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A non-native and a native fungal plant pathogen similarly stimulate ectomycorrhiza development but are perceived differently by a fungal symbiont

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Brief heading: Mycorrhizal fungi recognize plant pathogens as alien or native and are stimulated by both

Summary

- The effects of plant symbionts on host defence responses against pathogens have been extensively documented, but little is known on impact of pathogens on the symbiosis and if such impact may differ for non-native and native pathogens. Here, this issue was addressed by working on the model system comprising *Pinus pinea*, its ectomycorrhizal symbiont *Tuber borchii,* the non-native and native pathogens *Heterobasidion irregulare* and *H. annosum*, respectively.
- Through a six-months inoculation experiment and using both *in planta* and gene expression analyses, we tested the hypothesis that *H. irregulare* may have greater effects on symbiosis than *H. annosum*.
- Although the two pathogens induced the same morphological reaction in the plant-symbiont complex, with mycorrhizal density increasing exponentially with pathogen colonization of host, the number of target genes regulated in *T. borchii* was more than twice in plants inoculated with the native pathogen, i.e. 67% of tested genes, compared to that inoculated with the non-native one, i.e. 27% of genes.
- Despite the two fungal pathogens did not differentially affect the amount of ectomycorrhizae, the fungal symbiont perceived their presence differently. Results could suggest that the symbiont has the ability to recognize a self/native and a non-self/non-native pathogen, probably trough host plant-mediated signal transduction.

Keywords: biological invasions, gene expression, *Heterobasidion*, impact, *Pinus pinea*, RT-qPCR, *Tuber borchii*.

Introduction

More than 90% of plants form root interactions with mycorrhizal fungi that offer to the host plant water and mineral nutrients (nitrogen, phosphorous and magnesium), in exchange of photoassimilates (Smith & Read, 2008). In particular, the ectomycorrhizal (ECM) symbiosis involves fungi belonging to Basidiomycota or Ascomycota and plants encompassing Betulaceae, Cistaceae, Dipterocarpaceae, Fabaceae, Fagaceae, Myrtaceae, Pinaceae and Salicaceae (Martin, 2007; Smith & Read 2008). During the symbiotic phase, ECM fungi, including *Tuber* species, form a fungal sheath named the mantle, made of densely packed hyphae that surround the root surface. This mycelium is linked to extramatrical hyphae, which are responsible for the water uptake and mineral nutrition. From the inner zone of the mantle, some hyphae penetrate among the root cells, forming the Hartig net, a hyphal network inside the root tissues where metabolites are exchanged (Balestrini *et al.*, 2012). The ECM symbiosis encompasses different habitats such as boreal, temperate, Mediterranean and some subtropical forest ecosystems (Martin, 2007; Smith & Read 2008). In coniferous forests, such as pine forests, trees with ECM associations have been reported to be dominant (Brundrett, 2002).

In addition to nutrient exchanges, it has been shown that mycorrhizal fungi can lead to an improved tolerance against abiotic (e.g. salinity, drought, heavy metals) and biotic stresses, such as infectious plant diseases (Smith & Read, 2008). For example, the ECM fungi *Laccaria laccata* (Scop.) Cooke, *Hebeloma crustuliniforme* (Bull.) Quél., *H. sinapizans* (Paulet) Gillet and *Paxillus involutus* (Batsch) Fr. have been shown to reduce the impact of the ink disease pathogens *Phytophthora cambivora* (Petri) Buisman and *P. cinnamomi* Rands on the European chestnut (Branzanti *et al.*, 1999). The symbiosis of poplar with *H. mesophaeum* (Pers.) Quél. allowed to compensate the infection caused by the rust fungus *Melampsora laricis-populina* Kleb. (Pfabel *et al.*, 2012). Other ECM fungi were demonstrated to reduce the negative effects of pathogenic strains of Dark Septate Endophytes (DSEs) in conifers (Renienger & Sieber, 2012).

Accidentally introduced invasive fungal plant pathogens have increased exponentially in their number in the past 40 years and are currently the main cause of disease outbreaks, with dramatic ecological implications in forest ecosystems (Stenlid *et al*., 2011; Santini *et al.*, 2013). Dutch elm disease, chestnut blight, ash dieback, and the mortality of stone pine trees are just some of the few examples of relevant tree diseases caused by invasive fungal pathogens (Anderson *et al.*, 2004; Gonthier *et al.*, 2004; Liebhold *et al.*, 2012; Gross *et al.*, 2014). Although the invasion of fungal plant pathogens may be driven by ecological factors affecting transmission potential (Garbelotto *et al.*, 2010; Giordano *et al.*, 2014; Sillo *et al.*, 2015a), disease outbreaks caused by introduced pathogens have been often attributed to a disproportional pathogenicity of the pathogens on naïve hosts (Oliva *et al.*, 2013) consistent with the lack of coevolution hypothesis (Parker & Gilbert, 2004).

As mentioned previously, the role played by ECM fungi on the protection of forest trees has been extensively documented (Branzanti *et al.*, 1999; Zhang *et al.*, 2011; Pfabel *et al.*, 2012; Renienger & Sieber, 2012). Conversely, little is known on the effects of plant diseases on ECM symbiosis. Recently, by investigating different forest stands Gaitnieks *et al.* (2016) showed that the two native pathogens *H. annosum* (Fr.) Bref. and *H. parviporum* Niemelä & Korhonen 1998 have limited effects on fine root morphology, mycorrhizal colonization and composition of ECM communities in roots of Norway spruce. However, there is no information on whether invasive pathogens may be more harmful and may affect differently ECM symbiosis than native ones.

Heterobasidion irregulare Garbel. & Otrosina is a major fungal pathogen of pines in North America and was accidentally introduced in central Italy during World War II (Gonthier *et al.*, 2004), where it has become invasive by colonizing pine and oak stands (Gonthier *et al.*, 2007; Gonthier *et al.*, 2012). Although at low levels, the Eurasian sister species *H. annosum* is also present in pine stands in the invasion area of *H. irregulare* (Gonthier *et al.*, 2007; Gonthier *et al.*, 2012; Garbelotto *et al.*, 2013; Gonthier *et al.*, 2014). The two fungal pathogens diverged in allopatry 34-41 million years ago (Linzer *et al.*, 2008; Dalman *et al.*, 2010) and based on a recent phylogenomic investigation they must be regarded as clearly distinct species (Sillo *et al.*, 2015a).

Pathogenicity and fitness of *H. irregulare* and *H. annosum* were compared on various host plants through different inoculation experiments (Garbelotto *et al.*, 2010; Giordano *et al.*, 2014; Pollastrini *et al.*, 2015). Despite the fact that *H. irregulare* and *H. annosum* differ in their potential to colonize wood and sporulate (Garbelotto *et al.*, 2010; Giordano *et al.*, 2014), they are equally pathogenic on several pine species (Garbelotto *et al.*, 2010; Pollastrini *et al.*, 2015).

It could be hypothesized that pathogens might affect the ECM symbiosis process and also that the expression pattern of key fungal genes for functional symbiosis might change in diseased plants. Based on this hypothesis, the aims of this study were to assess and compare the effects of *H. irregulare* and *H. annosum* on the ECM symbiosis between *Tuber borchii* Vittad. and *Pinus pinea* L., in a three-actors model system including the invasive or native fungal pathogen, the symbiont and the host plant. Specific aims included: 1) to verify if *H. irregulare* and *H. annosum* might affect quantitatively the ECM symbiosis process, 2) to assess if changes may happen in the *T. borchii* gene expression, focusing on genes usually regulated during the ECM symbiosis, and 3) to test if differential gene expression might allow to discriminate the effects of the invasive *H. irregulare* compared to that of the native *H. annosum* on *T. borchii*.

Material and Methods

Heterobasidion spp. genotypes and inoculation experiment on mycorrhized pines

The three heterokaryotic (ploidy: n+n) genotypes of *H. irregulare* and the three heterokaryotic genotypes of *H. annosum* (Table 1) analyzed by Sillo *et al.* (2015b) were used in this study. Genotypes were deposited at the *Mycotheca Universitatis Taurinensis* (MUT) of University of Turin with the accession numbers listed in the Table 1. One month before fungal inoculations they were sub-cultured in Petri dishes containing MEA (Malt Extract Agar: 20 g malt extract, 20 g glucose, 20 g agar, 2 g peptone, 0.5 g citric acid, 1 l distilled water).

In order to mimic what happens in nature, where more than 90% of plants form mycorrhizal symbiosis since the earliest stages of development (Smith & Read, 2008), seedlings previously mycorrhized were inoculated with plant pathogens. The inoculation experiment was carried out on two-year-old *P. pinea* seedlings (about 60-70 cm length and 4-5 mm diameter) mycorrhized with *T. borchii* and provided in 9-cm diameter pots by Raggi Vivai (Cesena, FC, Italy). The provided plant stock was certified by Raggi Vivai with an average mycorrhization level of 71.5%±5.8. Such mycorrhization level was visually assessed and expressed as the ratio between mycorrhized apexes and the total number of root apexes. The mycorrhization level and its homogeneity among seedlings were confirmed by observations under a dissecting microscope (x 40 magnification) of root systems of 10 randomly selected seedlings. *P. pinea* was selected for this experiment because it is the most largely widespread host for both *Heterobasidion* species in the invasion area in central Italy (Garbelotto & Gonthier, 2013; Gonthier *et al.*, 2014) and could be easily mycorrhized with *T. borchii* (Zambonelli *et al.*, 2000).

