17**:
675 316 – 331.

676 **Schlaeppi K, Dombrowski N, Oter RG, Ver Loren van Themaat E, Schulze-Lefert P.**
677 **2013.** Quantitative divergence of the bacterial root microbiota in *Arabidopsis thaliana*
678 relatives. *Proceedings of the National Academy of Sciences of the United States of America*
679 **111**: 585 – 592.

680 **Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. 2015.** The ImageJ ecosystem: An open
681 platform for biomedical image analysis. *Molecular Reproduction and Development* **82**: 518 –
682 529.

683 **Schulz B, Boyle C, Draeger S, Römmert A-K, Krohn K. 2002.** Endophytic fungi: a source
684 of novel biologically active secondary metabolites. *Mycological Research* **106**: 996 – 1004.

685 **Schulz B, Boyle C. 2005.** The endophytic continuum. *Mycological research* **109**: 661 – 686.

686 **Schulz B. 2006.** Mutualistic interactions with fungal root endophytes. In: Schulz BJE, Boyle
687 CJC, Sieber TN, eds. *Microbial Root Endophytes*. Berlin, Germany: Springer Verlag, 261 –
688 279.

689 **Schulz B, Krohn K, Meier K, Draeger S. 2011.** Isolation of endophytic fungi for the
690 production of biologically active secondary metabolites. In: Pirttilä AM, Sovari S, eds.
691 *Prospects and Applications for Plant-Associated Microbes*. Turku, Finland: BioBien
692 Innovations, 88 – 95.

693 **Schulz B, Haas S, Junker C, Andrée N, Schobert M. 2015.** Fungal endophytes are involved
694 in multiple balanced antagonisms. *Current Science* **109**: 39 – 45.

695 **Shan X, Zhang Y, Peng W, Wang Z, Xie D. 2009.** Molecular mechanism for jasmonate-
696 induction of anthocyanin accumulation in *Arabidopsis*. *Journal of Experimental Botany* **60**:
697 3849 – 3860.

698 **Shapiro SS, Wilk MB. 1965.** An analysis of variance test for normality (complete samples).
699 *Biometrika* **52**: 591 – 611.

700 **Shoresh M, Harman GE, Mastouri F. 2010.** Induced systemic resistance and plant responses
701 to fungal biocontrol agents. *Annual Review of Phytopathology* **48**: 21 – 43.

Son SW, Kim HY, Choi GJ, Lim HK, Jang KS, Lee SO, Lee S, Sung ND, Kim J-C. 2008. Bikaverin and fusaric acid from *Fusarium oxysporum* show antioomycete activity against *Phytophthora infestans*. *Journal of Applied Microbiology* **104**: 692 – 698.

Song J, Bardeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, et al. 2003. Gene *RB* from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 9128 – 9133.

Stein E, Molitor A, Kogel K-H, Waller F. 2008. Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant and Cell Physiology* **49**: 1747 – 1751.

Strobel SA, Strobel GA. 2007. Plant endophytes as a platform for discovery-based undergraduate science education. *Nature Chemical Biology* **3**: 356 – 359.

Sturz AV, Christie BR, Matheson BG, Arsenault WJ, Buchanan NA. 1999. Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. *Plant Pathology* **48**: 360 – 369.

Suryanarayanan TS, Govinda Rajul MB, Vidal S. 2016. Biological control through fungal endophytes: gaps in knowledge hindering success. *Current Biotechnology* **5**: doi 10.2174/2211550105666160504130322.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution* **28:** 2731 – 2739.

Tellenbach C, Sieber TN. 2012. Do colonization by dark septate endophytes and elevated temperature affect pathogenicity of oomycetes? *FEMS Microbiology Ecology* **82**: 157 – 168.

Terhonen E, Sipari N, Asiegbu FO. 2016. Inhibition of phytopathogens by fungal root endophytes of Norway spruce. *Biological Control* **99**: 53 – 63.

Tukey J. 1949. Comparing individual means in the analysis of variance. *Biometrics* **5**: 99 – 114.

Verma VC, Kharwar RN, Strobel GA. 2009. Chemical and functional diversity of natural products from plant associated endophytic fungi. *Natural Product Communications* **4**: 1511 – 1532.

