Biocontrol potential of Pseudomonas protegens against Heterobasidion species attacking conifers in Europe

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Biocontrol potential of *Pseudomonas protegens* against *Heterobasidion* species attacking conifers in Europe

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Abstract

The biocontrol potential of a commercial product based on the bacterium *Pseudomonas protegens* (strain DSMZ 13134) and of its cell-free filtrate (CFF) were tested under controlled conditions *in vitro* and on wood discs against genotypes of the three native European *Heterobasidion* species and on the non-native *H. irregulare*, all reported as destructive forest pathogens in Europe. *In vitro* experiments through traditional dual culture assays and by incorporating CFF into the media showed thattreatments were effective in reducing significantly and regardless of the culture medium and incubation temperature both mycelial growth and rate of conidial germination of *Heterobasidion* spp., and that antibiosis could be the main mechanism involved in the inhibition of *Heterobasidion* spp. Outcomes of dual culture assays performed on two-divided Petri plates further suggest that antibiosis operates through the production of diffusible rather than volatile compounds. Based on comparative experiments on wood discs of preferential hosts of *Heterobasidion* spp., CFF performed significantly better than the commercial product against most of *Heterobasidion* spp., further confirming that the antagonistic activity hinges on antibiosis rather than on other mechanisms. While on wood discs the tested treatments seem poorly effective against the non-native *H. irregulare*, CFF significantly and substantially reduced infections of the native European *Heterobasidion* species compared to controls mostly to the level of the state of the art chemical treatment urea and of the biological treatment based on *Phlebiopsis gigantea*.

Keywords

Antibiosis

Cell-free filtrate

Phlebiopsis gigantea

Proradix®

Rotstop®

Urea

Declarations of interest: none.

66 **1. Introduction**

67 Root and butt rots caused by the fungal species complex *Heterobasidion annosum* (Fr.) Bref. *sensu*
68 *lato* (*s.l.*) stand among the most destructive diseases of conifers worldwide (Garbelotto and Gonthier,
69 2013). Financial losses associated with the three European species of *H. annosum s.l.*, i.e. *H.*
70 *abietinum* Niemelä & Korhonen, *H. annosum sensu stricto* (*s.s.*), hereafter referred to as *H. annosum*,
71 and *H. parviporum* Niemelä & Korhonen, were estimated a few decades ago at 790 million Euros per
72 year due to a reduction in both wood quality and productivity (Woodward *et al.*, 1998). As previously
73 recognized, losses are likely higher because this estimate did not take into account the reduction of
74 resistance of forest stands associated with the presence of these fungi during storms, which may be
75 locally relevant (Garbelotto and Gonthier, 2013; Woodward *et al.*, 1998).
76 In Europe, losses associated with *H. annosum s.l.* increased as a result of the introduction of the
77 north American species *H. irregulare* Garbel. & Otrosina, which became invasive and is now
78 distributed in coastal pine stands of central Italy (Gonthier *et al.*, 2004, 2007, 2012, 2014). Based on
79 its current and potential impact, *H. irregulare* is a quarantine plant pest recommended for regulation
80 under the European and Mediterranean Pant Protection Organization (EPPO) A2 list..
81 The distribution in Europe of the four *Heterobasidion* species was previously reviewed (Garbelotto
82 and Gonthier, 2013). Some of the species display distinct host preference. In fact, while both the
83 native *H. annosum* and the non-native *H. irregulare* are associated with pines (*Pinus* spp.), *H.*
84 *abietinum* and *H. parviporum* preferentially attack *Abies alba* Mill. and *Picea abies* (L.) Karst.,
85 respectively (Garbelotto and Gonthier, 2013).
86 Regardless of the *Heterobasidion* species, spreading occurs by means of both basidiospores (primary
87 infection) and mycelium (secondary infection) (Garbelotto and Gonthier, 2013). Primary infection
88 takes place as a result of landing and germination of airborne basidiospores on freshly cut stump
89 surfaces or, to a lesser extent, on wounds. Once the primary infection has occurred, mycelium
90 spreads to the root system and eventually to neighbouring trees if root contacts are present
91 (Garbelotto and Gonthier, 2013).

Silvicultural and prophylactic measures have been developed and are currently adopted to control *Heterobasidion* spp. (Garbelotto and Gonthier, 2013; Gonthier and Thor, 2013). However, silvicultural approaches, such as the use of mixed stands, stump removal, strategic planning of thinning operations, are not always implementable because often they do not meet the needs of intensive forest management (Asiegbu *et al.*, 2005; Vasaitis *et al.*, 2008). Prophylactic measures are focused on the use of chemical or biological treatments on freshly cut stump surfaces in the attempt to prevent *Heterobasidion* primary infection. One of the most effective and commonly used chemical treatment is that with urea, which protects the exposed stump surfaces by raising the pH to a level at which *Heterobasidion* spp. basidiospores are unable to germinate (Johansson *et al.*, 2002). The major biological treatments widely used in practical forestry are those based on *Phlebiopsis gigantea* (Fr.) Jülich, a wood decay basidiomycete outcompeting *Heterobasidion* spp. mainly thanks to its rapid colonization of stumps (Asiegbu *et al.,* 2005; Pratt *et al.*, 1999; Rönnberg *et al.*, 2006). Stump treatments with *P. gigantea* are available in different formulations, including Rotstop® (Gonthier and Thor, 2013; Pratt *et al.*, 1999). However, neither Rotstop® nor other products based on *P. gigantea* are currently registered for use in southern Europe. In addition, the registration of urea as a pesticide in Europe will expire August 2021 (EU Reg. 2020/1160). If this registration will not be renewed, there will be an urgent need to identify alternative treatments. This would be particularly important for southern Europe, where no prophylactic stump treatments will be available to manage the three native *Heterobasidion* species and the non-native invasive *H. irregulare*.

Despite extensive research on beneficial fungi and bacteria (Azeem *et al.*, 2019; Mesanza *et al.*, 2016, 2019; Nicolotti and Varese, 1996; Szwajkowska-Michałek *et al.*, 2012; Terhonen *et al.*, 2016), a comprehensive investigation on the effectiveness of a biocontrol agent against all *Heterobasidion* species present in Europe, including the invasive species *H. irregulare*, is still lacking.

The relevance of plant growth-promoting rhizobacteria (PGPR) for the control of plant pathogens is now recognized (Beneduzi *et al.*, 2012; Compant *et al.*, 2005; Mishra and Arora, 2018). Our study focuses on the soil bacterium *Pseudomonas protegens* (strain DSMZ 13134), which is the active

component of the bio fungicide Proradix® (SP Sourcon Padena GmbH, Tübingen, Germany). According to the manufacturer of Proradix®, this bacterial strain is a naturally occurring PGPR, it is not a genetically modified organism (GMO), and it is non-toxic and non-pathogenic to plants. Proradix® was effective against silver scab caused by *Helminthosporium solani* Durieu & Mont. and stem canker and black scurf caused by *Rhizoctonia solani* J.G. Kühn. Although Proradix® is currently targeting soil borne pathogens, some evidences point to the ability of its active component to synthesise antifungal compounds potentially effective against a range of fungal plant pathogens (Buddrus-Schiemann *et al.*, 2010; Compant *et al.*, 2005; Fröhlich *et al.*, 2012). Indeed, Roberti *et al.* (2012) reported antibiosis to be the main mode of action of *P. protegens* (DSMZ 13134) in the control of zucchini foot and root rot caused by the race 1 of *Fusarium solani* f.sp. *cucurbitae* W.C. Snyder & H.N. Hansen. Interestingly, Proradix® proved to be efficient against *H. parviporum in vitro* and in a pilot field study on Norway spruce stumps (Gžibovska, 2016; Rönnberg and Magazniece, 2018), making it a good candidate for further assessments against all *Heterobasidion* spp. present in Europe.