P. pinea stems were inoculated 10 cm above ground by placing a colonized plug of MEA (5 mm diameter) in a slit obtained by excising a portion of the bark, superficially cleaned with 70% ethanol, with a sterile scalpel according to the methods described by Garbelotto *et al.* (2007). Inoculation points were wrapped in Parafilm in order to avoid external contaminations and tissue dryness. A total of 60 *P. pinea* seedlings were inoculated, 10 for each *Heterobasidion* genotype. Ten additional pine seedlings were wounded as described above and mock-inoculated, using a plug of sterile MEA in order to serve as controls to be compared with inoculated pines.

Pine seedlings were randomly allocated in a growth chamber set at a temperature of 25±2°C with a photoperiod of 12 h day^{-1} and incubated for six months. The seedlings were inspected at regular intervals for the presence of crown symptoms including needle discoloration and needle loss. Whenever seedlings showed evident crown symptoms and a strong decline in vitality, they were scored as "symptomatic pines" and sectioned as described below. For these samplings taking place before the conclusion of the experiment, three randomly selected control *P. pinea* seedlings were sectioned and incubated as well. At the end of the experiment, i.e. six months after the inoculation, all remaining living seedlings were scored as "asymptomatic pines" and sectioned as described below.

Heterobasidion spp. colonization and _T. borchii_ mycorrhization rate assessment

All 60 inoculated pine stems, after needle removal, were sectioned in 3 mm-thick slices, which were incubated at room temperature (22±2°C) for one week in 15 cm diameter Petri dishes containing a sterile filter paper dampened with sterile distilled water. A dissecting microscope (x 40 magnification) was used to measure the extent of the stem portion colonized by the fungal pathogens (C, in mm) based on the number of slices displaying the characteristic *Heterobasidion* conidiophores (Swedjemark & Karlsson, 2006).

The root systems of seedlings were washed under tap water and subsequently observed under a dissecting microscope (x 40 magnification) in order to determine the total number of ectomycorrhizae (m) in the entire root system of each plant. The ectomycorrhizae were collected in 2 ml tubes by excising them from the root systems, through the use of sterile tweezers. Subsequently, they were directly frozen in liquid nitrogen and stored at -80°C. After collection of ectomycorrhizae, the total root system was lyophilized for 24 h and then weighted.

RNA extraction from ectomycorrhizae and cDNA conversion

RNA was extracted from the ectomycorrhizae of all symptomatic pines and from three *P. pinea* mock-inoculated seedlings as controls. At the end of the experiment, RNA was also extracted from the ectomycorrhizae of three randomly selected asymptomatic pines inoculated with each fungal genotype as well as from three randomly selected control seedlings.

All RNA extractions were carried out using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) following the manufacturer's instructions. RNA was cleaned of DNA by using Promega DNase (RQ1 RNase-Free DNase, Promega Corp., Madison, WI, USA), and then quantified by using a NanoDrop (Thermo Scientific, Hudson, NH, USA). The absence of genomic DNA was verified through a one-step retrotranscription PCR (One-Step RT-PCR, Qiagen) with primers for elongation factor 1-α (TboEF1af/TboEF1ar) (Table 2). Briefly, the same extracted RNA was used either as template for retro-transcription and for PCR amplification or for PCR amplification only (RT- reactions). The absence of a signal after PCR amplification without retrotranscription was regarded as an absence of DNA. Total RNA for each sample was used to synthesize the cDNA, according to the SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) procedure.

Primer selection and design for gene expression analyses

The genes analyzed in this study were selected based on their putative function during the symbiotic stage of the target fungus *T. borchii*. In particular, in addition to a secreted and surface-associated

phospholipase A_2 (Soragni *et al.* 2001; Miozzi *et al.*, 2005) and an ubiquitin-like protein (Zeppa *et al.*, 2006), genes involved in different metabolic pathway have been considered: nitrogen acquisition (Montanini *et al.*, 2003; Montanini *et al.*, 2006; Guescini *et al.*, 2007; Guescini *et al.*, 2009), mitochondrial binary division (Guidi *et al.*, 2003), glucose pathway (Polidori *et al.*, 2002; Polidori *et al.*, 2004), hexose uptake (Polidori *et al.*, 2007), signal mechanism (Polidori *et al.*, 2002), sulphur metabolism (Polidori *et al.*, 2002; Zeppa *et al.*, 2010) and protein biosynthesis (Polidori *et al.*, 2002). The specific putative function of these genes and the primers used for their amplification in RT-qPCR are reported in Table 2. Newly developed primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Before RT-qPCR, all primers were tested *in silico* on Primer-BLAST and in PCR reactions on genomic DNA extracted from *P. pinea* in order to verify the absence of cross amplification. The DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen Valencia, CA, USA) according to the manufacturer's instructions. In order to assess the validity of using genes of the target ECM fungus rather than genes of other mycorrhizal fungi, a cross-check was performed searching for homologs by blastn in the related congener species *T. melanosporum* Vittad. (Martin *et al*., 2010), subsequently verifying their putative differential expression based on the RNA-Seq data (available at Mycorweb site http://mycor.nancy.inra.fr/IMGC/TuberGenome/download.php).

Gene expression analyses

The RT-qPCRs were carried out with the Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR reaction was conducted on a total volume of 10 µl, containing 1 µl diluted cDNA (dilution 1:2), 5 µl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories), 3.4 µl of water and 0.3 µl of each primer (10 µM), using a 96 well plate. The following PCR program, which included the calculation of a melting curve, was used: 95°C for 30 s, 40 cycles of 95°C for 10 s, the optimal annealing temperature for 30 s, ramp from 65°C to 93°C with a temperature increment of 0.5°C and a read plate every 2 s. The temperatures of annealing (Ta) of primers are listed in Table 2. All the reactions were performed for two and three biological replicates for the symptomatic and the asymptomatic pines, respectively, and for three technical replicates. The baseline range and Ct values were automatically calculated using the Bio-Rad CFX Manager software. In order to compare the data from different PCR runs or cDNA samples, the Ct values of all the genes were normalized to the Ct value of the reference gene. The candidate gene expression was normalized to that of the reference gene by subtracting the Ct value of the reference gene from the Ct value of the candidate gene without efficiency correction, with the equation $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001), where ΔΔCt represents the ΔCt of inoculated seedlings-ΔCt of control seedlings.

Data interpretation and statistical analyses

For each seedling, the mycorrhizal density (d) was calculated as the log-transformed ratio between the total number of ectomycorrhizae (m) and the weight (in g) of the lyophilized root system. Mann-Whitney tests with exact P-value were performed to compare the averages of m and d between the 60 seedlings inoculated with either *H. irregulare* or *H. annosum* and the 10 randomly selected control seedlings (Crawley, 2013; Carsey & Harden, 2014). Similarly, Kruskal-Wallis and Mann-Whitney tests with the Benjamini-Hochberg P-value correction were performed to contrast the averages of m and d among the 10 control seedlings, the 30 seedlings inoculated with *H. irregulare* and the 30 ones inoculated with *H. annosum* (Benjamini & Hochberg, 1995; Crawley, 2013; Carsey & Harden, 2014). For each of the above cited averages, the 95% bootstrap bias-corrected and accelerated confidence interval (95% CI_{BCa}) was calculated based on 10^4 iterations (DiCiccio & Efron, 1996).

Markov Chain Monte Carlo Generalized Linear Mixed Models (MCMC GLMMs, hereafter defined as M) (Hadfield, 2010; Kéry, 2010; Crawley, 2013; Giordano *et al.*, 2014) were fitted to model the response variable d of the inoculated seedlings according to the following equation:

$$\mathbf{d} = \mathbf{X}\mathbf{b} + \mathbf{e} \quad \textbf{(equation 1)}$$

with:

d: 60 × 1 column vector of the mycorrhizal density d observed in the inoculated seedlings;

X: 60 × k design matrix. The number of columns of the design matrix is $k = 1 + c + \sum_{i=1}^{f}(l_i - 1)_i$, where the first term (i.e. 1) accounts for the parametrization including the intercept α, c is the number of continuous predictors and f the number of factors (i.e. categorical predictors), with each ith factor characterized by l_i levels;

b: k × 1 column vector of the predictors coefficients;

e: 60 × 1 column vector of the error term.