732 **Vleeshouwers VG, Raffaele S, Vossen JH, Champouret N, Oliva R, Segretin ME, Rietman**
733 **H, Cano LM, Lokossou A, Kessel G, et al. 2011.** Understanding and exploiting late blight
734 resistance in the age of effectors. *Annual Reviews of Phytopathology* **49**: 507 – 531.

735 **van der Vossen E, Sikkema A, te Lintel Hekkert B, Gros J, Stevens P, Muskens M,**
736 **Wouters D, Pereira A, Stiekema W, Allefs S. 2003.** An ancient *R* gene from the wild potato
737 species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans*
738 in cultivated potato and tomato. *The Plant Journal* **36**: 867 – 882.

739 **de Vries S, Kloesges T, Rose LE. 2015.** Evolutionarily dynamic, but robust, targeting of
740 resistance genes by the miR482/2118 gene family in the Solanaceae. *Genome Biology and*
741 *Evolution* **7**: 3307 – 21.

742 **de Vries S, von Dahlen JK, Uhlmann C, Schnake A, Kloesges T, Rose LE. 2017.** Signatures
743 of selection and host-adapted gene expression of the *Phytophthora infestans* RNA silencing
744 suppressor PSR2. *Molecular Plant Pathology* **18**: 110 – 124.

745 **White TJ, Bruns T, Lee S, Taylor JW. 1990.** Amplification and direct sequencing of fungal
746 ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ,
747 eds. *PCR Protocols: A Guide to Methods and Applications*. New York, USA: Academic Press,
748 Inc., 315 – 322.

749 **Zhang Y, Schoch CL, Fournier J, Crous PW, de Gruyter J, Woudenberg JHC, Hirayama**
750 **K, Tanaka K, Pointing SB, Spatafora JW, et al. 2009.** Multi-locus phylogeny of
751 Pleosporales: a taxonomic, ecological and evolutionary re-evaluation. *Studies in Mycology* **64**:
752 85 – 102.

753 **Zhang C, Liu L, Zheng Z, Sun Y, Zhou L, Yang Y, Cheng F, Zhang Z, Wang X, Huang**
754 **S, et al. 2013.** Fine mapping of the *Ph-3* gene conferring resistance to late blight (*Phytophthora*
755 *infestans*) in tomato. *Theoretical and Applied Genetics* **126**: 2643 – 2653.

756

757 **Table**

758 **Table 1.** Average relative growth inhibition of endophytes (first column) by *Phy. infestans*
759 (upper row). The relative inhibition is calculated from the average radii estimated for co-
760 cultivations and control plates.

761

	NL88069	IPO-C	NL90128	IPO428-2	NL10001	3928A	T20-2	D12-2	T15-2	Average+/-SEM
Phi. fortinii	0.060	0.109	0.119	0.139	0.143	0.145	0.016	0.188	0.077	0.111 +/-0.016
Pho. eupatorii	0.002	0.050	0.004	0.070	0.089	0.068	0.059	0.049	0.034	0.047 +/-0.009
9907	0.010	-0.024	0.016	-0.037	0.045	0.048	0.028	0.033	0.020	0.015 +/-0.009
Monosporascus sp.	0.066	0.116	0.079	-0.032	-0.026	0.015	0.033	0.110	0.052	0.046 +/-0.017

762

763 **Table 2.** Endophytic outgrowth from surface sterilized roots after inoculation with the
764 endophyte. Roots were surfaces sterilized and an imprint of each root was prepared to test for
765 efficiency of the treatment. The days after which the roots were surveyed is given as days post
766 sterilization (dps). Treatment 1, 2 and 3 indicate the type of surface sterilization as described
767 in the Material and Method section. The number of imprints and roots with fungal growth and
768 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