In general, before field trials, the efficacy of potential biocontrol agents against *Heterobasidion* spp. is assessed through experiments both *in vitro* and in controlled conditions on substrates such as wood (Holdenrieder *et al.*, 1998). Experiments have been conducted using either billets (Sun *et al*., 2009a and 2009b; Zaluma *et al*., 2019) or wood blocks (Nicolotti and Varese, 1996; Oliva *et al.,* 2015; Samils *et al.,* 2008). In this study, we used wood samples reported to simulate freshly cut stump surfaces, which are right the infection courts of *Heterobasidion* spp. and hence the target of treatments. These wood samples, known as wood discs, although never employed for testing the efficacy of treatments are widely used for investigating the epidemiology of *Heterobasidion* spp. (Gonthier *et al.*, 2001, 2007, 2012).

In this study, we tested the biocontrol potential of *P. protegens* (strain DSMZ 13134) towards the four *Heterobasidion* spp. currently occurring in Europe. This was carried out by (i) determining the inhibitory effect of both the bacterium itself and cell-free filtrate *in vitro*, (ii) assessing the role of diffusible and volatile compounds in the interaction between the bacterium and the fungal plant

144 pathogens, and (iii) performing a comparative analysis of the efficacy of the bacterium (Proradix®), its
145 cell-free filtrate and the state of the art treatments urea and *P. gigantea* (Rotstop®) on wood discs. Our
146 hypothesis was that *P. protegens* (strain DSMZ 13134) could negatively affect *Heterobasidion* spp.
147 through the mechanism of antibiosis.
148
149 **2. Materials and methods**
150 *2.1 Microorganisms and culture conditions*
151 *Pseudomonas protegens* (strain DSMZ 13134) was provided by SP Sourcon Padena GmbH
152 (Tübeningen, Germany) and stored in Luria-Bertani (LB) broth amended with 30% glycerol at -80 °C.
153 Fresh cultures were started from frozen stocks and refreshed in LB broth at 25 °C for 24 h with
154 shaking before use. Five genotypes for each species of *H. annosum s.l.* occurring in Europe, i.e. *H.*
155 *abietinum*, *H. annosum*, *H. irregulare* and *H. parviporum*, were randomly selected from the culture
156 collection of the University of Turin (Table 1). Although some genotypes of *H. annosum* originated
157 from the same area where the invasive and interfertile *H. irregulare* is present, none of them showed
158 significant genetic admixing based on more than 500 AFLP markers (Gonthier and Garbelotto, 2011).
159 Therefore, genotypes of *H. annosum* and *H. irregulare* selected for this study should be regarded as
160 pure genotypes of either species, as previously suggested (Gonthier and Garbelotto, 2011). All
161 genotypes of *H. annosum s.l.* derived from single spores collected on woody spore traps (i.e. wood
162 discs) placed in the field and isolated in the laboratory as previously described (Gonthier *et al.*, 2007).
163 The fungal cultures were maintained on ISP2 medium (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g
164 L^{-1} glucose and 20 g L^{-1} agar, pH 7.3) (Pridham *et al.*, 1957) at 4 °C.
165
166 *2.2 Determination of antifungal activity of P. protegens (DSMZ 13134) against Heterobasidion spp.*
167 *2.2.1 Dual culture assays*
168 Antifungal activity of *P. protegens* (DSMZ 13134) against *Heterobasidion* spp. was first assessed by
169 dual culture assays on both ISP2 medium and ISP2 medium supplemented with sawdust (ISP2-s)

obtained from fresh wood of preferential host species, i.e. *Pinus pinea* L. for *H. annosum* and *H. irregulare, A. alba* Mill. for *H. abietinum,* and *P. abies* (L.) H. Karst. for *H. parviporum*, to simulate the growth substrate of *Heterobasidion* spp. in natural conditions. Sawdust was prepared by cutting fresh stems using a circular saw and collecting the resulting sawdust in a polyethylene bag. Sawdust with a maximum granulometry of 5 mm was added to ISP2 medium at a dose of 10 g L^{-1} prior autoclaving for 20 min. at 121 °C. Fungal mycelial plug was taken from a 7 days old culture and inoculated towards one edge of a 6-cm diameter Petri plate. *Pseudomonas protegens* (DSMZ 13134) (optical density at 600 nm [OD_{600} = 0.4], approximately 10^7 CFU mL^{-1}) was streaked (streak length 20 mm) at 15 mm from the edge of the fungal plug on the opposite side of the Petri plate (treated plates). Control plates were prepared by using only the *Heterobasidion* spp. genotypes. Treated and control plates were incubated in the dark at both 25 and 10 °C until the fungal colonies in the control plates had grown to fill the plate. The radius of *Heterobasidion* spp. colonies was measured (in mm) in treated (r_T) and control (r_C) after 7 days in Petri plates incubated at 25 °C and after 11 days in Petri plates incubated at 10 °C. The mycelial growth inhibition (MGI) of *Heterobasidion* spp. was assessed by calculating (in %) the radial reduction observed in treated plates in relation to the corresponding control plates with the following equation:

$$\mathrm{MGI} = 100\% \cdot \frac{r_C - r_T}{r_C} \quad \text{(Equation 1)}$$

For each medium (ISP2 and ISP2-s) and temperature, 10 control and 10 treated plates were established per each *Heterobasidion* spp. genotype.

2.2.2 Effects of volatile compounds on mycelial growth

The antagonistic potential of volatile compounds produced by *P. protegens* (DSMZ 13134) against *Heterobasidion* spp. was explored using two-divided Petri plates. In treated plates, a 7-days-old fungal mycelial plug and a bacterial suspension (OD_{600} of 0.4) were spotted onto different halves of the same 9 cm-diameter two-divided Petri plates. In control plates only *Heterobasidion* spp.

genotypes were grown. The same growing media, incubation temperatures, *Heterobasidion* spp. genotypes, number of replicates and MGI assessment method described in section 2.2.1 were used.