The full set of predictors encompassed: I) the pathogenic fungal species inoculated (S, with l=2, 30 seedlings per level, *H. annosum* as reference level and associated coefficient β); II) the pathogenic fungal genotypes inoculated (G, with l=6, G_1, G_2 and G_3 for *H. irregulare* and G_4, G_5 and G_6 for *H. annosum*, 10 seedlings per level, G_6 as reference level and associated coefficients $\gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5$); III) the length of the stem portions of seedlings colonized by the pathogens (C, with associated coefficient δ). All combinations of S, G and C were tested, resulting in 8 models: M_{SGC} (with row

vector of predictors coefficients $\mathbf{b}^\top = [\alpha, \beta, \gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5, \delta]$), M_{SG} ($\mathbf{b}^\top = [\alpha, \beta, \gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5]$), M_{SC} ($\mathbf{b}^\top = [\alpha, \beta, \delta]$), M_{GC} ($\mathbf{b}^\top = [\alpha, \gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5, \delta]$), M_S ($\mathbf{b}^\top = [\alpha, \beta]$), M_G ($\mathbf{b}^\top = [\alpha, \gamma_1, \gamma_2, \gamma_3, \gamma_4, \gamma_5]$), M_C ($\mathbf{b}^\top = [\alpha, \delta]$) and the null model M_0 ($\mathbf{b}^\top = [\alpha]$). All predictors were treated as fixed and their coefficients were estimated along with their associated 95% credible intervals (95% $CI_{0.95}$) and P_{MCMC} values (Hadfield, 2010; Kéry, 2010; Crawley, 2013). The identity link and the Gaussian distribution were used to fit Ms to *d* (Kéry, 2010). Depending on the model, the number of iterations ranged from $3 \cdot 10^5$ to $5 \cdot 10^6$, with a burn-in ranging between $6 \cdot 10^4$ and $1 \cdot 10^6$ and a thinning interval set to 10 (Kéry, 2010). The convergence of the Markov Chains was assessed with the Heidelberger and Welch test for stationarity, while the absence of autocorrelation was verified by checking that the absolute values of the autocorrelation function were lower than 0.1 at lags 10, 50, 100, 500 (Heidelberger & Welch, 1981; Heidelberger & Welch, 1983; Kéry, 2010; Taylor *et al.*, 2012). The chain and burn-in length were increased until the convergence and the lack of autocorrelation above defined were achieved. For all models, the default uninformative priors proposed by Hadfield (2010) were used, with parameters μ and V set to 0 and 10^2, respectively, in agreement with the null hypothesis (i.e. regression coefficients not different from 0) (Kéry, 2010). For each model, the corrected Akaike Information Criterion (AICc) and the Deviance Information Criterion (DIC) were calculated along with their weights AICcw and DICw. Models selection was performed according to the minimum value of the information criteria and the maximum value of the associated weights (Wagenmakers & Farrell, 2004; Grueber *et al.*, 2011). The 95% credible and prediction intervals were calculated for the best model identified by the information criteria, if at least one significant coefficient other than the intercept was present (Hadfield, 2010; Kéry, 2010). The elements of the column vector **e** were tested for normality with the Shapiro-Wilk test (Crawley, 2013). The stem portion colonized by *H. irregulare* and *H. annosum*, a parameter which is often used as a proxy of the pathogen virulence (Garbelotto *et al.*, 2007; 2010), was compared between the two fungal species with a Mann-Whitney test.
All tests were run with a cut-off significance threshold set to 0.05 on R version 3.2.3, with the libraries boot, CODA, MCMCglmm and MuMIn (Plummer *et al.*, 2006; Hadfield, 2010; R Core Team, 2015; Bartoń, 2016; Canty & Ripley, 2016). Data, matrices and vectors associated with models and R algorithms are provided as Supporting Information (Notes S1, S2, S3 and S4).
Concerning gene expression, statistical analyses were carried out using Rest 2009, version 2.0.13, considering 0.05 as the *P*-value. Only significant expression values ($P < 0.05$) were considered. A custom R script (provided as Supporting Information Note S5) was used to visualize gene expression values as HeatMaps. This representation of the transcript levels coupled to a hierarchical clustering was performed in order to group genes with similar expression profiles.

Results

T. borchii mycorrhization rate and its association with *Heterobasidion* spp. colonization

Fifty-five days after inoculation, eight seedlings showed evident crown symptoms and a strong decline in vitality. Additional three seedlings became symptomatic 91 days after inoculation and one after 147 days . These 12 seedlings, four inoculated with two different genotypes of *H. irregulare* and eight inoculated with three different genotypes of *H. annosum*, were therefore scored as symptomatic pines. In all seedlings, successful pathogen inoculation was confirmed by the presence of *Heterobasidion* conidiophores. In such symptomatic seedlings, the mean length of stem colonized by the fungal genotypes ranged from 30.0 to 288.0 mm, with an average of 112.5 mm for *H. irregulare*, and from 6.0 to 348.0 mm, with an average of 145.5 mm for *H. annosum*.

At the end of the experiment, the presence of *Heterobasidion* spp. was confirmed for 41 out of 48 inoculated seedlings. No control seedlings displayed crown symptoms or *Heterobasidion* spp. conidiophores. In these asymptomatic seedlings, the mean length of stem colonized by the fungal genotypes ranged from 0 to 165.0 mm, with an average of 46.3 for *H. irregulare*, and from 0 to 201.0 mm, with an average of 67.6 mm for *H. annosum*. Neither *H. irregulare* nor *H. annosum* were observed at the root level of pine seedlings.

The average value of total number of ectomycorrhizae (*m*) was 43 (33-52 95% CI_{BCa}) in control seedlings and 77 (66-96 95% CI_{BCa}) in seedlings inoculated with either *H. irregulare* or *H. annosum*, while mycorrhizal density (*d*) was on average 3.77 (3.30-4.90 95% CI_{BCa}) in control seedlings and 4.54 (4.37-4.72 95% CI_{BCa}) in inoculated seedlings. The average values of both *m* and *d* were significantly different ($P < 0.05$) between inoculated and control seedlings (Fig. 1). No significant differences ($P > 0.05$) were detected between the averages of *m* in seedlings inoculated with *H. irregulare* (77, 62-114 95% CI_{BCa}) and *H. annosum* (78, 64-98 95% CI_{BCa}). Similarly, the averages of *d* did not display significant differences ($P > 0.05$) between seedlings inoculated with *H. irregulare* (4.61, 4.36-4.88-95% CI_{BCa}) and *H. annosum* (4.48, 4.24-4.70 95% CI_{BCa}) (Fig. S1).

In MCMC GLMMs modelling of the response variable *d* the only significant predictor detected was C in M_C and M_{SC}, indicating a positive linear correlation ($P > 0.05$) between C and *d* (Table 3). The corrected Akaike Information Criterion (AICc) ranged between 130.2 and 148.1 and the Deviance Information Criterion (DIC) was comprised between 126.7 and 136.2, in both cases with the minimum values shown by the model M_c (Fig. 2), whose weights were maximum, attaining 0.504 and 0.452 for AICc and DIC, respectively. For all MCMC GLMMs, the number of iterations, the burn-in and the thinning interval satisfied the Heidelberger and Welch test ($P > 0.05$) and provided values of the autocorrelation function lower than 0.1 at all lags. The Shapiro-Wilk test showed that

residuals were normally distributed in all models ($P > 0.05$). The stem portion colonized by *H. irregulare* and *H. annosum* genotypes attained on average 55.1 mm and 88.4 mm, respectively, yet no significant differences between the two fungal pathogens were detected ($P > 0.05$) (Fig. 3).

Gene expression analyses

Among symptomatic pines, RNA extraction did not properly work for ectomycorrhizae collected from seedlings inoculated with *H. irregulare* G_2 genotype or with *H. annosum* G_4 genotype; therefore, these samples were discarded. Fifteen *T. borchii* genes were considered (Table 2). In general, the number of genes significantly differentially expressed with respect to the controls was higher in the symptomatic pines (11 genes, Fig. 4) compared to the asymptomatic pines (4 genes, Fig. 5).

The ΔCt for each biological replicate was shown in Supporting Information Table S1 (symptomatic pines) and Supporting Information Table S2 (asymptomatic pines) coupled with mean and standard deviation. In Supporting Information Table S3 the fold change was shown for genes that were significantly ($P < 0.05$) and not significantly differentially expressed ($P > 0.05$) in all the analyzed seedlings.