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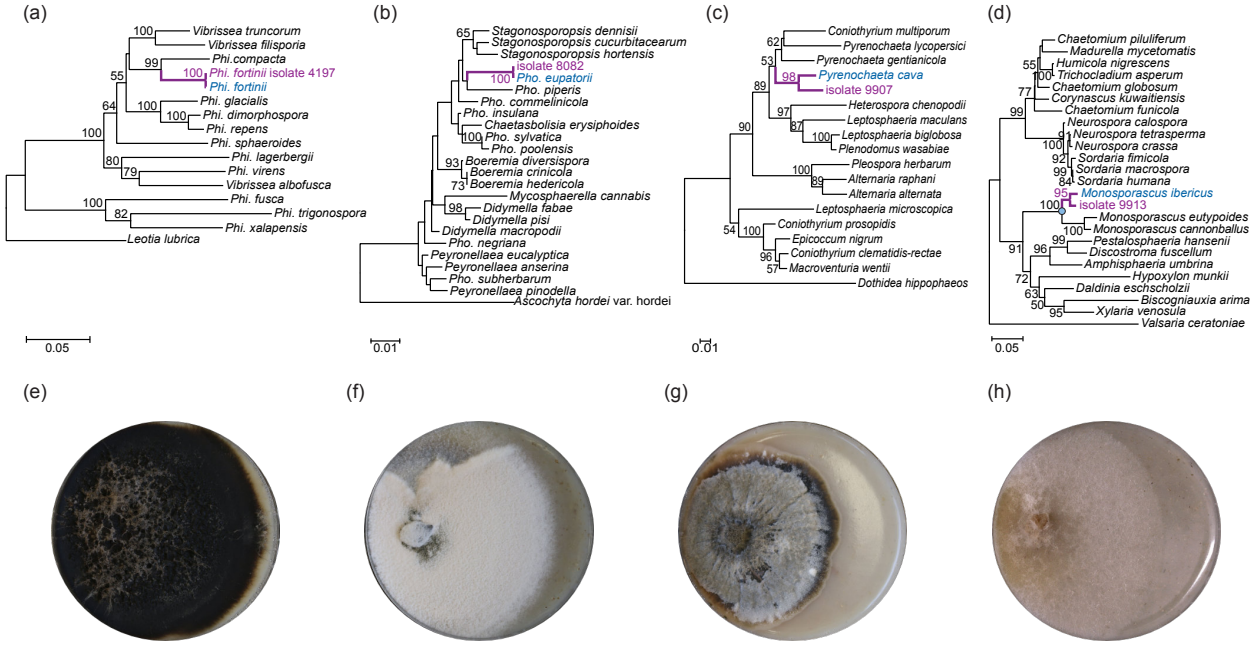

Figure 1. Phylogenetic placement of fungal endophytes.

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).