2.2.3 Inhibition of mycelial growth by cell-free filtrate (CFF)

The CFF of *P. protegens* (DSMZ 13134) containing extracellular metabolites of the bacterium was tested for its inhibitory activity against mycelial growth of *Heterobasidion* spp. at 25 °C. The incubation temperature of 10 °C was omitted in this and in subsequent experiments because very little *Heterobasidion* spp. mycelial growth was observed at this temperature in dual culture assays. The CFF was prepared by culturing *P. protegens* (DSMZ 13134) in LB broth with constant shaking for 24 h at both 25 °C (OD_{600} of 1.1) and 10 °C (OD_{600} of 0.3) to determine whether CFF prepared at different temperatures may perform differently towards *Heterobasidion* spp. Cells were pelleted by centrifugation at 4,000 rpm for 10 min, and the supernatant was filtered aseptically through a 0.22 µm filter membrane to obtain CFF free from bacterial cells (CFF_{25} and CFF_{10}). CFF was incorporated into the water agar medium (17 g L^{-1}) maintained at 50 °C to the final concentration of 80% (v/v). Petri plates filled with 80% (v/v) LB broth instead of CFF served as controls. Mycelial plugs of *Heterobasidion* spp. genotypes were individually placed at the centre of Petri plates. Petri plates were then incubated until the fungal colonies in the control plates had grown to fill the plate. Experiments were performed with ten replicates per each *Heterobasidion* genotype. MGI was determined as described in section 2.2.1 after 7 days of incubation.

2.2.4 Inhibition of conidial germination by cell-free filtrate (CFF)

The CFF_{25} and CFF_{10} described in section 2.2.3 were also used to assess the inhibition potential of extracellular metabolites of *P. protegens* (DSMZ 13134) on *Heterobasidion* spp. conidial germination. The conidia of *Heterobasidion* spp. were obtained as follows. 500 µL of sterile water was loaded on the surface of 7-10 days old fungal colonies previously incubated at 25 °C in the dark. The water was gently shaked and collected by using a pipette. The concentration of conidia in the suspension was

assessed by using a Bürker chamber, and the conidial suspension was subsequently adjusted to 10^5 conidia mL^{-1}. The same growing media described in section 2.2.3 were used and poured as a thin layer (2 mm) in Petri plate. 100 µL of conidial suspension was loaded in Petri dishes and uniformly distributed using an L-shaped sterile Drigalski spatula. Three replicates per *Heterobasidion* spp. genotypes were used for each treatment (i.e. CFF_{25} and CFF_{10}) and for controls. After 48 h incubation at 25 °C, at least 100 conidia per Petri plate were inspected by direct observation on the underside of plates through a microscope at 200X magnification. Conidia were scored as germinated when the length of the germ tube exceeded the small-end diameter of the conidia. The conidial germination of treated (g_T) and control (g_C) plates was calculated as the ratio (in %) between the number of conidia germinated and the total number of conidia examined. Finally, the conidial germination inhibition (CGI) of *Heterobasidion* spp. was assessed by calculating the germination reduction (in %) observed in treated plates compared to control plates with the following equation:

$$\mathrm{CGI} = 100\% \cdot \frac{g_C - g_T}{g_C} \quad \text{(Equation 2)}$$

2.3 Comparative performances of P. protegens (DSMZ 13134) and state of the art treatments against Heterobasidion spp. on wood discs

Comparative experiments on wood discs were conducted using both Proradix® (SP Sourcon Padena GmbH, Tübeningen, Germany) and CFF_{25}, and the two main treatments used against *Heterobasidion* spp. in Europe, the biocontrol product Rotstop® (Verdera Oy, Espoo, Finland) and aqueous urea (Fluka, Cologno Monzese, Italy) solution (30% w/v) (Gonthier and Thor, 2013). The CFF_{25} was chosen instead of CFF_{10} due to its greater inhibitory effect against mycelial growth and conidial germination of *Heterobasidion* spp. (see results).

Three genotypes for each species of *H. annosum s.l.* displaying highest growth rates in control plates in dual culture assays were selected for this comparative experiment on wood discs (Table 1). Freshly cut woody discs 9 to 12 cm in diameter and 1-2 cm thick were obtained from healthy branches of *P.*

246 *pinea, A. alba,* and *P. abies* to mimic the substrate for natural infection, i.e. freshly cut stumps, of *H.*
247 *annosum* or *H. irregulare, H. abietinum* and *H. parviporum,* respectively. One surface (S_T) of each
248 disc was sprayed by using a sterilized Pirex® sprayer with either a treatment or with sterile water to be
249 used as a control until the surface became uniformly wet, i.e. approximately 1 mm thick layer.
250 Proradix® was prepared by using methods and doses (12 g L^{-1}) reported on the product label 12 hours
251 before the application on wood discs to foster the growth of bacteria. The CFF_{25} was prepared as
252 described in section 2.2.3, while Rotstop® was suspended in sterile water, according to the
253 instructions provided by the manufacturer. The urea treatment was prepared by dissolving urea in
254 sterile water. Four hours after treatment application, the S_T of discs was uniformly sprayed as
255 described above with a conidial suspension of *Heterobasidion* spp. Conidial suspensions were
256 prepared as described in section 2.2.4 at the concentration of 10^4 conidia mL^{-1}. Wood discs were
257 singly placed onto sterilized microscope slides in Petri plates (15 cm in diameter) containing pieces of
258 sterile filter paper dampened with sterile water. Petri plates were incubated at room temperature in the
259 dark while filter papers were dampened periodically with 1 mL of sterile water to provide an adequate
260 relative humidity for fungal growth.
261 After 8 days of incubation, discs were reversed and incubated at room temperature for additional 5
262 days. Hence, the untreated surface (S_{UNT}) of discs was inspected after 13 days of incubation under a
263 dissecting microscope (20X magnification) for the presence of *Heterobasidion* conidiophores as
264 previously described (Gonthier *et al.,* 2001, 2007). The area covered with *Heterobasidion*
265 conidiophores was delimited with a marker (Fig. 1) and redrawn on a transparent sheet. The sheet
266 was scanned at 1200 dpi and the area colonised by *Heterobasidion* spp. conidiophores was
267 measured (in mm^2) for each disc by using a virtual planimeter. A total of 600 wood discs were
268 analysed (10 repetitions per each combination of five treatments, four *Heterobasidion* species and
269 three genotypes per species).
270 To avoid using branches already infected by *Heterobasidion* spp. prior to the establishment of the
271 experiments, two discs from each branch were also incubated in Petri plates containing dampened

filter papers as described above. They were also inspected for the presence of *Heterobasidion* spp. conidiophores as previously described.

2.4 Statistical analyses

Data on antifungal activity (i.e. dual culture assays, effects of volatile compounds on mycelial growth, inhibition of mycelial growth and conidial germination by CFF, comparative experiments on wood discs) of *P. protegens* (DSMZ 13134) were analysed by using conditional inference tree models whose algorithm (Hothorn and Zeileis, 2015; Hothorn *et al.*, 2006) was set as described in Lione *et al.* (2020). For the dual culture assay (see section 2.2.1) and for the assessment of the effects of volatile compounds on mycelial growth (see section 2.2.2), the average MGI was calculated for each *Heterobasidion* spp., temperature level (10 °C and 25 °C) and culture medium (ISP2 and ISP2-s), along with the associated 95% Bias Corrected and accelerated (BCa) confidence interval ($CI_{95\%}$) (DiCiccio and Efron, 1996). The above confidence interval was obtained through the bootstrap iterative resampling method (Carsey and Harden, 2014) set as described in Lione *et al.* (2020). Conditional inference tree models were fitted separately for the different growing media, comparing the averages of MGI among the four *Heterobasidion* spp. for each temperature level, and between the two temperature levels for each *Heterobasidion* spp. The same statistical analyses were performed on data of inhibition of mycelial growth by CFF (see section 2.2.3), although in this case no models were fitted to compare the averages of MGI among *Heterobasidion* spp. at 25 °C and between temperature levels within species, because of the constant value displayed by MGI at 25 °C (see results). Conditional inference tree models and BCa $CI_{95\%}$ were applied as described for sections 2.2.1 and 2.2.2 to analyse the inhibition of conidial germination by CFF (see section 2.2.4), replacing the input variable MGI with CGI and the temperature levels with the two filtrates types CFF_{10} and CFF_{25}. To compare the efficacy of the different treatments on wood discs, conditional inference tree models and BCa $CI_{95\%}$ were applied separately for each *Heterobasidion* spp. by contrasting the