In the symptomatic pines, genes were significantly differentially expressed compared to the controls especially in pines colonized by *H. annosum* (Fig. 4, Supporting Information Table S3), i.e. 10 differentially regulated genes (67% of the tested genes), rather than in pines colonized by *H. irregulare*, where only 4 genes (27% of the tested genes) were differentially regulated, i.e. *TbNre1*, *tbhos*, *tbsul1* and *TpTbCl28*. Two genes, i.e. *tbnir1* and *Tbeno-1*, were significantly down-regulated in symptomatic pines, irrespectively to the inoculated fungal genotype. Hierarchical clustering analysis of gene expression data allowed to identify three clusters (Fig. 4). The first cluster included genes not differentially expressed or down-regulated in pines colonized by both fungal pathogens, while the second and third mostly included up-regulated genes in seedlings inoculated with *H. annosum* only. Each cluster comprised genes deriving from different pathways. In particular, genes related to sulphur metabolism were not differentially expressed from the control with the exception of *TpTbCl5* that was up-regulated in seedlings inoculated with *H. annosum*, while the genes related to nitrogen and carbon metabolism were down-regulated with the exception of *TbNre1* and *TpTbCl28*, which were not differentially regulated in comparison to the control seedlings. The glutamine synthase coding gene (*TbGS*) was down-regulated in two out of three combinations. Conversely, *TbSP1*, *TpTbCl3* and *TpTbCl5* genes were mainly up-regulated. In the symptomatic pines the most up-regulated gene was *TpTbCl5* (fold 5.76) in pines inoculated with *H. annosum* G_5 genotype, while the most down-regulated was *tbnir1* (fold 0.003) in presence of the same genotype (Supporting Information Table S3).

In the asymptomatic pines, fold change values showed a general not significantly different gene expression compared to control seedlings, with some exceptions including up-regulated genes, i.e., *tbsul1*, *TbSP1*, *TbGS* and *TpTbCl5* (Fig. 5, Supporting Information Table S3). *Tbsul1* was up-regulated only in seedlings inoculated with *H. annosum* G_4, while *TbSP1* was up-regulated only in seedlings inoculated with *H. annosum* G_5. *TbGS* was up-regulated twice in presence of *H. irregulare* and once in presence of *H. annosum*, while *TpTbCl5* was up-regulated in seedlings inoculated with all fungal genotypes with the exception of *H. annosum* G_4. Also in the asymptomatic pines, genes related to sulphur metabolism (*tbhos, tbsul1*, *TpTbCl5*) were not differentially expressed from the control with the exception of *TpTb*Cl5. In the asymptomatic pines, the most up-regulated gene was *TpTbCl5* (fold 8.46) in pines inoculated with *H. annosum* G_5, while there were no down-regulated genes (Supporting Information Table S3).
All 15 genes selected in *T. borchii* owned a homolog in *T. melanosporum*, but of these, only three were up-regulated in *T. melanosporum* ectomycorrhizae (Supporting Information Table S4).

Discussion

In this work we described the effects of plant pathogenic fungi on both the ECM symbiosis development and the expression of genes putatively involved in the ECM symbiosis functioning, in a three-actors model system including a non-native invasive fungal pathogen or a native related one, an ECM symbiont and a common host plant species. Through a six-months inoculation experiment and by using both *in planta* as well as molecular analyses, we tested the hypothesis that a selected invasive fungal pathogen may have greater effects on ECM symbiosis than a closely related native one. In this study, such issue was addressed by working on the model system comprising *P. pinea*, its symbiont *T. borchii* and the non-native and native pathogens *H. irregulare* and *H. annosum*. Although simultaneous inoculations of pathogens and symbionts in the same hosts have been occasionally reported (Shukla *et al*., 2015), the largest body of literature dealing with the interaction between plant pathogens and symbionts on a common host, reports separate inoculations of pathogens and symbiont at different times, with the former inoculated after the latter (reviewed by de Souza *et al*., 2016). In our study, the delayed inoculation of the pathogen with respect to the symbiont is also supported by the natural dynamics of the ecosystem. It should be noted that in the *Heterobasidion* spp. – *P. pinea* pathosystem the mycorrhization process is unlikely to occur after pathogen infections as the disease generally affects adult trees (Garbelotto & Gonthier, 2013). The well-characterized pathosystem involving *H. irregulare* and *H. annosum* represents a unique opportunity to test specific ecological interactions among non-native or native fungal pathogens and native ecosystem components such as symbionts. Although allopatrically differentiated and clearly distinct species

(Sillo *et al.*, 2015a), *H. irregulare* and *H. annosum* appear to be similar in their biology, displaying a clear preference for the genus *Pinus* (Garbelotto & Gonthier, 2013). Despite the inoculation method used in this work may not completely mirror what occurs in nature where *Heterobasidion* spp. are generally associated with the root system of adult pines, stem inoculations of potted host seedlings in the laboratory or in the greenhouse are generally employed to study pathogenicity of *Heterobasidion* spp. (Garbelotto *et al.*, 2007; Garbelotto *et al.*, 2010; Pollastrini *et al.*, 2015) and may thus be deemed as good starting points to decipher the effects of these pathogens on both host plant and its symbiont. It is worth considering that both in this experiment and in nature, the effects of *Heterobasidion* spp. on ECM fungi should be regarded as indirect since they generally do not come into contact, the former being associated with stems or larger diameter roots and the latter with fine roots.

Only 12 seedlings out of 60 inoculated with the fungal pathogens showed crown symptoms and a strong decline in vitality during the experiment. The remaining inoculated seedlings, although the large majority of them was proved to be infected at the end of the experiment, did not show symptoms of disease six months after inoculations. These differences in disease expression may be due to the starting plant material that was not clonal but instead was a pool of different seeds (genotypes) resulting from sexual reproduction.

In order to gather an accurate quantification of mycorrhizae present in the seedlings, the total count of mycorrhized apexes was performed on the whole root system instead of using only a rough visual estimate or an assessment based on a subsample of the root system. The total number of ectomycorrhizae and their density were, on average, significantly and substantially higher in seedlings inoculated with either *H. irregulare* or *H. annosum* than in control seedlings. The results are unlikely to have been influenced by the sampling size and by the unbalanced design (i.e. 60 inoculated and 10 control seedlings), since the *P*-values were calculated through an exact approach based on permutation (Mehta & Patel, 2011; Lione & Gonthier, 2016). Interestingly, by working in the field and using a different experimental design, Gaitnieks *et al.* (2016) failed to detect any significant effect of *Heterobasidion* spp. on ECM fungal communities. In our study, the differences observed in the total number of ectomycorrhizae and in the mycorrhizal density between inoculated and control seedlings suggest that the occurrence of an infection can trigger the mycorrhization process. The average increase of the number of ectomycorrhizae and of the mycorrhizal density detected in inoculated seedlings is likely to be a response to the stress induced by the pathogen, at least during the earliest stage of the infection, which is the timeframe analysed in this study. However, we cannot exclude that the diseased plants might also provide a more favourable environment, thus enhancing the ECM colonization process as suggested by Fitter & Garbaye (1994). Despite no physiological and biochemical assays were performed, a higher amount of ectomycorrhizae might

have improved the trophic balance and the water availability for the inoculated seedlings, acting as compensation mechanism attempting to thwart the effects of the infection, i.e. stem colonization. It is worth noting that mycorrhizal symbiosis, in particular arbuscular mycorrhizal (AM) symbiosis, has been reported to exert a protection role towards the plants by compensating the damage induced by biotic stresses through an enhanced phosphorous supply, even though other biological mechanisms could be involved (Filion *et al.*, 1999; Pozo & Azcón-Aguilar, 2007).

Since the initial mycorrhization level of the seedlings was homogeneous, the number of ectomycorrhizae observed at the end of the experiment was deemed to be associated with the effects of the inoculation. Despite both the total number of ectomycorrhizae and the mycorrhizal density express the amount of ectomycorrhizae, the density seems to be more adequate for comparison purposes, since it is mathematically defined so as to include the lyophilized weight of the plant root system as normalization factor. Moreover, the log scaling of the density improves the numerical tractability of the data, compensating the eventual tail derived from the Poisson distribution of the numerator and reducing the effect of outliers, if present (Crawley, 2013).

The comparison between the number of ectomycorrhizae and their density between seedlings inoculated with the non-native and the native pathogens and the MCMC GLMMs showed that the mycorrhizal density in inoculated seedlings was not influenced either by the fungal pathogen species, or by the fungal genotype inoculated. This finding might be partially explained by considering that the non-native and the native fungal pathogens do not differ in their pathogenicity (Garbelotto *et al*., 2010; Gonthier *et al*., 2014). The lack of significant differences between the length of stem portions of seedlings colonized by *H. irregulare* and *H. annosum* furthermore confirms their comparable virulence on the common host. Moreover, the results presented in this study are consistent with those recently obtained with an *in vitro* dual culture experiment showing that both pathogenic species have equally affected the growth of the ECM symbiont *Suillus luteus* (L.) Roussel (Sillo *et al.*, 2015b). As mentioned above, under the assumption that the mycorrhization process is a response of the plant to the biotic stress associated with the infection, *H. irregulare* and *H. annosum* seem to induce the same macroscopic reaction in the plant-symbiont complex, i.e. the development of new mycorrhizae (see Fig. 1, Fig. S1). This response has been previously observed in the presence of helper bacteria that promote mycorrhizal development through an increase of root receptivity (Garbaye, 1994). However, since both pathogenic and symbiotic processes may be triggered by the plant's immune system (Gust *et al.,* 2012), it cannot be excluded that the pathogen could have modulated the plant's immunity, thus favouring ectomycorrhizal colonization. During symbiosis, i.e. AM interaction, modulation of plant immunity may lead to a primed state of the host plant allowing a functional activation of defence response mechanisms against pathogens (Jung *et al.,* 2012). To date, this is the first study analysing

quantitatively the total number of mycorrhizae associated with the whole root system of the seedlings in a model including a non-native invasive and a native pathogen, an ECM symbiont and the host plant.