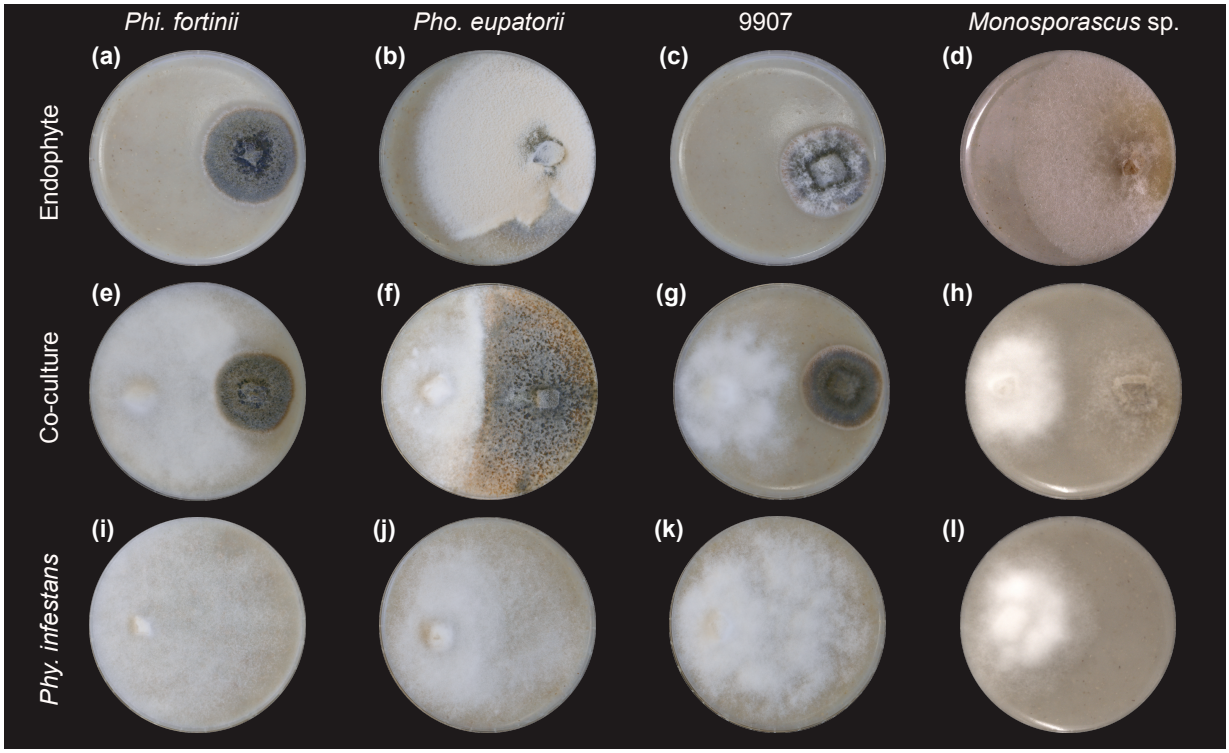

Figure 2. Co-cultivation of fungal endophytes with *Phytophthora infestans* on plate.

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.

