Comparative transcriptional and metabolic responses of Pinus pinea to a native and a non-native Heterobasidion species

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Comparative transcriptional and metabolic responses of *Pinus pinea* to a native and a non-native *Heterobasidion* species

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Running Title: Responses of *P. pinea* to *Heterobasidion* species.

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Abstract

Heterobasidion irregulare is a causal agent of root and butt-rot disease in conifers, and is native to North America. In 1944 it was introduced in central Italy in a *Pinus pinea* stand, where it shares the same niche with the native species *H. annosum*. The introduction of a non-native pathogen may have significant negative effects on a naïve host tree and the ecosystem in which it resides, requiring a better understanding of the system. We compared the spatio-temporal phenotypic, transcriptional and metabolic host responses to inoculation with the two *Heterobasidion* species in a large experiment with *P. pinea* seedlings. Differences in length of lesions at the inoculation site (IS), expression of host genes involved in lignin pathway and in cell rescue and defence, and analysis of terpenes at both IS and 12 cm above the IS (distal site, DS), were assessed at 3, 14 and 35 days post inoculation (dpi). Results clearly showed that both species elicit similar physiological and biochemical responses in *P. pinea* seedlings. The analysis of host transcripts and total terpenes showed differences between inoculation sites and between pathogen and mock inoculated plants. Both pathogen and mock inoculations induced antimicrobial peptide and phenylalanine ammonia-lyase overexpression at IS beginning at 3 dpi; while at DS all the analyzed genes, except for peroxidase, were overexpressed at 14 dpi. A significantly higher accumulation of terpenoids was observed at 14 dpi at IS, and at 35 dpi at DS. The terpene blend at IS showed significant variation among treatments and sampling times, while no significant differences were ever observed in DS tissues. Based on our results, *H. irregulare* does not seem to have competitive advantages over the native species *H. annosum* in terms of pathogenicity towards *P. pinea* trees; this may explain why the non-native species has not widely spread over the 73 years since its putative year of introduction into central Italy.

Introduction

Over the past century, the introduction and establishment of non-native pathogens, aided by the increasing trade of plants for planting, has turned into a major global threat to forest ecosystem stability around the globe (Garrett et al. 2006, Hulme 2009, Jump et al. 2009, Santini et al. 2013, Ghelardini et al. 2016). Climate change probably contributes to such invasions as a priming factor, by stressing the native hosts, which are then unable to respond effectively to the new threat (Santini and Ghelardini 2015).

Due to co-evolutionary relationships with their hosts, many non-native pathogens are harmless in their original range and often unknown to science, but when they reach new environments they can establish and spread on novel (naïve) hosts with which they have a lack of coevolution (Parker and Gilbert 2004).

Once established, alien pathogen species can spread rapidly and threaten or affect the native hosts and the organisms depending on them, including native pathogens. For example, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, the non-native causal agent of ash dieback in Europe, is replacing the native European species *H. albidus* (Gillet) W. Phillips (McKinney et al. 2012, Baral and Bemman 2014). Thus, alien species can have very strong, negative evolutionary, ecological, and oftentimes economic effects on the invaded ecosystems (Mooney and Cleland 2001, Didham et al. 2007, Loo 2009). Indeed, usually the non-native species are considered competitively superior to the autochthonous species, with the strongest competition expected in species that showed similar niches (Čuda et al. 2015). However, in some cases such competition is not very clear. For example, *Heterobasidion irregulare* Garbel. & Otrosina, a non-native basidiomycete introduced into Italy from North America (Gonthier et al. 2004) in the last century, shares the same niche with the congeneric native species *H. annosum* (Fr.) Bref., a common agent of root and butt rot of pines as well as broadleaved species in Europe (Woodward et al. 1998, Asiegbu et al. 2005), but does not outcompete it and is not more destructive on its host.

Both the native and the non-native Heterobasidion species coexist in the same *Pinus pinea* L. (Italian stone pine) plantations in central Italy (Latium region) (Gonthier et al. 2007, Gonthier et al. 2012, Gonthier et al. 2014) but display different lifestyles: e.g. *H. irregulare* sporulates significantly more profusely and shows higher saprobic activity than *H. annosum* (Giordano et al. 2014). Sillo et al. (2015a) found that genes involved in sporulation are more variable between the two species, compared with genes involved in pathogenesis. It is likely that the speciation between *H. annosum* and *H. irregulare* has influenced the transcriptional activity of conserved genes, but this hypothesis has not been validated (Sillo et al. 2015a).

Oftentimes, the lack of coevolution between a host and an invasive pathogen is expressed in ineffective host defence systems (Stenlid and Oliva 2016). In conifers, multiple and overlapping mechanical and chemical constitutive defences may constrain initial insect invasions and fungal colonization (Franceschi et al. 2005, Keeling and Bohlman 2006). Inducible resistance enhances the host defence capacity to subsequent attacks both locally and on distal parts of a tree, reducing the damage caused by a pest (Franceschi et al. 2005, Bonello et al. 2006, Fossdal et al. 2007, Wallis et al. 2008, Eyles et al. 2010).