297 average values of area covered with *Heterobasidion* conidiophores on S_{UNT} of discs among the five
298 treatments.
299 All the analyses were carried out in R version 3.6.0 (R Core Team, 2019) with packages *bootstrap*
300 (Efron and Tibshirani, 1994), *partykit* (Hothorn and Zeileis 2015), and *strucchange* (Zeileis *et al.,*
301 2002). The significance threshold level was set at 0.05 for all tests comparing averages, while
302 comparisons between an average value (e.g. MGI, CGI) and a predefined threshold (i.e. 0) was
303 deemed significant at $P < 0.05$ if the threshold was located below or above the lower or upper bounds
304 of the BCa $CI_{95\%}$ (Crawley, 2013; Hosmer and Lemeshow, 1989). For the MGI and CGI indexes
305 reported in equations 1 and 2, the threshold 0 indicates no difference between treatment and control.
306

3. Results

3.1 Antifungal activity of P. protegens (DSMZ 13134) against Heterobasidion spp.

3.1.1 Dual culture assays

Pseudomonas protegens (DSMZ 13134) inhibited mycelial growth regardless of *Heterobasidion* species, culture medium and incubation temperature (Fig. 2.). Mycelial growth inhibition (MGI) varied depending on *Heterobasidion* species and was significantly lower at 10 °C than that observed at 25 °C (Fig. 2.). On ISP2 medium after 7 days of incubation at 25 °C, MGI was highest for *H. abietinum* and *H. parviporum* (83% and 81%, respectively), and significantly lower for *H. annosum* and *H. irregulare* (75% and 76%, respectively) (Fig. 2a.). On ISP2 after 11 days at 10 °C, the MGI was significantly higher for *H. abietinum* and *H. irregulare* (68% and 60%, respectively) compared to *H. annosum* and *H. parviporum* (40% and 47%, respectively) (Fig. 2a.). On ISP2-s after 7 days of incubation at 25 °C, MGI was highest for *H. abietinum, H. irregulare* and *H. parviporum* (73%, 68%, 69%, respectively), and significantly lower for *H. annosum* (59%) (Fig. 2b.). On the same medium after 11 days of incubation at 10°C , again MGI was highest for *H. abietinum, H. irregulare* and *H. parviporum* (48%, 57%, 54%, respectively) and significantly lower (29%) for *H. annosum* (Fig. 2b.).

3.1.2 Effects of volatile compounds on mycelial growth

On ISP2, MGI varied depending on *Heterobasidion* species and was significantly lower at 25 °C than at 10 °C for *H. abietinum* and *H. parviporum* (Fig. 3a.). At both incubation temperatures, *H. annosum* was the least inhibited species (4 and 12% of MGI at 25 °C and 10 °C, respectively) (Fig. 3a.). On ISP2-s, MGI was significantly higher at 25 °C than at 10 °C (Fig. 3b.). After 7 days at 25 °C, the MGI was highest for *H. annosum* and *H. parviporum* (24 and 21%, respectively), compared to the other two species.

3.1.3 Inhibition of mycelial growth by cell-free filtrate (CFF)

CFF_{25} and CFF_{10} effectively inhibited mycelial growth of *Heterobasidion* spp. after 7 days of incubation (Table 2). CFF_{25} resulted in 100% MGI for all tested fungal pathogens (Table 2 and Fig. 4.). Inhibitory effect of CFF_{10} was significantly higher for *H. abietinum* and *H. parviporum* (76% and 75%, respectively), and lower for *H. annosum* and *H. irregulare* (70%, and 66%, respectively) (Table 2).

3.1.4 Inhibition of conidial germination by cell-free filtrates (CFF)

The addition of CFF_{25} and CFF_{10} in water agar medium had a drastic effect on conidial germination compared to controls, regardless of *Heterobasidion* species (Table 3). Conidial germination inhibition (CGI) was significantly lower with CFF_{10} than with CFF_{25}. After 48 h of incubation, CFF_{25} resulted in at least 98% CGI without significant differences among pathogens. CFF_{10} resulted in values of CGI significantly higher for *H. irregulare* and *H. parviporum* (92% and 88%, respectively), compared to *H. abietinum* and *H. annosum* (84% and 87%, respectively).

3.2 Comparative performances of P. protegens (DSMZ 13134) and state of the art treatments against Heterobasidion spp. on wood discs

In general, treatments resulted in a significant reduction of colonised areas of wood discs by the fungal pathogens compared to controls, with the exception of Proradix® for *H. annosum*, and of Proradix® and *P. protegens* (DSMZ 13134) CFF_{25} for *H. irregulare* (Fig. 5a-d.). CFF_{25} performed significantly better than Proradix® when used against all *Heterobasidion* species, with the exception of *H. irregulare*, against which they performed similarly. In experiments against *H. abietinum*, CFF_{25} reduced to a significantly greater extent than Rotstop® the area colonised by the pathogen compared to controls, and was almost as effective as the best treatment urea (Fig. 5a). Against *H. annosum*, CFF_{25} was as effective as the state of the art treatments Rotstop® and urea (Fig. 5b). Against *H. irregulare*, CFF_{25} was not effective as state of the art treatments (Fig. 5c), and the same was true for *H. parviporum*, despite in this case there was a significant and substantial reduction of colonised area

358 compared to controls (Fig. 5d). Treatment with urea always resulted in 0 mm^2 of area colonised by all
359 the tested *Heterobasidion* species.
360
361 **4. Discussion**
362 This study is the first dealing with the effects of *P. protegens* (DSMZ 13134) against all
363 *Heterobasidion* species present in Europe. Previous pioneering reports referred to pilot studies
364 conducted against a single *Heterobasidion* species, i.e. *H. parviporum* (Gžibovska, 2016; Rönnberg
365 and Magazniece, 2018).
366 In dual culture experiments, *P. protegens* (DSMZ 13134) proved to be a strong antagonist of all the
367 tested *Heterobasidion* species regardless of culture medium and incubation temperature.
368 Nevertheless, the antifungal activity of the bacterium was significantly affected by the incubation
369 temperature. The higher antagonistic activity at 25 °C than at 10 °C than may be due to environmental
370 conditions closest to the optimum for the bacterium. It should be noted that the optimum growth
371 temperature of *P. protegens* (DSMZ 13134) was recorded at 26 °C (Giovanni Amenta, personal
372 communication).
373 Biological control involves different mechanisms including competition for nutrients and space,
374 induction of plant defensive mechanisms, and antibiosis (Compant *et al.,* 2005; Mérillon and
375 Ramawat, 2012). The clear inhibition zone in dual culture assays precluding contact between the
376 bacterium and the fungal genotypes, suggests that antibiosis through the production of antifungal
377 compounds could be the main mechanism involved in the inhibition of *Heterobasidion* spp. by *P.*
378 *protegens* (DSMZ 13134), as previously documented for other plant pathogens (Roberti *et al*., 2012).
379 The production of antibiotics by bacteria has been reported to be affected by several abiotic factors,
380 including temperature (Raaijmakers *et al*., 2002; Shanahan *et al*., 1992). Further, the production of
381 antibiotics may be regulated by multiple genes that could possess different temperature thresholds
382 (Daskin *et al*., 2014). In our case, the observed reduction in mycelial growth inhibition (MGI) of