The MCMC GLMMs showed a significant correlation between the mycorrhizal density and the length of the stem portion colonized by the pathogens. The weights of the information criteria confirmed that the model with the length of the stem portion colonized by the pathogens as the only predictor was the best one. This model outperformed the competing models at least from 1.3- to 2.5-fold according to DIC and AICc, respectively, following the evaluation criteria reported in Wagenmakers & Farrell (2004). The value of the significant δ coefficient associated with the stem portion colonized by the pathogens was always positive, indicating that the response of the plant, in terms of mycorrhizal density, increases linearly with the progression of the pathogen colonization along the stem. However, it is worth noting that linearity is associated with a logarithmic response variable, which explains the low absolute value of the δ coefficient and implies an exponential, rather than linear, raise of the number of mycorrhizae per gram of lyophilized root in response to the pathogen colonization. This finding supports the hypothesis that the plant compensates the stress induced by the pathogen colonization by increasing the mycorrhization rate.

The similarity in the response of the plant-symbiont complex to the inoculation of either *H. irregulare* or *H. annosum* was detected on the basis of observable macroscopical traits, such as the number of mycorrhizae and their density. However, the gene expression analysis, focusing mainly on genes potentially involved during the symbiotic stage, showed that the number of genes regulated in the ECM fungus *T. borchii* was more than twice in seedlings inoculated with the native fungal pathogen compared to those inoculated with the non-native one.

In the symptomatic pines, genes encoding a ribosomal protein (*TpTbCl3*) and a sulphonate/ α-ketoglutarate synthase (*TpTbCl5*), previously reported as most expressed in *T. borchii* ECMs (Polidori et al. 2002), were up-regulated, while a nucleoside diphosphate kinase gene (*TpTbCl16*) and *Tbsmt3* were down-regulated only when seedlings were inoculated with *H. annosum*. In particular, *Tbsmt3* codes for an ubiquitin-like protein and it was reported as expressed in the different stages of *T. borchii* life cycle (Zeppa et al., 2006). This protein can play a regulatory role in several mechanisms related to signal transduction, cell cycle progression, differentiation and cell response (Laney & Hochstrasser, 1999). Ubiquitin proteins bind with a covalent attachment and favor the degradation of transcription factors, cell growth modulators, signal transducers, cell cycle proteins, and damaged, mis-folded, or mis-assembled proteins (Laney & Hochstrasser, 1999). The different expression of this fungal gene in the seedlings inoculated with the native pathogen, compared to the control, suggests that the proteolytic system involving ubiquitin was altered. By contrast, when seedlings were

inoculated with the non-native pathogen, this gene showed the same expression profile of the wounded control plants. Although intriguing, the hypothesis that a native pathogen was perceived by a native symbiont, while the infection of a non-native pathogen was perceived as a wound needs further studies as our experiment was not designed to specifically compare the effects of mechanical injuries and fungal infections. Despite the fact that in this study a global transcriptomic analysis was not performed, it is worth noting that the cross-check carried out between *T. borchii* and *T. melanosporum* pointed out the validity of using well-characterized genes of the target species, as we did, instead of searching for homologs in other mycorrhizal fungi.

In symptomatic pines, the *T. borchii* down-regulated genes were *Tbeno-1* and *Tbnir1* with all tested pathogen genotypes. *Tbeno-1* codes for an enolase, which allows to convert the 2-phosphoglycerate to phosphoenolpyruvate in the glycolysis pathway (Polidori *et al.*, 2004). The gene was found in the free living mycelium as constitutively expressed under several environmental conditions, like carbon starvation or when pyruvate was used as carbon source (Polidori *et al.*, 2004), and it was found up-regulated during the symbiotic phase (Polidori *et al.*, 2002). The down-regulation of this fungal putative symbiosis related gene in symptomatic pines could suggest that the symbiosis may be affected by pathogenic processes. *Tbnir1* codes for a nitrite reductase, which plays a crucial role to improve the host plant capacity in using nitrite in its nitrogen nutrition (Guescini *et al.*, 2007). It is worth noting that the homolog of this gene in *T. melanosporum* (*Tmelnir1* GSTUMT00010229001) showed an up-regulation in symbiotic stage (Supporting Table S4) (Martin *et al.,* 2010). Up-regulation of this gene was found to be associated with the availability of nitrate and the absence of other nitrogen sources, while its down-regulation was found to be related to a carbon starvation condition (Guescini *et al.*, 2007). The reduced photosynthetic activity in symptomatic host plants and the resulting photoassimilate (carbon compound) limitation may explain the down-regulation of this gene.

In the asymptomatic pines most of the *T. borchii* genes were not differentially expressed compared to the control with the exception of four up-regulated genes, i.e. *TbSP1*, *TpTbCl5*, *TbGS,* and *tbsul1*, coding for a phospholipase A_2 (Soragni *et al.*, 2001; Miozzi *et al.*, 2005), a putative sulfonate/α-ketoglutarate dioxygenase (Polidori *et al.*, 2002), a glutamine synthase (Montanini *et al.*, 2003) and a putative sulphate transporter (Zeppa *et al.*, 2010). It should be noted that the homolog of *TbSP1* in *T. melanosporum* (*TmelPLA2* GSTUMT00003580001) was one of the most up-regulated genes in the ectomycorrhizae (Supporting Table S4) (Martin *et al.*, 2010). Also for these genes, the up-regulation only occurred when seedlings were inoculated with a specific pathogen genotype.

The overall down-regulation of genes related to signaling, carbon and nitrogen assimilation could suggest a lack of communication with the plant that was dying (symptomatic pines) compared to the

ectomycorrhizae collected from asymptomatic pines where these genes were not differentially regulated. The reduction of expression levels of these fungal genes could be due to the activation of stress programs aimed at saving energy and substrates as suggested by Guescini *et al.* (2007). It is worth noting that while the observed increase of mycorrhizal density is probably associated with the mycorrhizal colonization process, the above supposed lack of communication between host and symbiont is likely to have occurred only once the mycorrhizae had developed.

The understanding of pathogen-symbiont-host plant interaction has become an area of recent research focus (Anderson *et al.,* 2010). Until now researches have been mainly focused on the effects of AM symbiosis on the stimulation of plant defence responses against pathogens (Filion *et al.*, 1999; Pozo & Azcón-Aguilar, 2007) and only a few studies have considered the effects of pathogens on the symbiosis establishment (de Souza *et al.*, 2016). To our knowledge, this is the first work focused on evaluating the effects of a non-native invasive and a native pathogen on the ECM symbiosis in a three-actors model system including the host plant. Although *H. irregulare* and *H. annosum* seem to induce the same macroscopic reaction in the plant-symbiont complex, i.e. the development of new mycorrhizae, whose density is correlated to the virulence of pathogen genotypes (Fig. 2), target gene expression analysis showed that the number of genes regulated in *T. borchii* ectomycorrhizae was more than twice in seedlings inoculated with the native fungal pathogen compared to those inoculated with the non-native invasive one.

Although our gene expression analysis was focused only on some target genes, making it difficult to fully understand the consequences on a native ECM fungus triggered by a non-native invasive and a native fungal pathogen, results suggest that a recognition mechanism through a host plant-mediated signal transduction may be involved between the native symbiont and the native pathogen, but not between the native symbiont and the non-native pathogen. We recognize that the results presented in this study do not allow to fully determine the yet poorly known impact that non-native invasive fungal pathogens have on ECM symbiosis, however, outcomes open new questions that could be successfully investigated through further phenotypic analyses coupled with -omics approaches. Such further analysis would also allow to investigate the effector-mediated signaling among the three actors of the system. Interestingly, a cerato-platanin-like protein of *H. annosum* has been recently characterized as an effector inducing defence responses in *P. sylvestris* L. (Chen *et al.,* 2015). In addition, it is known that colonization of roots by ECM fungi may involve the activation of genes coding for effectors that can overcome plant defences and that may resemble those of pathogenic fungi (Plett *et al.,* 2011). In fact, the early plant response to mycorrhizal fungi involves a broad-spectrum of defence related proteins, such as chitinases and peroxidases, but this response is generally only transient and it is attenuated in mature ectomycorrhizae (Albrecht *et al.,* 1994; Tarkka *et al.,*

2013). On the other hand, a non-transient response of the plant can be observed as a reaction to infection by pathogens. For instance, preliminary analyses in *P. pinea* inoculated with *H. irregulare* and *H. annosum* suggest that pine gene expression can be compromised by both pathogens (Pepori *et al.,* 2015). Interestingly, the infection by the invasive and native *Heterobasidion* species seemed to affect similarly both photosynthesis and stomatal conductance (Pepori *et al.,* 2015). A global survey of effectors as well as of plant pathogenesis related proteins in a tripartite system will allow to elucidate the dialogue among pathogen, symbiont and host plant. Finally, this work may hopefully promote research focused not only on invasive plant pathogens, but also on invasive plants and even on invasive symbionts, whose presence was recently documented (Murat *et al*., 2008; Bonito *et al*., 2011).