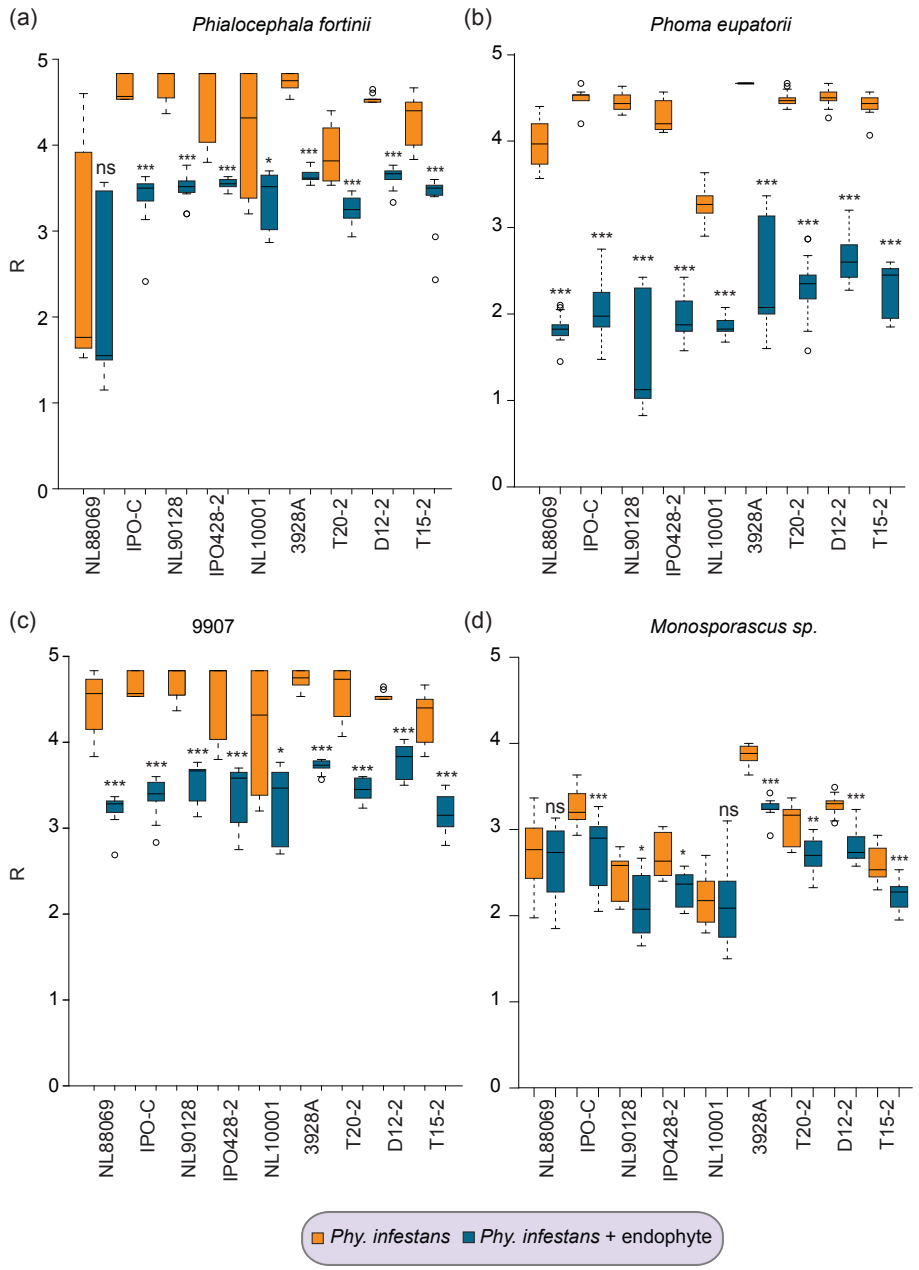

Figure 3. Radial growth inhibition of *Phytophthora infestans* isolates by fungal endophtyes.

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.