Many different compounds are involved in conifer defence, including terpenoids and phenylpropanoids, with a number of them acting as a chemical defence and a physical barrier against herbivorous and microbial attacks (Eyles et al. 2010). Conifer oleoresin is a defensive secretion composed of different classes of terpenoid and phenolic compounds (Gershenzon and Croteau 1993, Michelozzi 1999). Complex oleoresin mixtures can magnify terpene function since the defensive role of a single compound can be further enhanced by interaction with other substances (Gershenzon and Dudareva 2007). Terpenoid oleoresins can act as both inducible and constitutive defenses (Langenheim 1994). A great defensive potential is provided by variations in constitutive and induced terpenoid mixtures; for example, certain combinations of constitutive monoterpenes in slash pine and loblolly pine clones were indicative of fusiform rust resistance (Michelozzi et al. 1990, Michelozzi et al. 1995), while the concentrations of several mono- and diterpenes increase markedly in Italian cypress infected by *Seiridium cardinale* (Wagener) B. Sutton & I.A.S. Gibson and are presumably involved in resistance to the pathogen (Achotegui-Castells et al. 2015).

The molecular bases of plant-microbe interactions can be explained by the 'Zig-Zag model' of the plant immune system, where the perception of elicitors of microbial origin can lead to host resistance toward non-adapted or maladapted pathogens (incompatible plant-pathogen interaction), while adapted microorganisms are able to avoid or disable host recognition and response, and promote virulence (compatible plant-pathogen interaction) (Jones and Dangl 2006). Thus, the success of infection and the ability of the pathogen to bypass host defences implies active signalling involving the up- and down-regulation of differentially expressed genes (Birch and Whisson 2001). Knowledge of the host transcriptional and metabolic responses to pathogen infection is therefore crucial to better understand fungal-tree interactions.

Some of these genes are known to be involved in general defence mechanisms, e.g., xyloglucan endo-transglycosylases (XET) are implicated in cell wall modification (Bourquin et al. 2002), antimicrobial peptides (AMP) act as constitutive or inducible defence against microbial infections (Padovan et al. 2010, Liu et al. 2013), and chitinases (CHI) defend the host by degrading the chitin of fungal cell wall (Wan et al. 2008). Both AMP and CHI, provide durable resistance in plants because they are active against a wide range of pathogens, and contribute to the modulation of the plant defence signalling pathways (Wan et al. 2008, Bolouri Moghaddam et al. 2016). XET, CHI, and AMP are generally thought of as part of a host's response related to cell rescue and defence (Adomas et al. 2007, Likar and Regvar 2008, Yaqoob et al. 2012, Kolosova et al. 2014, Oliva et al. 2015).

H. annosum infections are known to induce candidate genes coding for enzymes involved in secondary metabolism that includes lignin, flavonoid and stilbene biosynthesis (Adomas et al. 2007). In the lignin biosynthesis pathway the entry point is phenylalanine ammonia-lyase (PAL), while cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POX) are part of the downstream pathways. The expression of PAL, CAD, POX, XET, CHI, and AMP has been quantified after *Heterobasidion* inoculation in Norway spruce (*Picea abies* (L.) H. Karst.) (Fossdal et al. 2012, Lundén et al. 2015, Oliva et al. 2015), Sitka spruce (*P. sitchensis* Bongard (Carrière)) (Deflorio et al. 2011), and Scots pine (*Pinus sylvestris* L.) (Adomas et al. 2007).

Recently several studies have been carried out to compare the outcome of infections with the native and the non-native *Heterobasidion* species on *P. pinea*. Comparative inoculation trials were performed in an ozone-enriched atmospheric environment (Pollastrini et al. 2015); to study the differential systemic induction of resistance to a shoot blight pathogen (Bonello et al. 2008); and their comparative effect on fungal symbionts (Zampieri et al. 2017). However, to our knowledge, no published records are available on whether host transcriptional and metabolic responses induced by inoculation with a native fungus (coevolved with the host) differ from those induced by inoculation with a non-native (non-coevolved) congeneric fungal species. Therefore, the aim of this preliminary study was to investigate i) what is the response of *P. pinea* to inoculations with native and non-native *Heterobasidion* species; ii) how the response varies over time; and iii) if there are differences in systemic defence induction between the native and the non-native pathogen. To meet these aims we selected a number of genes involved in different steps of the lignin biosynthesis and in defence reaction, as well as terpenoid production analysis.

Material and methods

Plant material

In early spring 2012, 96 3-year-old Italian stone pines (*Pinus pinea* L.) were obtained from 'Alto Tevere' nursery (Carabinieri Forestali - Pieve Santo Stefano, Italy). Plants were grown from seed of natural stone pine forests in Central Italy (Cecina, 43º 18' N, 10º 31' E). Each single seedling was transferred to 7.6 l plastic pots filled with a 1:1 (v:v) sand:peat mixture, and grown outdoors at the IPSP-CNR experimental nursery in Florence, Italy. Plants were irrigated daily to field capacity. Three weeks after transplanting, the potted pine trees were grouped based on size and overall appearance. *P. pinea* seedlings had a mean stem height of 75.9 ± 1.2 cm and a mean stem diameter, measured 3 cm above soil, of 1.3 ± 0.1 cm.