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Author Contribution

E.Z. designed and conducted the gene expression analyses. L.G. designed and conducted the inoculation experiment. A.V. and F.S. performed the mycorrhization rate assessment. G.L. performed statistical analyses. E.Z. and F.S. wrote the first draft of the paper. L.G. and G.L. contributed to manuscript writing. R.B. contributed to the interpretation of the molecular experiments and critically revised the manuscript. P.G. designed and supervised the work, and critically revised the manuscript.

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Figure legends

Fig. 1 Barplots comparing (a) the average number of the total mycorrhizae m and (b) their average density d (expressed as the log-transformed ratio between m and root weight) between inoculated and control seedlings. Different letters indicate a significant Mann-Whitney exact P-value ($P < 0.05$). Error bars represent the lower and upper limits of the 95% bootstrap bias-corrected and accelerated confidence interval of the average.

Fig. 2 Graph of the model M_c relating the mycorrhizal density (d) expressed as the log-transformed ratio between m and root weight to the length of the stem portion colonized by the pathogens (C, in mm) in inoculated seedlings. The continuous black line represents the equation derived from the fit of the model ($d = 0.002 \cdot C + 4.366$), the dark grey area included between the dashed lines delimits the 95% credible interval, the light grey region comprised between the dotted lines identifies the 95% prediction interval and the dots of the scatterplot indicate the location of the observed values of C and d used for the model fitting.

Fig. 3 Boxplots of the stem portion of pine seedlings colonized (C, mm) by *H. irregulare* (light grey) and *H. annosum* (dark grey) at species (a) and genotype (b) levels. Rectangles show C values included between the 25th and 75th percentiles, the vertical black lines represent the median values, the T-shaped lines mark the minimum and maximum values, the circles are outliers. The same letter (a) indicate a not significant Mann-Whitney exact P-value ($P > 0.05$).

Fig. 4 HeatMap representation of the transcript levels coupled to a hierarchical clustering in symptomatic pines. Each column represents a fungal genotype, while each row represents a gene. Expression levels are coloured green for low intensities and red for high intensities (see scale at the top-left corner). The black cells represent genes not significantly different from the control. For inoculated *Heterobasidion* genotype codes see Table 1.

Fig. 5 HeatMap representation of the transcript levels coupled to a hierarchical clustering in asymptomatic pines. Each column represents a fungal genotype, while each row represents a gene. Expression levels are coloured green for low intensities and red for high intensities (see scale at the top-left corner). The black cells represent genes not significantly different from the control. For inoculated *Heterobasidion* genotype codes see Table 1.