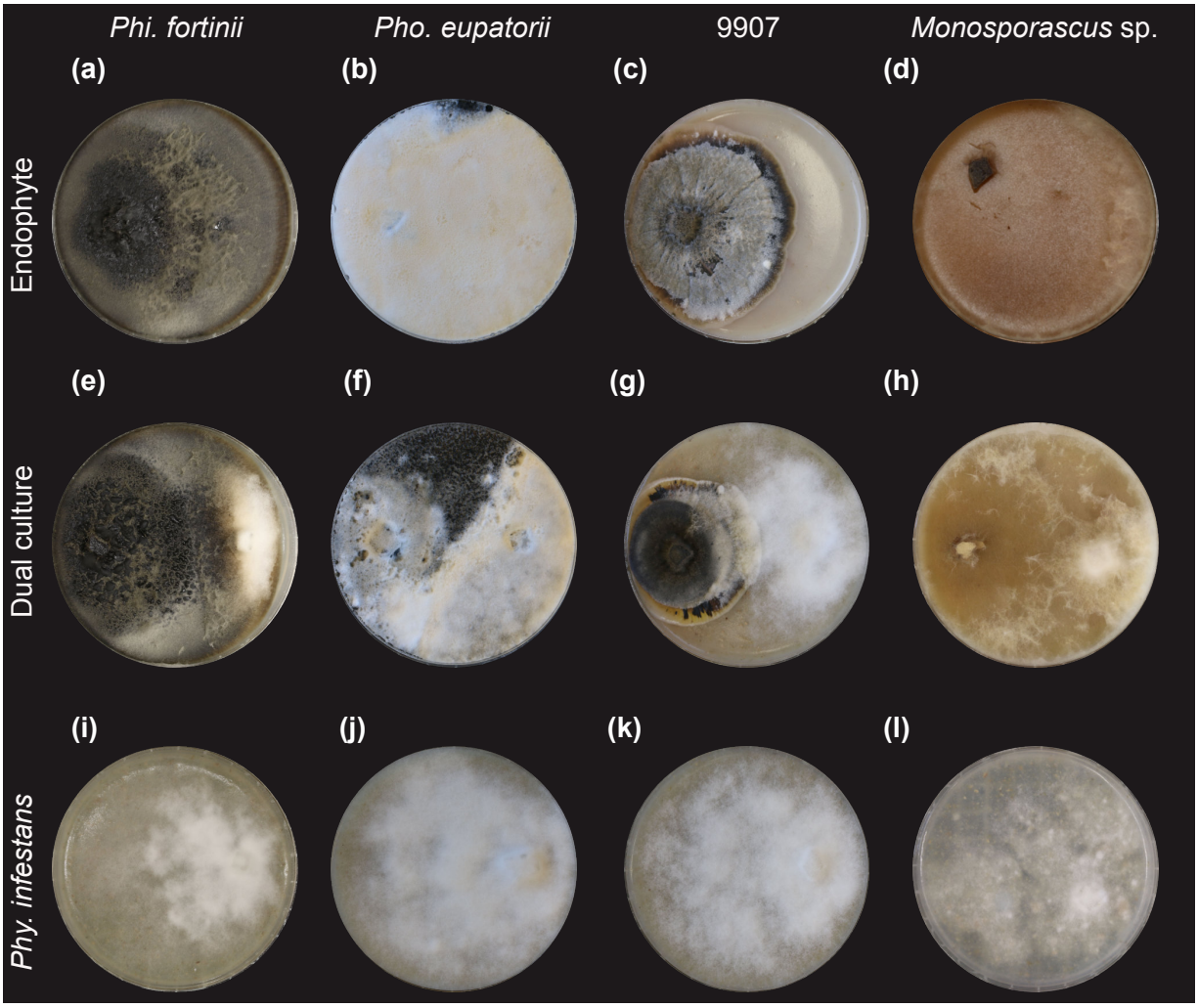

Figure 4. Long-term co-cultivation of fungal endophytes with *Phytophthora infestans* on plate

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.

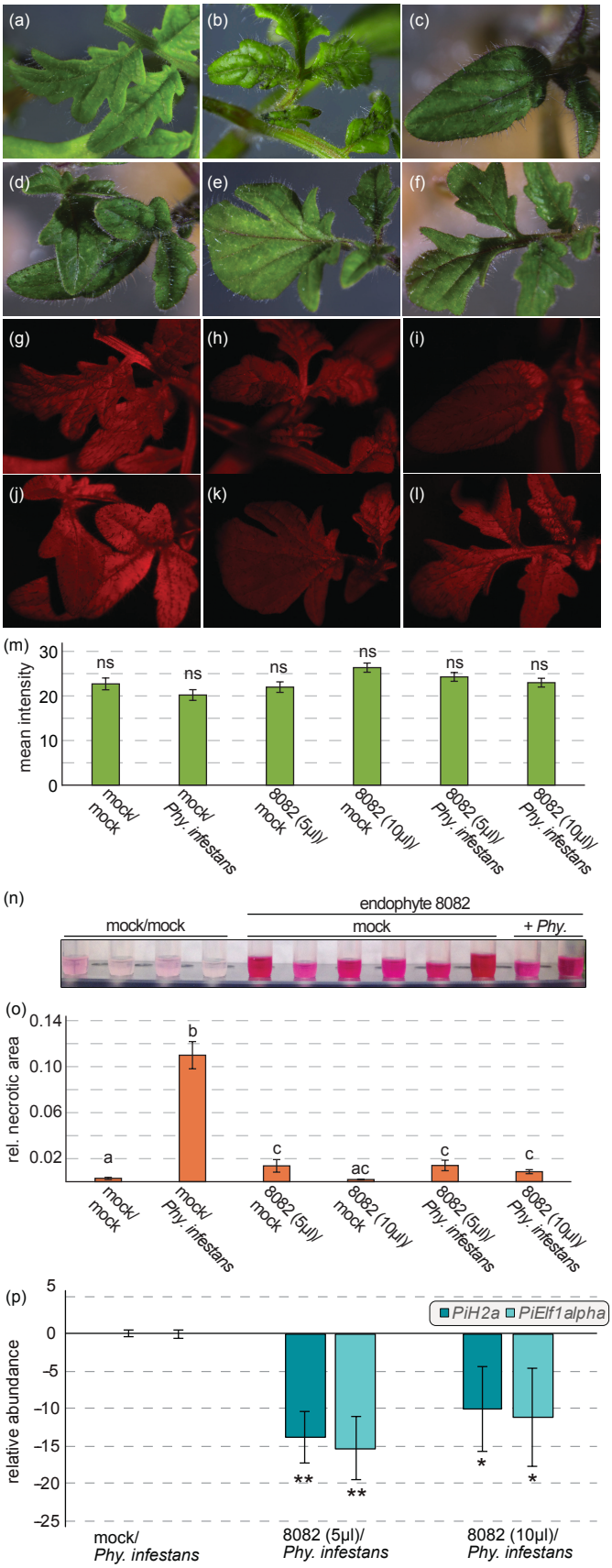

Figure 5. *In planta* co-inoculations of *Phoma eupatorii* isolate 8082 and *Phytophthora infestans*

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.