Experimental design and fungal inoculation

Pine seedlings were used in each of the following treatments: (a) *H. annosum* (Ha) (three isolates: HaCarp, 142OH-137OC and 43NA; 4 plants for each strain - total 12 plants); (b) *H. irregulare* (Hi) (three isolates: 45SE, 39NE and CP15; 4 plants for each strain- total 12 plants); (c) mock inoculation (wounding treatment – W; total 4 plants); (d) unwounded (UW; total 4 plants). Three different sampling time 3, 14, 35 dpi: 36 plant Hi; 36 Ha; 12 W and 12 UW. The isolates used in this work were selected based on their high pathogenicity on *P. pinea* seedlings from a sample of 8 isolates per species (data not shown). All six *Heterobasidion* isolates used in this study were deposited at the Mycotheca Universitatis Taurinensis (MUT) (University of Turin, Italy) with the accession numbers listed in Table 1. These isolates were previously characterized as heterokaryons (Sillo et al., 2015b) and used in other inoculation experiments (Zampieri et al., 2017). None of the used cultures represent a hybrid.

Stem inoculations with *Heterobasidion* spp. were carried out by using 8-day-old fungal cultures growing on 2% malt extract agar (MEA, Thermo Fisher Scientific Inc.) at 20°C. A disk of bark and phloem was removed 8 cm above the soil line with a 5 mm diameter cork borer, previously dipped in 95% ethanol and rinsed in sterile water, and replaced with a colonized plug of agar collected from the margins of the actively growing cultures. Agar plugs were placed mycelium side directly against the sapwood. Mock inoculation consisted of application of non-colonized sterile plugs of MEA. All inoculation sites were sealed with Parafilm (American National Can Co., Chicago, IL) to minimize desiccation and environmental contamination. Plants were arranged in a complete randomized block design in the greenhouse.

Tissue sampling, lesion measurement and fungal re-isolation

A strip of bark (3-cm-long bark samples) containing phloem and cambium from each seedling was removed (as described in Bonello et al. 2008) from around the stem at 3, 14 and 35 days post inoculation (dpi) and from two locations on the stem: a) at the inoculation site (IS), and b) approx. 12 cm above the inoculation site (distal site, DS). Each strip was longitudinally split in two parts: one for RNA extraction and the other for terpene analyses. Immediately after excision, the samples were placed in 2 ml microtubes (Eppendorf), frozen in liquid nitrogen and stored at -80 °C. Lesion size was only measured above the IS, starting at the margin of the wound, and served as a measure of resistance to the pathogens (Blodgett et al. 2007). The presence of the pathogens in the IS was confirmed by re-isolation, placing small fragments of necrotic tissue on 2% MEA. Plates were incubated at 20 °C for 10 days.

168 *RNA isolation and cDNA synthesis*

169 Total RNA was extracted from bark samples following the protocol reported by Chang et al.
170 (1993). Briefly, plant tissue (approx. 100 mg) was ground in liquid nitrogen in a mortar to a
171 fine powder, lysed with CTAB extraction buffer and β-mercaptoethanol, and incubated at 65°C
172 for 10 min. Then, isoamyl-alcohol (24:1) was added to separate the phases by centrifugation.
173 The upper phase was then mixed with 1/4 volume 10 M LiCl and incubated overnight on ice
174 in a cold room. After centrifugation, was discarded the supernatant and the pellet was then
175 washed in 70% EtOH and then dried. Total RNA was eluted in 50 µl of RNase free sterile
176 MilliQ H_2O. Contaminating DNA was removed by using RQ1 RNase-Free DNase (Promega,
177 USA). RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA),
178 while purity was assessed by electrophoresis using a RiboRuler High Range RNA Ladder
179 (Fermentas). Reverse transcription was performed using the iScript™ cDNA Synthesis Kit
180 (Bio-Rad, USA) from 200 ng of total RNA following the manufacturer's instructions.

181 *Identification of candidate genes and primer design*

182 PCR primers for *P. pinea* candidate genes involved in the lignin pathway (PAL, CAD,
183 POX), and those involved in general defence mechanisms (CHI, AMP, XET) were designed
184 based on *P. taeda* sequences (Figure S1) by using Primer Express® Software 3.0 (Applied
185 Biosystems, Forster City, CA, USA). For each target gene, the identity of each amplicon
186 sequence was verified using BLASTN on the NCBI website. Newly designed primer pairs and
187 fragment sizes are listed in Table 2. The PCR products were cloned in pGEM-T easy vector
188 (Promega) according to the manufacturer's instructions and sequenced with the Universal
189 Primers (M13) flanking the inserts, using a commercial DNA sequencing service (Macrogen).
190 All sequences were deposited in GenBank (for accession numbers see Table 2). In order to
191 verify the orthology of *P. pinea* derived sequences and *P. taeda* genes, pairwise alignments
192 were generated with MUSCLE 3.8 (Edgar 2010). The annotations of *P. pinea* sequences were
193 confirmed by BLAST analysis.