Fig. 1

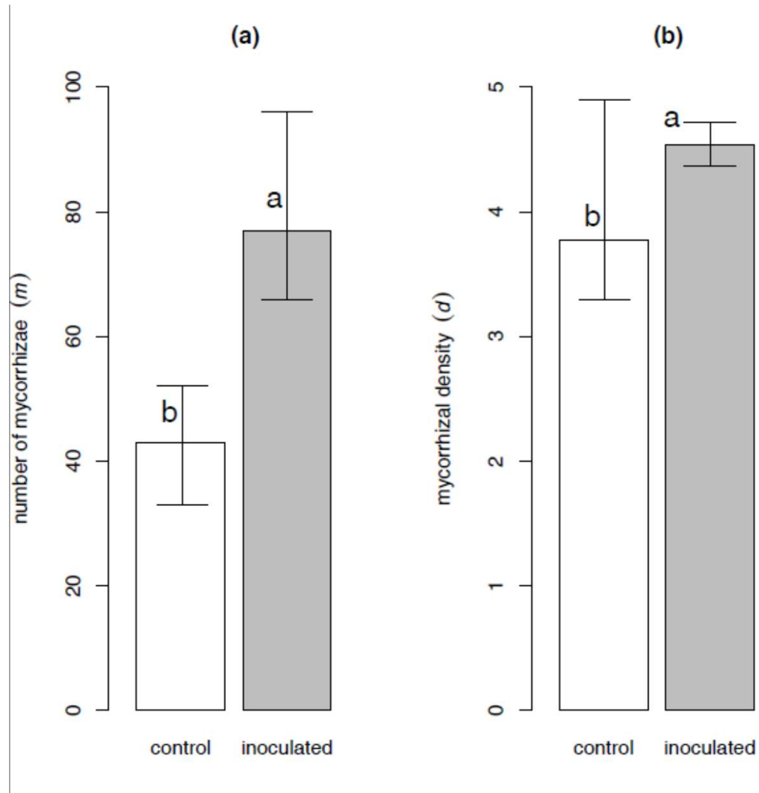


Fig. 2

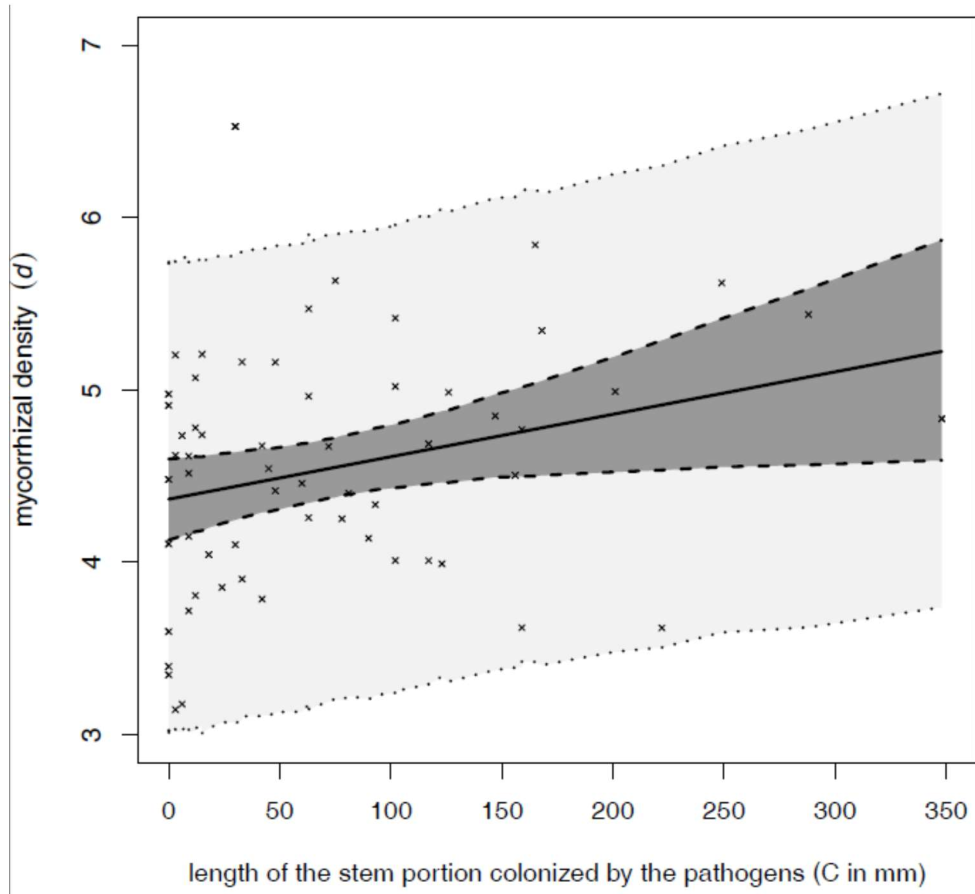


Fig. 3

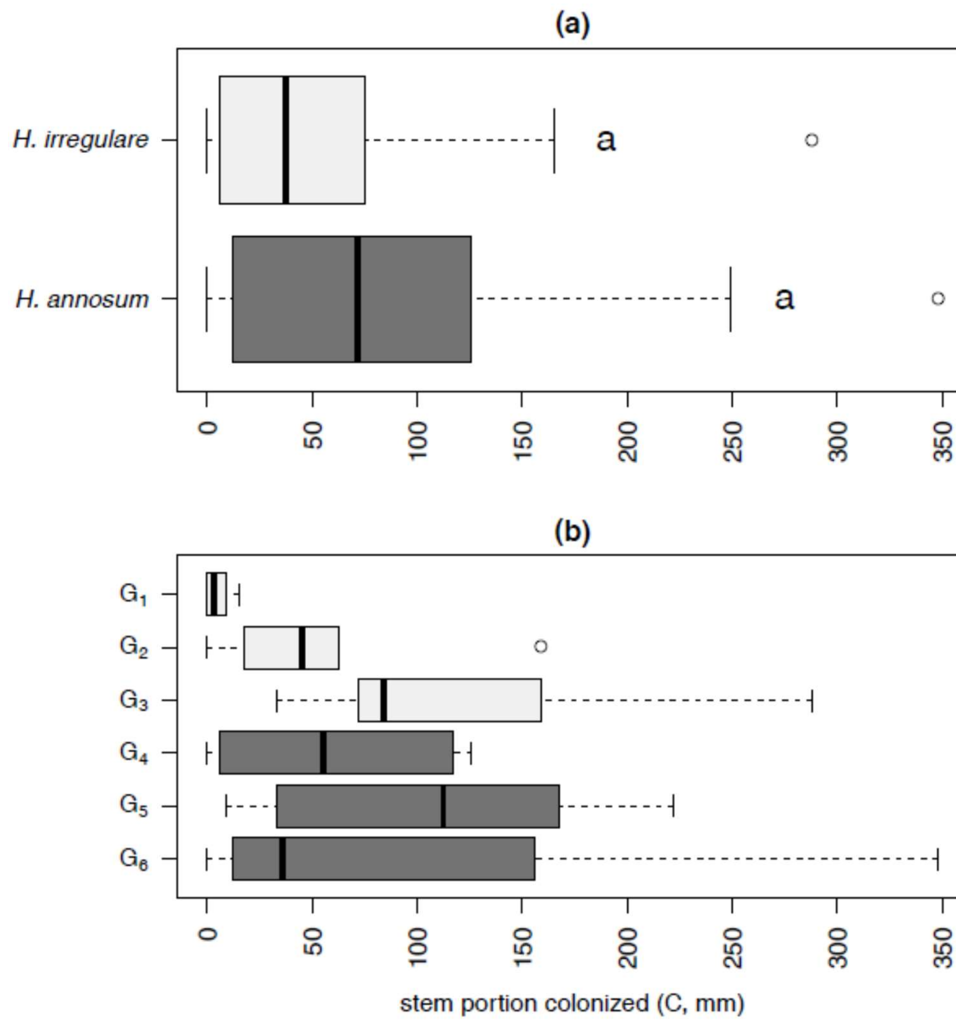


Fig. 4

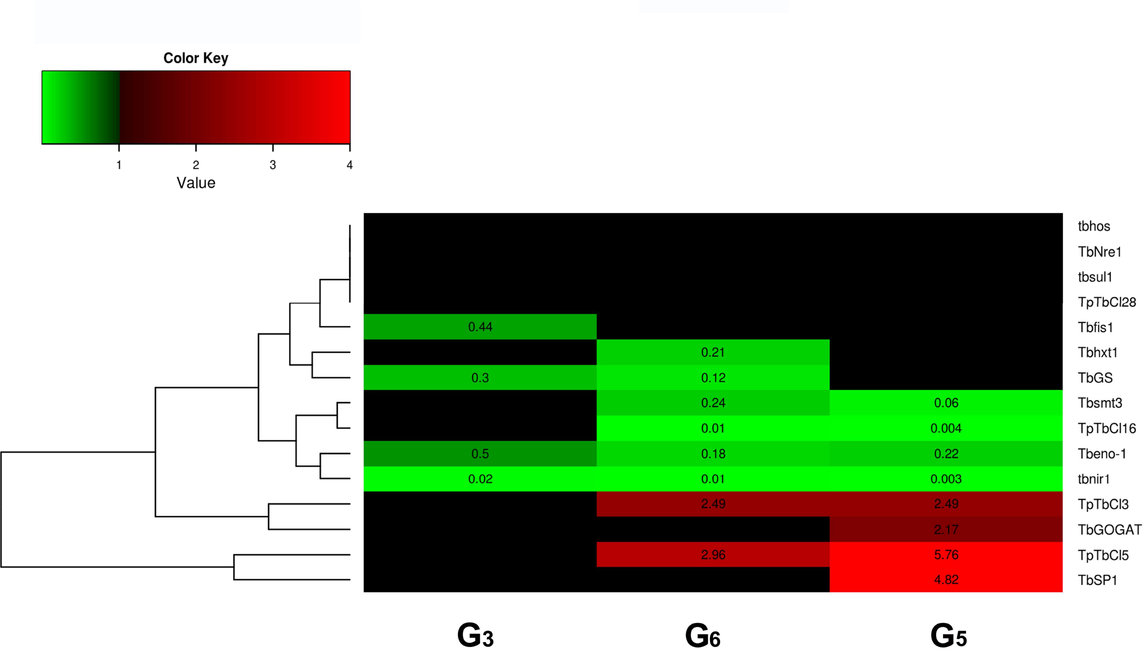
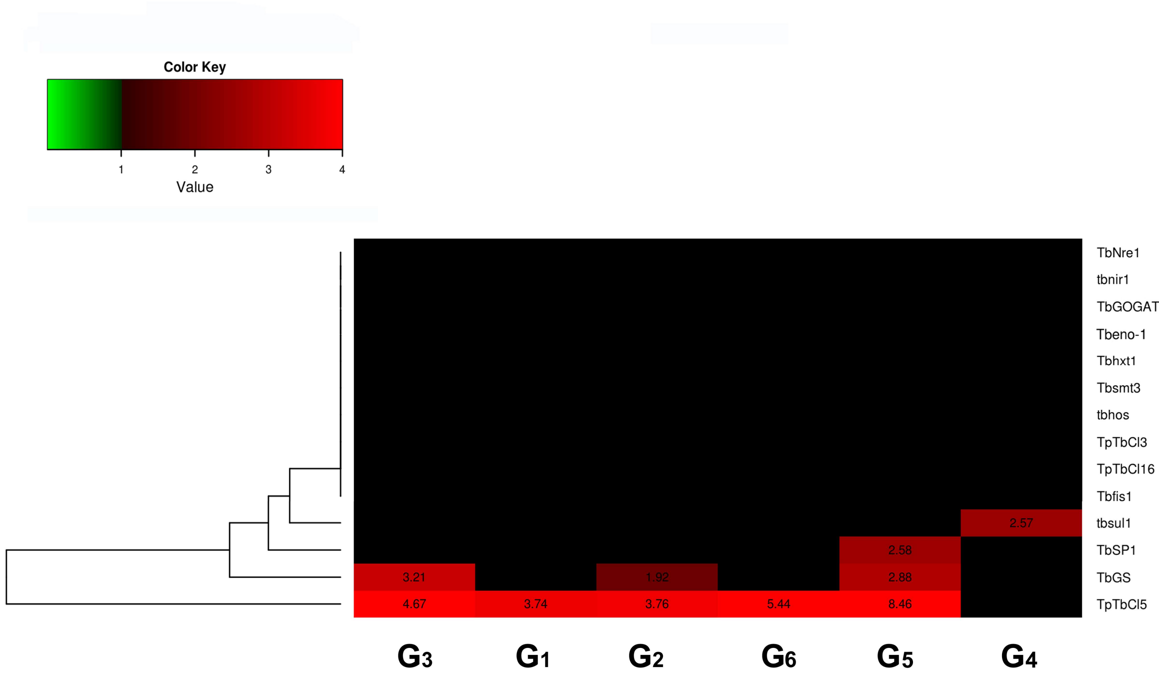


Fig. 5



Tables

Table 1 Genotypes of *Heterobasidion* spp. used in the present study.

Genotype code	**Isolation date**	**Geographic origin**	**Species**	**MUT[1] accession N.**
G_1	2005	Castelfusano, RM, Italy	*H. irregulare*	MUT00001193
G_2	2005	Sabaudia, LT, Italy	*H. irregulare*	MUT00001151
G_3	2002	Castelporziano, RM, Italy	*H. irregulare*	MUT00003560
G_4	2007	Sabaudia, LT, Italy	*H. annosum*	MUT00001143
G_5	2005	Sabaudia, LT, Italy	*H. annosum*	MUT00001204
G_6	2013	Artificial heterokaryotic genotype	*H. annosum*	MUT00005731