383 *Heterobasidion* spp. may be caused by different antifungal compounds, whose production by *P.*
384 *protegens* (DSMZ 13134) and/or activity is mediated by temperature.
385 It has been previously suggested that the culture medium supplemented with sawdust is the closest
386 medium to the woody natural substrate (Mgbeahuruike *et al.,* 2011), providing a realistic approach to
387 understand the fungal behaviour in woody substrates in the field (Woods *et al.*, 2005). Overall, *P.*
388 *protegens* (DSMZ 13134) showed a lower inhibition activity in culture medium supplemented with
389 sawdust compared to the non-supplemented medium. This may be due either to the stimulation of
390 *Heterobasidion* spp. operated by sawdust in the dual culture assay or by the inhibition of the
391 bacterium or the suppression of production of antibiotics, or by all the above hypotheses since these
392 are not mutually exclusive. It should be noted that while *Heterobasidion* spp. are fungi strictly
393 associated with wood (Garbelotto and Gonthier, 2013), *P. protegens* is a PGPR associated with soil
394 (Ramette *et al*., 2011), hence it is not surprising that dual culture assays on medium supplemented
395 with sawdust resulted in a lower inhibition activity of the bacterium compared to the non-
396 supplemented medium.
397 Regardless of the temperature of incubation and the culture medium, mycelial growth inhibition (MGI)
398 determined by *P. protegens* (DSMZ 13134) in dual culture assays was constantly lower for *H.*
399 *annosum* than for the other *Heterobasidion* species, suggesting that the outcomes of the interaction
400 are not uniform across *Heterobasidion* species, but rather they may be taxon-specific.
401 Previous studies demonstrated the antifungal nature of volatile compounds produced by bacteria,
402 including *Pseudomonas* spp., suggesting a potential role of these compounds in the management of
403 fungal plant pathogens (Cornelison *et al.,* 2014*;* Fernando *et al.,* 2005; Gabriel *et al.,* 2018; Yuan *et*
404 *al.,* 2012). In the current study, *P. protegens* (DSMZ 13134) showed a much weaker ability to inhibit
405 *Heterobasidion* species through volatile compounds compared to the combined exposure of both
406 diffusible and volatile compounds in dual culture assays. This finding supports the hypothesis that the
407 antifungal compounds responsible for the inhibition of *Heterobasidion* spp. are more likely diffusible
408 than volatile. It is worth noting that an opposite effect of incubation temperatures was observed on

ISP2 culture medium. In fact, while MGI of *Heterobasidion* spp. was greater at 25 °C than at 10 °C in dual culture assays, the opposite was true when only the effect of volatile compounds was tested. As discussed previously for antibiotics, also the production and effectiveness of antifungal volatile compounds are influenced by several abiotic factors, including temperature (Gabriel *et al.,* 2018). These observations stress the importance of considering environmental conditions when assessing the effectiveness of biocontrol agents. Again, weaker performances of volatile compounds were observed on medium supplemented with sawdust compared to the non-supplemented medium. The reasons for that may be similar to those described previously for the dual culture assays.

The prominent role of antibiotics and diffusible antifungal compounds produced by *P. protegens* (DSMZ 13134) in the interaction with *Heterobasidion* spp. is also demonstrated by the outcomes of experiments using cell free filtrates (CFF), both CFF_{25} and CFF_{10}, and both on mycelial growth and conidia germination. These findings prompt a comprehensive analysis of specific compounds produced by *P. protegens* (DSMZ 13134) and involved in the inhibition of *Heterobasidion* spp. Several strains of *Pseudomonas* spp. have been reported to produce a plethora of antifungal compounds, such as hydrogen cyanide, pyoluteorin, phenazines, siderophores, cyclic lipopeptides, 2,4-diacetylphloroglucinol, and pyrrolnitrin (Compant *et al.,* 2005, Mishra and Arora, 2018; Zhang *et al.,* 2010). Whether these compounds, or other antifungal compounds are produced by *P. protegens* (DSMZ 13134), and whether some of these alone or in combination with each other may be responsible for the inhibition of *Heterobasidion* spp. will deserve further investigation.

The inhibition of conidial germination by CFF, which is almost complete when CFF is produced at 25 °C (CFF_{25}), suggests a potential application of CFF on fresh stump surfaces during logging operations in the attempt to prevent *Heterobasidion* spp. airborne infections. Although in nature conidia may not be as abundant as spores (Garbelotto and Gonthier, 2013), such infectious propagules have been widely used in artificial inoculation studies to mimic *Heterobasidion* basidiospore infection on logs, on billets and on stumps (Annesi *et al.,* 2005; Sun *et al.,* 2009a, 2009b; Lehtijärvi *et al*., 2011).

In this study we used conidial suspensions not only to test the effects of CFF on conidia germination, but also to mimic natural spore deposition on treated or untreated wood discs simulating stumps. On untreated control wood discs, the areas colonised by *H. abietinum* and *H. parviporum* on discs of *A. alba* and *P. abies*, respectively, were much larger than those colonised by *H. annosum* and *H. irregulare* on discs of *P. pinea*. This may be due to the wood colonisation ability of the fungal species, to the host wood, or to the interaction between fungal and host wood species. While the elucidation of factors associated with the colonisation of wood by *Heterobasidion* spp. was not listed in the aims of this study, it is worth noting that on the same wood substrate (i.e. *P. pinea*), *H. irregulare* colonised a larger area than *H. annosum* did, which is consistent with previous findings pointing to a greater saprobic ability of the non-native *H. irregulare* compared to the native *H. annosum* on pine wood (Giordano *et al*., 2014). In these comparative experiments on wood discs, treatments with *P. protegens* (DSMZ 13134) CFF performed significantly better than Proradix® against most of the *Heterobasidion* species, further confirming on wood that the antagonistic activity hinges on antibiotics produced by the bacterium and present in the CFF rather than on other mechanisms.

P. protegens (DSMZ 13134) CFF significantly reduced the areas colonised by *H. annosum* and *H. abietinum* to the level of state of the art treatments. Against this latter species, *P. protegens* (DSMZ 13134) CFF performed even significantly better, although not substantially, than Rotstop®. Although *P. protegens* (DSMZ 13134) CFF did not performed as well as state of the art treatments against *H. parviporum*, the reduction of the colonised area occupied by the fungus compared to control was significant and substantial.