194 *Quantification of gene expression in P. pinea*

195 Expression of the candidate genes was measured by using real-time quantitative reverse
196 transcription PCR (qRT-PCR). The reference gene was actin (ACT), as described previously
197 (Adomas et al. 2007, Alonso et al. 2007) (Table 2). To evaluate the stability of ACT itself, we
198 quantified its transcription over all treatments (UW,W, Ha, Hi).

199 The qRT-PCR reactions were performed in triplicate in 96-well plates (Applied
200 Biosystem, Forest City, CA). Each reaction in a final volume of 12.5 µl contained: 6.25 µl

SYBR Green Mastermix (Applied Biosystems), 1.5 µl cDNA, a final concentration of 60 nM forward and reverse primer, and water to the final volume. The thermal profile was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were run in a StepOne™ Real-Time PCR System (Applied Biosystem, Forest City, CA). The threshold cycle value (Ct) for the real-time PCR reactions was determined for four biological replicates based on three technical replicates each by using the sequence detection system (SDS) Software version 1.9 (Applied Biosystem, Forest City, CA). To confirm that the signal was the result of single PCR product the melting curve analysis was performed after the PCR reaction.

Analysis of terpenoids

For terpenoids analysis, pine bark was ground in a mortar with liquid nitrogen. The obtained fine powder (approx. 100 mg) was placed in 2 ml glass vials with a teflon-coated screw cap (Perkin-Elmer, Norwalk, Connecticut, USA), and extracted in 1 ml of n-pentane with tridecane as an internal standard (Raffa and Smalley 1995).

Gas chromatographic-flame ionization detection (GC-FID) analysis was performed using a Perkin-Elmer Autosystem XL GC and separation of enantiomeric monoterpenes performed on a 30 m Cyclodex-B capillary column, 0.25-mm-diameter (J & W Scientific, CA, U.S.A.). Analysis was carried out following the conditions described in Pollastrini et al., (2015).

Terpenoids (mono and sesquiterpenes) were identified by comparison of retention times with those of standards under the same conditions. Absolute amounts of terpenoids were determined by comparison with the tridecane internal standard, and expressed as mg g^{-1} fresh weight. Relative amount (proportion of profile) of each monoterpene was expressed as a percentage of total monoterpenes, while each sesquiterpene was calculated as a percentage of total monoterpenes plus sesquiterpenes.

Statistical analysis

For each sampling time *H. annosum* and *H. irregulare* lesion size were compared by using Student's t test.

To determine the stability of the ACT as reference gene the threshold cycle (Ct) values were compared among different sampling time (3, 14 and 35 dpi) and treatments (Hi, Ha, W and UW) by one-way analysis of variance (ANOVA). All qRT-PCR data were normalized to the reference actin gene and the relative gene expression ($2^{-\Delta\Delta Ct}$ or fold change value) has been calculated according to Pfaffl (2001), comparing the data for each gene in the Ha, Hi, and W samples to each gene in the UW samples. To achieve the homogeneity of variance transcripts data were subjected to log transformation. For each sampling site (DS and IS) and sampling

time (3, 15 and 35dpi) differences of transcript data where determined among different treatments (Hi, Ha, W) with one-way ANOVA analyses Whenever ANOVA was significant, Fisher's least significant differences (LSD) test was calculated.

Terpenoid levels were not normally distributed (Kolmogorov-Smirnov one sample test) and normality was not achieved after an arcsine square-root transformation; therefore, statistical tests were performed using Kruskall-Wallis and Mann-Whitney non-parametric tests.

All data (lesion size, qRT-PCR and terpenoids) were presented as mean ± standard error (SE). For all analysis, $p < 0.05$ was considered as statistically significant. All statistical analyses were carried out by using SYSTAT 12.0 software (Systat Software Inc., USA).

Results

Fungal inoculations and cross-induction of systemic susceptibility

No lesions or resin flow were observed in any of the control plants (UW plants). The presence of resin was observed at IS for mock-inoculated (W) and *H. annosum* and *H. irregulare* collected at 3dpi, while no necrosis has been observed. All seedlings inoculated with *H. annosum* and *H. irregulare* and collected at 14 and 35 dpi showed necrosis and abundant resin flow from the inoculation sites. Fungal mycelium was re-isolated at IS from all plants infected with *H. irregulare* and *H. annosum*, confirming the stem colonization of each pathogen at different sampling time (3, 14 and 35 dpi). Mock-inoculated and unwounded seedlings did not yield either pathogen. Since no differences in lesion length were observed among the three isolates of each pathogen, we considered different isolates as repeats of *H. annosum* and *H. irregulare*. *H. annosum* caused significantly shorter lesion than *H. irregulare* at 14 dpi (10.3 ± 0.6 mm vs. 13.6 ± 0.79 mm, respectively; $t_{22} = -3.24$, $p = 0.003$; for Hi, n=12; for Ha n=12), and at 35 dpi (13.4 ± 0.5 vs. 15.6 ± 0.6 mm, respectively; $t_{22} = -2.79$, $p=0.012$; for Hi, n=12; for Ha n=12) (Figure 1).