[1]*MUT Mycotheca Universitatis Taurinensis*, University of Torino

1 **Table 2** List of primers used in this study and their temperature of annealing (Ta).
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Primer name	**Primer sequence**	**Gene name**	**Putative function (and reference)**	**Ta**	**Reference**
HOS1for	CGATGATATCAGCACCGTGTTC	*tbhos*	Homocysteine synthase (Zeppa *et al.*, 2010)	60°C	Zeppa *et al.* (2010)
HOS1rev	CTAGTCGTTGATAACACCTTCG				
TbLP4	CCGCTTCACAGAGGCTAATC	*TbSP1*	Phospholipase A_2 (Miozzi *et al.*, 2005)	60°C	Miozzi *et al.* (2005)
TbRP4	AAGTGTTCGCGATTTTACGG				
TboEF1af	CAAGGCTGGAAAGTCCTCTG	*TboEF1-α*	Elongation factor 1-α (Bonito *et al.*, 2013)	60°C	This work
TboEF1ar	CCGTTCCAATACCACCAATC				
Tbfis1pf	TTTGTCTGCCCTGGGATAAG	*Tbfis1*	Putative mitochondrial fission protein (Guidi *et al.*, 2003)	60°C	This work
Tbfis1pr	CGAACTGTCCCTCTTTCTCG				
TbGSf	AGGGAGGCATGAAGGCTATT	*TbGS*	Glutamine synthetase (Montanini *et al.*, 2003)	60°C	This work
TbGSr	AGACGGAGGGTGTTGTCATC				
TbGlutamatesyntf	TGTGCCCATCTCGAACATTA	*TbGOGAT*	Glutamate synthase (Montanini *et al.*, 2003)	60°C	This work
TbGlutamatesyntr	CCAGAACACAAGCACCTTCA				
Tb60Sf	ACAAAGAAGGTTGGCGTGAC	*TpTbCl3*	Ribosomal protein (60S subunit) (Polidori *et al.*, 2002)	60°C	This work
Tb60Sr	CAGGTGTATCTGGCGTGTTG				
Tbaketoglutaratesulff	ACTGGTGGAGACACCCTTTG	*TpTbCl5*	Sulfonate/ α-ketoglutarate dioxygenase (Polidori *et al.*, 2002)	60°C	This work
Tbaketoglutaratesulfr	CAAGCCCTCAAGAGTTTTGC				
Tbsorbitodehydrf	TAGTCCTCGGGCATGAGTCT	*TpTbCl28*	Sorbitol dehydrogenase (Polidori *et al.*, 2002)	60°C	This work
Tbsorbitodehydrr	TCCCCAACACTCCATTTCTC				
Tbenolasef	CGATAGCAGCAAGTGGTTGA	*Tbeno-1*	Enolase (Polidori *et al.*, 2004)	60°C	This work
Tbenolaser	TCAATGCTGACAATGGGGTA				
Tbsmt3f	CATCGGAAAGTTCAGCCAAT	*Tbsmt3*	Ubiquitin-like protein (Zeppa *et al.*, 2006)	60°C	This work
Tbsmt3r	CAAACTGCAAAAGTCGTCCA				
TbST2newf	TATGTGTTCCGCTGGAATGA	*tbsul1*	Putative sulphate transporter (Zeppa *et al.*, 2010	60°C	This work
TbST2newr	CGTTGACAGGGTTTCCCTAA				
TBNRE1Fnew	ATTTTCTTTCGGCTGGGTTT	*TbNre1*	Nitrogen regulator element (Guescini *et al.*, 2009)	60°C	This work
TBNRE1rnew	ACCTCCACGAAAGACCACAC				

TBNR1f new	GGGATGTATTCGGGAATGTG	*tbnir1*	Nitrite reductase (Guescini *et al.,* 2007)	60°C	This work
TBNR1r new	GTTTCCGCCAACAAAGATGT				
TbhxtF new	GCTCCAATTTCCCTCTTTCC	*Tbhxt1*	Hexose transporter (Polidori *et al.,* 2007)	60°C	This work
TbhxtrR new	AACATACCCAGTGCGGAGAC				
TbNDKf	GCGAACCTCATCCTCACATT	*TpTbCl16*	Nucleoside diphosphate kinase (Polidori *et al.,* 2002)	62°C	This work
TbNDKr	AGATGATGGGACCAACAAGC				

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22 **Table 3** Markov Chain Monte Carlo Generalized Linear Mixed Models (MCMC GLMMs) modelling the mycorrhizal density (*d*). For each model M
23 the predictors coefficients with their associated 95% credible intervals (95% $CI_{0.95}$) and P_{MCMC} values are reported along with the information criteria
24 and weights. Significant coefficients ($P < 0.05$) are marked with the symbol *, while coefficients of predictors not included in the models are indicated
25 with the symbol -. See the text for predictors, vectors and coefficients acronyms as well as for the sample sizes of inoculated seedlings.
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	Coefficients	Model M_{SGC}	M_{SG}	M_{SC}	M_{GC}	M_S	M_G	M_C	M_0
Intercept	α	4.135* (3.635; 4.641) $P_{MCMC} < 10^{-4}$	4.377* (3.934; 4.824) $P_{MCMC} < 10^{-4}$	4.236* (3.920; 4.554) $P_{MCMC} < 10^{-4}$	4.135* (3.643; 4.646) $P_{MCMC} < 10^{-4}$	4.481* (4.222; 4.735) $P_{MCMC} < 10^{-4}$	4.377* (3.930; 4.820) $P_{MCMC} < 10^{-4}$	4.366* (4.134; 4.064) $P_{MCMC} < 10^{-4}$	4.544* (4.368; 4.726) $P_{MCMC} < 10^{-4}$
Species (S)	β	0.024 (-9.568; 10.006) $P_{MCMC} = 0.961$	0.168 (-9.585; 10.087) $P_{MCMC} = 0.973$	0.217 (-0.136;0.577) $P_{MCMC} = 0.224$	-	0.126 (-0.235; 0.485) $P_{MCMC} = 0.487$	-	-	-
Genotype (G)	γ_1	0.005 (-9.866; 9.771) $P_{MCMC} = 0.993$	-0.200 (-10.017; 9.647) $P_{MCMC} = 0.968$	-	0.198 (-0.448; 0.867) $P_{MCMC} = 0.548$	-	-0.032 (-0.676; 0.591) $P_{MCMC} = 0.921$	-	-
	γ_2	0.012 (-9.739; 9.891) $P_{MCMC} = 0.979$	0.083 (-9.668; 9.999) $P_{MCMC} = 0.987$	-	0.366 (-0.269; 0.983) $P_{MCMC} = 0.248$	-	0.251 (-0.380; 0.881) $P_{MCMC} = 0.428$	-	-
	γ_3	0.018 (-9.629; 10.000) $P_{MCMC} = 0.969$	0.302 (-9.473; 10.202) $P_{MCMC} = 0.952$	-	0.428 (-0.186; 1.043) $P_{MCMC} = 0.168$	-	0.469 (-0.154; 1.106) $P_{MCMC} = 0.140$	-	-
	γ_4	0.024 (-0.038; 0.087) $P_{MCMC} = 0.436$	0.151 (-0.482;0.779) $P_{MCMC} = 0.633$	-	0.243 (-0.376; 0.872) $P_{MCMC} = 0.434$	-	0.150 (-0.471; 0.788) $P_{MCMC} = 0.636$	-	-
	γ_5	0.019 (-0.050; 0.074) $P_{MCMC} = 0.704$	0.161 (-0.470; 0.788) $P_{MCMC} = 0.612$	-	0.118 (-0.502; 0.728) $P_{MCMC} = 0.702$	-	0.158 (-0.480;0.782) $P_{MCMC} = 0.617$	-	-
Stem	δ	0.003	-	0.003*	0.003	-	-	0.002*	-

colonization (C) (mm)		(-8.731·10^{-5}; 0.005) P_{MCMC} = 0.058		(5.024·10^{-5}; 0.005) P_{MCMC} = 0.019	(-1.013 ·10^{-4}; 0.005) P_{MCMC} = 0.56			(2.732 ·10^{-4}; 0.005) P_{MCMC} = 0.031	
AICc		148.0	148.1	132.0	145.2	134.5	145.4	130.2	131.7
AICcw		0.000	0.000	0.204	0.000	0.059	0.000	0.504	0.233
DIC		134.2	136.2	127.2	134.2	131.0	136.1	126.7	129.5
DICw		0.011	0.004	0.353	0.011	0.053	0.004	0.452	0.112

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34 **Supporting Information**

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36 **Fig. S1** Barplots comparing (a) the average number of the total mycorrhizae *m* and (b) their average
37 density *d* (expressed as the log-transformed ratio between *m* and root weight) among control
38 seedlings, seedlings inoculated with *Heterobasidion irregulare* and with *H. annosum*.

39 **Table S1** Relative expression of 15 different genes in the nine used biological replicates
40 (symptomatic pines).

41 **Table S2** Relative expression of 15 different genes in the 21 used biological replicates (asymptomatic
42 pines).

43 **Table S3** Expression values in RT-qPCR of *Tuber borchii* genes.

44 **Table S4** Expression data of the *Tuber melanosporum* homologs in the symbiotic stage based on
45 RNA-Seq data.

46 **Notes S1** Database (data1) used to perform the Mann-Whitney tests comparing the averages of
47 number of ectomycorrhizae (*m*) and mycorrhizal density (*d*) between seedlings inoculated with either
48 *Heterobasidion irregulare* or *H. annosum* and the control seedlings.

49 **Notes S2** Database (data2) used to fit the Markov Chain Monte Carlo Generalized Linear Mixed
50 Models (MCMC GLMMs) modeling mycorrhizal density (*d*).

51 **Notes S3** R script performing the statistical analyses on databases data1 and data2.

52 **Notes S4** Design matrices **X**, column vectors of the predictors coefficients **b** and of the error term **e**
53 of Markov Chain Monte Carlo Generalized Linear Mixed Models (MCMC GLMMs) modeling
54 mycorrhizal density (*d*).

55 **Notes S5** R script used to visualize gene expression data (Table S3) as HeatMaps.

56