While both *P. protegens* (DSMZ 13134) treatments or at least CFF proved to be effective against native European *Heterobasidion* spp., neither CFF nor Proradix® showed significant effects against the non-native *H. irregulare* on wood discs. This observation is in contrast with the efficacy of treatments against *H. irregulare* recorded in *in vitro* experiments, suggesting a possible role played by the wood disc in the interaction between the biological control agent and the fungal pathogen. We cannot exclude that the observed response could be related to the chemical-physical properties of the

P. pinea wood used in this experiment. However, this hypothesis should be tested through appropriate experiments.

Comparative experiments on wood discs provided few additional side information: 1- the suitability of using wood discs not only for studying the epidemiology of *Heterobasidion* spp. but also for testing the efficacy of treatments against these fungal plant pathogens; 2- the overall efficacy of state of the art treatments Rotstop® and, especially, urea. While this last treatment was already proved to be effective against native European *Heterobasidion* spp. on stumps of several tree species (Gonthier, 2019), data presented in this paper are the first pointing to the efficacy of urea against the non-native *H. irregulare*.

The efficacy of treatments is commonly evaluated in terms of average performance, but the variability of data displayed on wood discs by both Proradix® and *P. protegens* (DSMZ 13134) CFF against all the *Heterobasidion* species may imply that their application in the forest could lead to variable and perhaps uncertain results. However, this hypothesis needs to be tested by *ad hoc* experiments on stumps in the forest.

In conclusion, *P. protegens* (DSMZ 13134) and especially the CFF of the bacterium showed for the first time a potential against all the native European *Heterobasidion* species, by performing mostly as well as state of the art chemical or biological treatments. Based on experiments on wood discs in controlled conditions, the tested treatments seem poorly effective against the non-native *H. irregulare*. Nevertheless, as the results point to the antibiosis as the main mode of action, the efficacy of treatments may be significantly improved by identifying the active molecules and/or by optimizing their production or application, making *P. protegens* (DSMZ 13134) a suitable candidate for the biological control of root and butt rots caused by *Heterobasidion* spp.

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732

733 **Tables**

734

735 **Table 1**

MUT[a] accession number	Isolation date	*Heterobasidion* species	Geographic origin
6,198*	2016	*H. abietinum*	Chabodey, AO, Italy
6,194*	2016	*H. abietinum*	Nus, AO, Italy
6,195	2018	*H. abietinum*	Chiusa di Pesio, CN, Italy
6,196	2018	*H. abietinum*	Chiusa di Pesio, CN, Italy
6,197*	2018	*H. abietinum*	Chiusa Pesio, CN, Italy
1,204	2005	*H. annosum*	Sabaudia, LT, Italy
3,538*	2006	*H. annosum*	Ansedonia, GR, Italy
3,656	2006	*H. annosum*	Sabaudia, LT, Italy
3,543*	2006	*H. annosum*	Mesola, FE, Italy
6,191*	2015	*H. annosum*	Saint-Denis, AO, Italy
5,666	2006	*H. irregulare*	Nettuno, RM, Italy
3,627	2005	*H. irregulare*	Sabaudia, LT, Italy
1,197*	2005	*H. irregulare*	Sabaudia, LT, Italy
1,151*	2005	*H. irregulare*	Sabaudia, LT, Italy
1,193*	2005	*H. irregulare*	Castelfusano, RM, Italy
6,192*	2016	*H. parviporum*	Chabodey, AO, Italy
5,605*	2006	*H. parviporum*	Druogno, VB, Italy
5,612*	2006	*H. parviporum*	Trasquera, VB, Italy
5,615	1999	*H. parviporum*	Charvensod, AO, Italy
6,193	2016	*H. parviporum*	Chabodey, AO, Italy

736 [a] MUT: Mycotheca Universitatis Taurinensis

737

738

739 **Table 2**

740

CFF	*Heterobasidion* species	MGI (%)
CFF_{25}	*H. abietinum*	100 (-)
	H. annosum	100 (-)
	H. irregulare	100 (-)
	H. parviporum	100 (-)
CFF_{10}	*H. abietinum*	76.35a (74.64-78.18)
	H. annosum	70.32b (69.11-71.36)
	H. irregulare	66.37c (64.66-68.14)
	H. parviporum	74.96a (73.50-76.01)

741

742

743 **Table 3**

744

CFF	*Heterobasidion* species	CGI (%)
CFF_{25}	*H. abietinum*	99.08a;A (98.46-99.59)
	H. annosum	98.40a;A (97.74-98.99)
	H. irregulare	98.93a;A (98.24-99.44)
	H. parviporum	99.01a;A (98.57-99.37)
CFF_{10}	*H. abietinum*	83.74b;B (82.45-85.31)
	H. annosum	86.71b;B (82.30-91.53)
	H. irregulare	91.87a;B (88.07-94.78)
	H. parviporum	88.40a;B (86.33-90.67)

745
746

747 **Table captions**

748

749 **Table 1** *Heterobasidion* genotypes used in this study. Asterisks after the accession numbers indicate
750 genotypes selected for comparative experiments on wood discs.

751

752 **Table 2** Comparison of the average values of mycelial growth inhibition (MGI) of *Heterobasidion* spp.
753 genotypes treated with cell-free filtrate (CFF) of *Pseudomonas protegens* (DSMZ 13134) produced at
754 25 °C (CFF_{25}) and 10 °C (CFF_{10}). For each combination of CFF and *Heterobasidion* species, the
755 average values of MGI are reported along with the associated 95% Bias Corrected and accelerated
756 (BCa) confidence interval ($CI_{95\%}$), if available. The symbol – indicates unavailable confidence interval
757 due to constant values displayed by MGI. Different letters after numbers indicate significant
758 differences ($P < 0.05$) among average values.

759

760 **Table 3** Comparison of the average values of conidial germination inhibition (CGI) of *Heterobasidion*
761 spp. genotypes treated with cell-free filtrate (CFF) of *Pseudomonas protegens* (DSMZ 13134)
762 produced at 25 °C (CFF_{25}) and 10 °C (CFF_{10}). For each combination of CFF and *Heterobasidion*
763 species, the average values of CGI along with the associated 95% Bias Corrected and accelerated
764 (BCa) confidence interval ($CI_{95\%}$) are reported. Significant differences ($P < 0.05$) of CGI values
765 between *Heterobasidion* species for each CFF are identified by lowercase letters, while between CFF
766 for each *Heterobasidion* species by uppercase letters.

767

768

769 **Figure captions**

770

771 **Fig. 1.** Wood disc of *Abies alba* with areas colonised by *Heterobasidion abietinum* (marked areas).

772

773 **Fig. 2.** Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI)
774 resulting from the dual culture assays with *Pseudomonas protegens* (DSMZ 13134) on ISP2 (panel a)
775 and ISP2-s (panel b) culture media at 10 °C and 25 °C. For each combination of culture medium,
776 temperature and *Heterobasidion* species, the average values of MGI are reported (circular and
777 triangular dots) along with the associated 95% Bias Corrected and accelerated (BCa) confidence
778 interval ($CI_{95\%}$) (whiskers). Significant differences ($P < 0.05$) of MGI values between *Heterobasidion*
779 species at each temperature level are identified by lowercase letters, while between temperature
780 levels for each *Heterobasidion* species by uppercase letters.