Quantification of gene expression in P. pinea

Pairwise alignments of newly obtained *P. pinea* sequences with orthologous *P. taeda* transcripts showed high similarity (Figure S1). The putative function of each target gene is reported in Table 2.

Statistical analysis of ACT gene showed no significant differences of Ct values for each treatment at different sampling time ($p > 0.05$), thus for each treatment Ct values from different sampling time were pooled. However, ANOVA showed no statistical differences ($F_{(3, 92)} = 2.6$, $p = 0.07$) among treatments (Ha, Hi, W, UW), confirming the stability of ACT gene as reference gene (Figure S2).

The real-time PCR analysis of all tested genes showed no significant differences between infected (both *H. annosum* and *H. irregulare*) and mock-inoculated seedlings at IS for all sampling time (Figure 2; Table S1). However, after 14 dpi both pathogens showed similar trend in the DS, with most of the candidate gene (CHI, POX, AMP, CAD and XET) significantly up-regulated than mock-inoculted (W) samples (Figure 2; Table S1, S2). These difference are also present, for the same sampling site, at 35 dpi for CAD while CHI and XET showed differences in the expression: while CHI is significantly up-regulated in *H. irregulare* and Wounding, XET is down-regulated for *H. annosum* in comparison with *H. irregulare* and Wounding (Figure 2; Table S1, S2).

At 3 dpi POX and PAL genes were up-regulated at the IS position by both *H. annosum* and *H. irregulare*, and the expression of these genes dropped at 14 dpi and 35 dpi (Figure 3; Table S3). At 3 dpi the PAL transcript levels were significantly higher than at the other two time points (Figure 3; Table S3). CAD was low at 3 dpi and significantly up-regulated at 14 dpi, in both sampling sites, compared to 3 and 35 dpi; the difference in expression between 3 and 14 dpi was about 5- to 8-fold at the IS and nearly 35-fold in the DS (Figure 3; Table S3). At 3 dpi, there was no change in expression of PAL and CAD lignin related genes in the distal site for all treatments tested, while POX was significant up-regulated by both pathogens (Figure 3). A highly significant difference was shown in POX and CAD levels in the distal site at 14 dpi between inoculated and W seedlings. The highest fold differences for AMP in the IS samples were at 3 dpi for all treatments (the level of expression was about 58- to 43-fold for *H. annosum* and *H. irregulare*, respectively) and at 14 dpi in the inoculated seedlings (16-fold for *H. annosum* and 34-fold for *H. irregulare*) (Figure 3; Table S3). At 3 dpi XET was not differentially expressed in inoculated tissues but it was highly up-regulated (about 25-fold change) at 14 dpi at both sites (with no significant differences between the pathogens) (Figure 3; Table S3). Later on, at 35 dpi, XET levels decreased. Inoculations induced significantly higher XET gene expression levels in DS tissues at 14 dpi in comparison with both W samples. CHI was slightly down-regulated by *H. annosum* and *H. irregulare* in IS tissues at 3 dpi and 35 dpi. CHI expression was much higher in DS tissues of inoculated plants compared to

transcripts levels in IS samples. Furthermore, expression levels in DS tissues of inoculated plants were up to 6-fold higher than in wounded at 14 dpi (Figure 3; Table S3).

Temporal and spatial variation of total terpenoids

Temporal variation in the accumulation of terpenoids was observed in IS samples in seedlings treated with *H. irregulare*, *H. annosum,* and W, while no significant differences were detected in DS tissue samples (see Tables S4-S7). For all these treatments, the highest value of total terpenoids present in bark tissue was observed at 14 dpi. No statistical differences were observed among the different sampling times in the IS tissues of UW plants (Figure 4 and Table S4). Significantly higher terpenoid accumulation was observed in DS than IS tissues at 35 dpi, both in inoculated and W plants. No significant differences in DS vs. IS tissues were observed at 3dpi, while at 14 dpi a significant higher terpenoids accumulation was observed in IS for *H. irregulare* inoculated and W plants.

Changes in terpenoid profiles

A number of different terpenoids were detected: eight monoterpenes [(+)-α-pinene, (-)-α-pinene, myrcene, (+)-β-pinene, (-)-β-pinene, (+)-limonene, (-)-limonene, β-phellandrene], three sesquiterpene (α-humulene, β-caryophyllene and longifolene), and three unknown compounds (unknown1, unknown2, unknown3) (Figure 5). Some of these terpenoids differed among treatments and sampling times in IS tissues (Figure 6 and Tables S4-S7). No significant differences were observed for any of the terpenoids in UW samples among sampling times. At 14 dpi inoculated plants showed a significantly higher percentage ($P < 0.01$) of (-)-limonene, β-caryophyllene and unknown3 in comparison with W and UW (Figure 6; Tables S4-S7). Similarly, significant differences ($P < 0.05$) were also observed at 35 dpi for (-)-limonene and unknown3, while β-caryophyllene showed significantly higher percentages in inoculated and W tissues in comparison with UW tissues (Figure 6 and Tables S4-S7). At 35 dpi the amount of longifolene was significantly higher in inoculated pines than UW and W plants (Figure 6 and Tables S4-S7). (-)-β-pinene in inoculated plants showed a different profile: between 14 and 35 dpi there was a significant decrease of this terpene compared with W and UW. The percent of α-humulene was significantly higher in infected and W than UW tissue at 14 and 35 dpi. In DS tissues, no significant differences were observed in terpenoid percentages among sampling times and treatments (Tables S4, S5, S7).