781

782 **Fig. 3.** Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI)
783 resulting from the trials testing the effects of volatile compounds of *Pseudomonas protegens* (DSMZ
784 13134)on mycelial growth on ISP2 (panel a) and ISP2-s (panel b) culture media at 10 °C and 25 °C.
785 For each combination of culture medium, temperature and *Heterobasidion* species, the average
786 values of MGI are reported (circular and triangular dots) along with the associated 95% Bias
787 Corrected and accelerated (BCa) confidence interval ($CI_{95\%}$) (whiskers). Significant differences ($P <$
788 0.05) of MGI values between *Heterobasidion* species at each temperature level are identified by
789 lowercase letters, while between temperature levels for each *Heterobasidion* species by uppercase
790 letters.

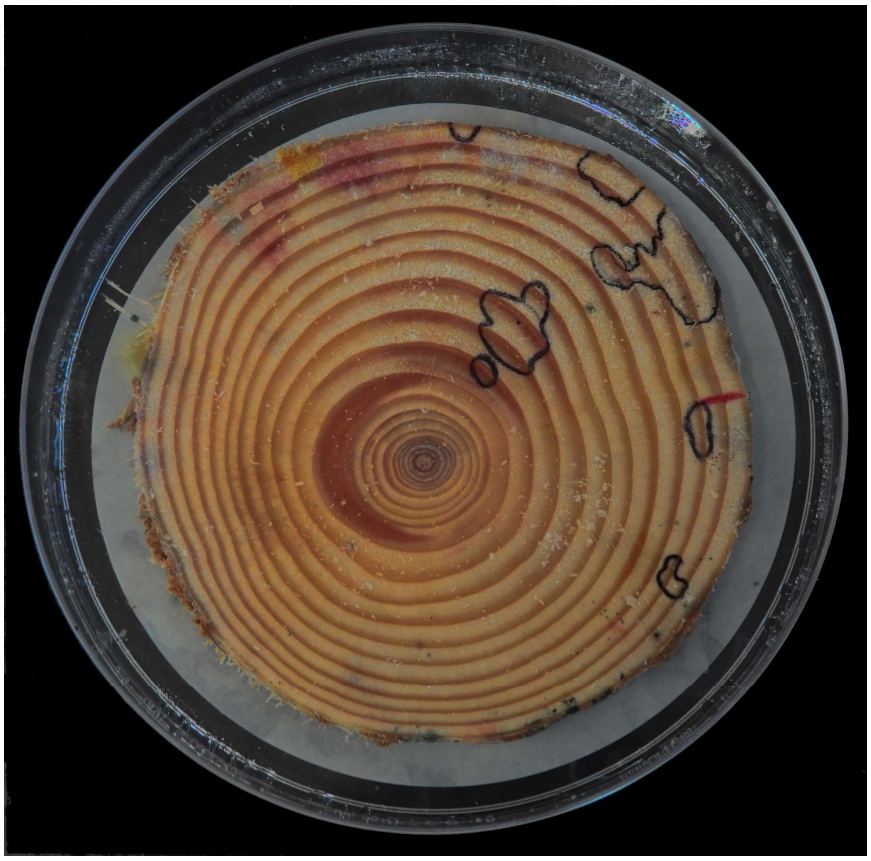
791

792 **Fig. 4.** Inhibition of mycelial growth of *Heterobasidion* spp. caused by CFF_{25} of *Pseudomonas*
793 *protegens* (DSMZ 13134). The mycelial growth of a single genotype of *H. abietinum* (a), *H. annosum*

(b), *H. irregulare* (c), and *H. parviporum* (d) on 80% CFF_{25} culture medium (left side) and on 80% LB culture medium (right side) after 7 days of incubation at 25 °C.

Fig. 5. Comparative performances of *Pseudomonas* sp. DSMZ 13134 and state of the art treatments against *Heterobasidion abietinum* (panel a), *H. annosum* (panel b), *H. irregulare* (panel c) and *H. parviporum* (panel d) on wood discs. For each treatment (wa - water; pr - Proradix®; cff - CFF_{25}; ro - Rotstop®; and ur - urea) the average area colonised by *Heterobasidion* spp. (mm^2) on the untreated surface is shown (circular dots) along with the associated 95% Bias Corrected and accelerated (BCa) confidence interval ($CI_{95\%}$) (whiskers). Different letters indicate a significant difference ($P < 0.05$) among the average values associated with each treatment.