Discussion

The native pathogen causes somewhat smaller lesions than the non-native pathogen in the inner bark of the pine host

The results of the present study show that the native pathogen *H. annosum* caused marginally, but significantly, smaller stem lesions on *P. pinea* than the non-native *H. irregulare* at 14 and 35 dpi. Lesion length in the inner bark has previously been used as indicator of *Heterobasidion annosum* strain virulence (Swedjemark et al. 2001), and more recently to evaluate the differences in severity of infection caused by *H. annosum* and *H. irregulare* (Bonello et al. 2008, Garbelotto et al. 2010, Scirè et al. 2011, Pollastrini et al. 2015). In this study we found that there is a difference in lesion length between these two pathogens. This is in contrast to Garbelotto et al. (2010), who found that susceptibility to *H. annosum* and *H. irregulare* did not vary in European and North American pine cuttings and seedlings and Pollastrini et al. (2015), who found that the mean basal lesion lengths were not significantly different between *P. pinea* seedlings inoculated with *H. irregulare* and *H. annosum* in ozone exposure experiments.

The two Heterobasidion species elicited similar host gene expression patterns both locally and systemically

Gene expression responses were observed throughout the experiment, including at 35 dpi, when differences in lesion length became evident, but the expression of specific defence genes elicited at each sampling site (IS and DS), were not significantly different between *H. annosum* and *H. irregulare*.

Several studies have demonstrated early host defence transcript accumulation after 24 h, 3 and 5 dpi in Norway spruce and *P. sylvestris* in response to *H. annosum* infection (Hietala et al. 2004, Adomas et al. 2007, Jaber et al. 2014, Lundén et al. 2015).

Both *H. annosum* and *H. irregulare*-infected seedlings showed rapid expression of lignin related genes (POX and PAL) at the inoculation site. Up-regulation of POX at 3 dpi was also reported in *P. abies* and *P. sylvestris* sapwood and/or bark after wounding and inoculation with *Heterobasidion* spp. (Johansson et al. 2004, Nagy et al. 2004b, Likar and Regvar 2008). BLAST analysis of the sequences obtained herein showed that POX had the highest identity with the Norway spruce plant class III peroxidase (PX2), which is involved in the formation of lignin and maturation of cell walls (Marjamaa et al. 2006). More recently Yaqoob et al. (2012)

365 found local up-regulation of lignin related peroxidases (*PaPX2* and *PaPX3*) in both Norway
366 Spruce bark and sapwood infected with *H. parviporum*.
367 Up-regulation of PAL, the enzyme that constitutes the initial key step in the
368 phenylpropanoid metabolic network, was not unexpected at 3 dpi. These results are consistent
369 with many studies, e.g. Deflorio et al. (2011), who also found up-regulation of this gene in
370 Sitka Spruce bark infected with *H. annosum*.
371 On the other hand, CAD, which is considered a more direct step for lignin biosynthesis
372 (Baucher et al. 1998), showed down-regulation at the IS for both pathogens at 3 dpi and 35 dpi,
373 while being up-regulated at 14 dpi. This was particularly true at the DS position. If elevated
374 CAD expression at the DS position is indeed followed by enhanced lignification, this outcome
375 may point to induction of systemic resistance (Sherwood and Bonello, 2013).
376 XET is involved in the mechanical reinforcement of cell walls under pathogen attack, and
377 resulted up-regulated at 14 dpi, while its level decreased at 35 dpi, down to a comparable level
378 of early stage of infection (3 dpi). Our observations concur with those of Adomas et al. (2007),
379 who found XET weakly up-regulated in the first stage of infection (5 dpi) and highly down-
380 regulated later (at 15 dpi) in 14-day-old *Pinus sylvestris* seedlings. In addition, a similar
381 response has also been observed in a mutualistic association, *Pinus sylvestris* - *Laccaria*
382 *bicolor*, where a decrease of XET has been observed after 30 dpi (Heller et al. 2008).
383 The AMP sequences obtained in this study show very high homology to a known
384 antimicrobial peptide (AMP3) from *Pinus sylvestris* infected with *Heterobasidion annosum*
385 (Asiegbu et al., 2003). AMP was the most abundant transcript observed at 3 and 14 dpi at IS,
386 suggesting a possible direct role in host defence against invading hyphae at the inoculation site,
387 as reported in previous studies (Fossdal et al. 2003, Pervieux et al. 2004, Adomas et al. 2007),
388 and perhaps representing a mechanism supporting systemically induced resistance (SIR)
389 (Bonello et al. 2006). Interestingly, the AMP gene was up-regulated soon after inoculation and
390 the up-regulation was sustained until at least 35 dpi, while in the mock-inoculated trees the up-
391 regulation was sustained only until 14 dpi at the IS and was never upregulated at the DS. This
392 suggests that AMP synthesis is elicited by PAMPs (Jones and Dangl 2006). AMP is thought to
393 act synergistically with other PR-proteins, contributing to constitutive and induced resistance
394 to different microorganisms (Padovan et al. 2010, Liu et al. 2013). In previous studies, the
395 expression of AMP was observed at the beginning of the interaction between pine seedlings
396 and *Cronartium ribicola* (Liu et al. 2013) and after 1-5 days in Scots pine inoculated with *H.*
397 *annosum* (Sooriyaarachchi et al. 2011).
398 Another defence related gene, CHI, showed similarities to the basic class I chitinase

PaCHI1 (Hietala et al., 2004). CHI was down-regulated in IS tissues inoculated with *Heterobasidion* at 3 dpi but up-regulated at 14 dpi in DS tissues. Our results are in accordance with Hietala et al. (2004), who showed that the local level of PaCHI1 decreased in Norway spruce infected with *H. annosum*. CHI is hypothesized to inhibit fungal growth by degrading chitin in fungal cell while the chitinolytic breakdown products are thought to elicit further defence responses in the plant (Wan et al. 2008). It is possible that at IS the fungus overwhelmed this particular host defence and the induction of CHI synthesis at a systemic level may be synergistic to AMP accumulation in the context of SIR and is consistent with a study of Norway spruce seedlings infected with *Rhizoctonia* sp. (Nagy et al. 2004a).

In summary, treatments induced significant up-regulation at the systemic level of all the genes analysed herein. Lastly, a systemic response was observed mostly in infected trees, suggesting that specific pathogen recognition processes are operating in the host. This is consistent with the findings of Bonello and Blodgett (2003) who showed that killed mycelium of *Diplodia sapinea* (ex. *Sphaeropsis sapinea*) was a close proxy for a live infection in Austrian pine (*Pinus nigra*) in terms of induction of specialized metabolites.

Differences in terpene composition were found locally while no significant differences were observed systemically

Infected *P. pinea* seedlings showed significant variation between the two pathogens in relative content and total amount of terpenes at different sampling times at the IS, while no significant differences were observed at the DS.

In our study both pathogens induced essentially the same temporal variation in relative content of terpenes. We obseved a different trend between the two main monoterpenes at 14 dpi: the relative amount of (-)-β-pinene decreased in response to attack by both pathogens, while the proportions of (-)-limonene significantly increased. These results are in accordance with a Pollastrini et al. (2015) study where (-)-β-pinene and (-)-limonene represented the main monoterpenes detected in oleoresins of *P. pinea* infected with *Heterobasidion* spp. These monoterpenes were shown to be both able to inhibit mycelial growth of *H. annosum* and *D. sapinea* in pure culture (Blodgett and Stanosz 1997, Zamponi et al. 2007). In addition, limonene was shown to significantly inhibit the growth of many bacterial and fungal strains (Lis-Balchin et al. 1996, Sokovic and Van Griensven 2006, Chutia et al. 2009, Razzaghi-Abyaneh et al. 2009, Amri et al. 2012). Furthermore, Sokovic and Van Griensven (2006) demonstrate that limonene and α-pinene have strong antimicrobial activity against *Verticillium*

433 *fungicola* and *Trichoderma harzianum*, and Chang et al. (2008) showed a higher toxic effect
434 of limonene than α- and β-pinene against fungi.
435 The other terpenes, longifolene, β-caryophyllene, α-humulene and unknown3 showed
436 lower abundance, ranging between 0.5% and 3.5% of the total terpene content. The amount of
437 these terpenes in response to attack by both pathogens significantly increased with respect to
438 UW plants and, in some cases, also to W seedlings. Similar results were found in Norway
439 spruce bark following wounding and infection by *Heterobasidion spp.* (Danielsson et al. 2011).
440 The accumulation of total terpenes at the IS location in *P. pinea* seedlings in response to *H.*
441 *annosum* and *H. irregulare* inoculations has also been observed in previous studies (Bonello
442 et al. 2008, Pollastrini et al. 2015).
443 Other papers (Bonello et al. 2008, Pollastrini et al. 2015) have described accumulation
444 of total terpenes at the stem base in *P. pinea* seedlings in response to *H. annosum* and *H.*
445 *irregulare* inoculations. Our results showed systemic increase of total amounts of terpenes in
446 DS cortical tissue samples at 14 and 35 dpi following wounding and infection with
447 *Heterobasidion* spp.; on the other hand, no systemic changes in terpene profiles, i.e., the
448 relative proportions (percentages) of volatile oleoresin constituents, were observed in response
449 to different treatments. A possible explanation could be that there are two phases of terpene
450 biosynthesis: systemic defensive reactions associated with total amounts of terpenes might
451 represent the first nonspecific chemical barrier to reduce subsequent pathogen attack. Only
452 subsequently, particularly if the spread of the infection is not blocked, a specific terpene profile
453 for a particular pathogen-plant system (Traw 2002, Arimura et al. 2009) might emerge.
454
455 **Conclusions**
456 Contrary to expectations, our study suggests that *H. irregulare* does not have a notable
457 competitive advantage over the native species *H. annosum* on *P. pinea* trees. Small, yet
458 statistically significant differences, were observed in lesion length between the two pathogens.
459 We have also shown that several defense-related genes and terpenes were highly induced both
460 locally and systemically by both *Heterobasidion* species. However, the expression patterns for
461 all six genes and the levels and profiles of terpenes did not differ between the two pathogens.
462 The up-regulation of transcripts as well as the accumulation of terpenoids at the distal site
463 revealed that a systemic signal is inducible in *P. pinea* by both pathogens.
464 Generally, it is well known that alien invasive species can have massive impacts on
465 native ecosystems, including by altering the evolutionary pathway of native species occupying
466 the same or similar ecological niches through competitive exclusion, hybridization,

introgression, and finally extinction (Mooney and Cleland 2001). In Italy *H. irregulare* was introduced by the US Army during World War II (Gonthier et al. 2004). The same pathway has also been proposed for two other important forest pathogens: *Ceratocystis platani* and *Seiridium cardinale*, the agents of canker stain disease of plane trees and Cypress canker, respectively (Cristinzio et al. 1973, Grasso 1951). While these two pathogens are now firmly established and widely spread in Europe, *H. irregulare* is still confined to a small area along the Latium coast (Gonthier et al. 2014). The difference in invaded territory among these pathogens supports our idea that *H. irregulare* does not have a competitive advantage over *H. annosum* on European pines, at least on *Pinus pinea*, even though these two pathogens have evolved and differentiated in allopatry for at least 34 million years (Otrosina et al. 1993, Linzer et al. 2008, Dalman et al. 2010; Otrosina and Garbelotto 2010).

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Table and Figure captions

Table 1. *Heterobasidion* isolates (all heterokaryotic) used in this study and deposited at Mycotheca Universitatis Taurinensis (MUT).

Table 2. qRT-PCR primers and functional annotations of the corresponding candidate genes tested on *Pinus pinea* in this study.

Figure 1: Mean lesion size caused by *H. annosum* and *H. irregulare* on 3-y-old *P.pinea* seedlings. For each treatment (*H. annosum* and *H. irregulare*) lesion lengths were measured from 12 seedlings at 14 and 35 days after inoculation (14dpi and 35 dpi, respectively). Different letters indicate significant differences between lesion size based on Student's t test ($P<0.05$). Error bars indicate the standard error (SE).

Figure 2. Relative gene expression (Mean Log_2 fold-change) of transcripts expressed in Italian stone pine bark tissue infected with *Heterobasidion annosum*, *H. irregulare*, and mock-inoculated at 3, 14 and 35 dpi at the inoculation site (IS) and at the distal site (DS) (approx. 12 cm above inoculation). Asterisk (*) mean statistical differences ($P \leq 0.05$) among treatments (*H. irregulare*, *H. annosum* and mock-inoculated). The qPCR results and statistical analysis are reported in Supplementary Data (Table S1, S2).

Figure 3. Expression of candidate gene during different sampling time (3, 14, and 35dpi) within each treatment (*H. annosum* - Ha; *H. irregulare*- Hi; Mock-inoculation- W) at the inoculation site (IS) and at the distal site (DS). The histogram bars show mean relative expression and the error bars represent the SE. Different letters indicate significant differences ($P \leq 0.05$) among the sampling days within treatment by LSD test**. S**tatistical analysis are reported in Supplementary Data (Table S3).

Figure 4. Total absolute amounts of terpenoids in seedlings of *Pinus pinea* (mean ± SE) among different treatments (Hi, *H. irregulare*; Ha, *H. annosum*; W, mock-inoculated; UW, unwounded). Terpenoids at the inoculation site (IS) and at the distal site (DS) (approx. 12 cm above IS) were extracted from the bark at 3, 14 and 35 dpi. Different letters indicate significant differences ($P \leq 0.05$) by Kruskal-Wallis Test: lowercase letters refer to the analysis among treatments within the sampling days; upper case letters refer to the analysis within a treatment combination among sampling days.

Figure 5. Chromatogram showing the complete separation of chiral and non-chiral monoterpene compounds present in liquid extracts from Italian stone pine cortical tissues.

Figure 6. Mean percentage (± SE) of terpenes from the reaction zones of *Heterobasidion* spp. or the control wounds at the stem base (IS site). Ha: *H. annosum*; Hi: *H. irregulare*; W: wounded (Mock-inoculated); UW: unwounded controls; dpi: days post inoculation. (See also Table S4-S7).