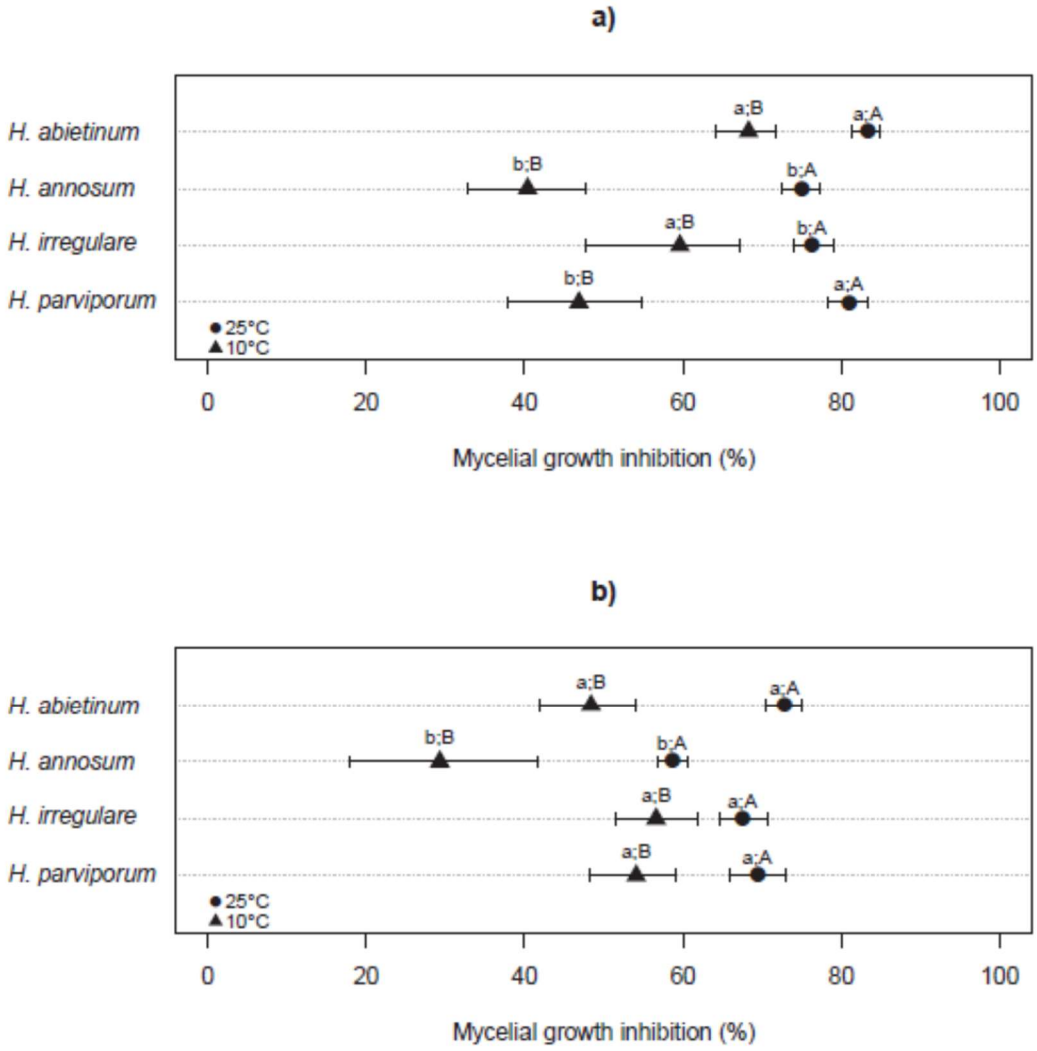
806 FIG. 1



807

808

809 FIG. 2



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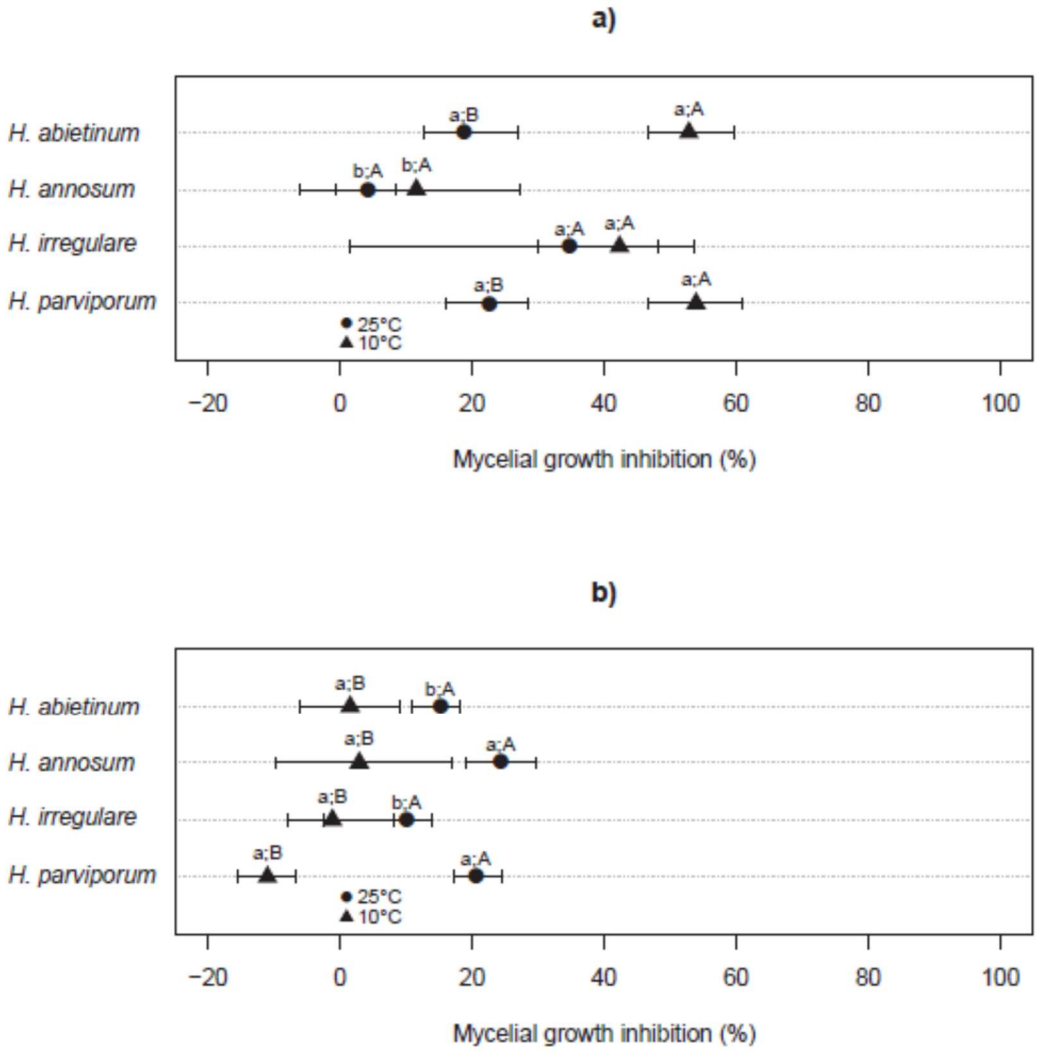
811

812 FIG. 3



813

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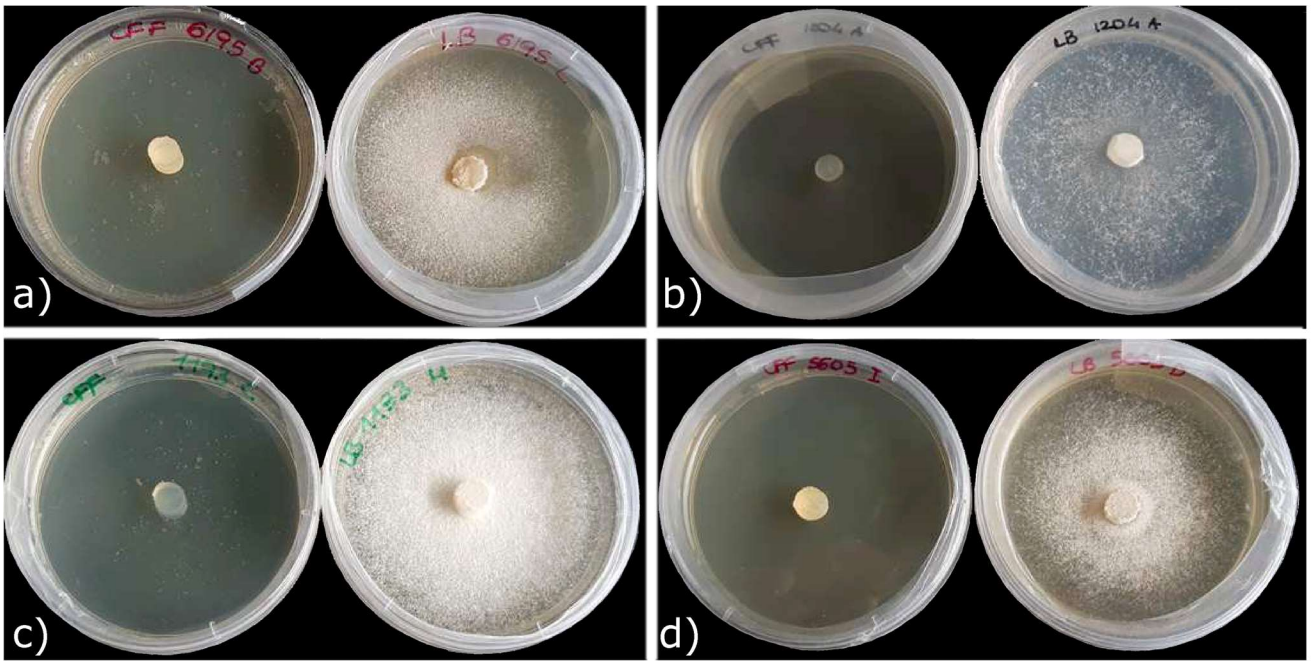
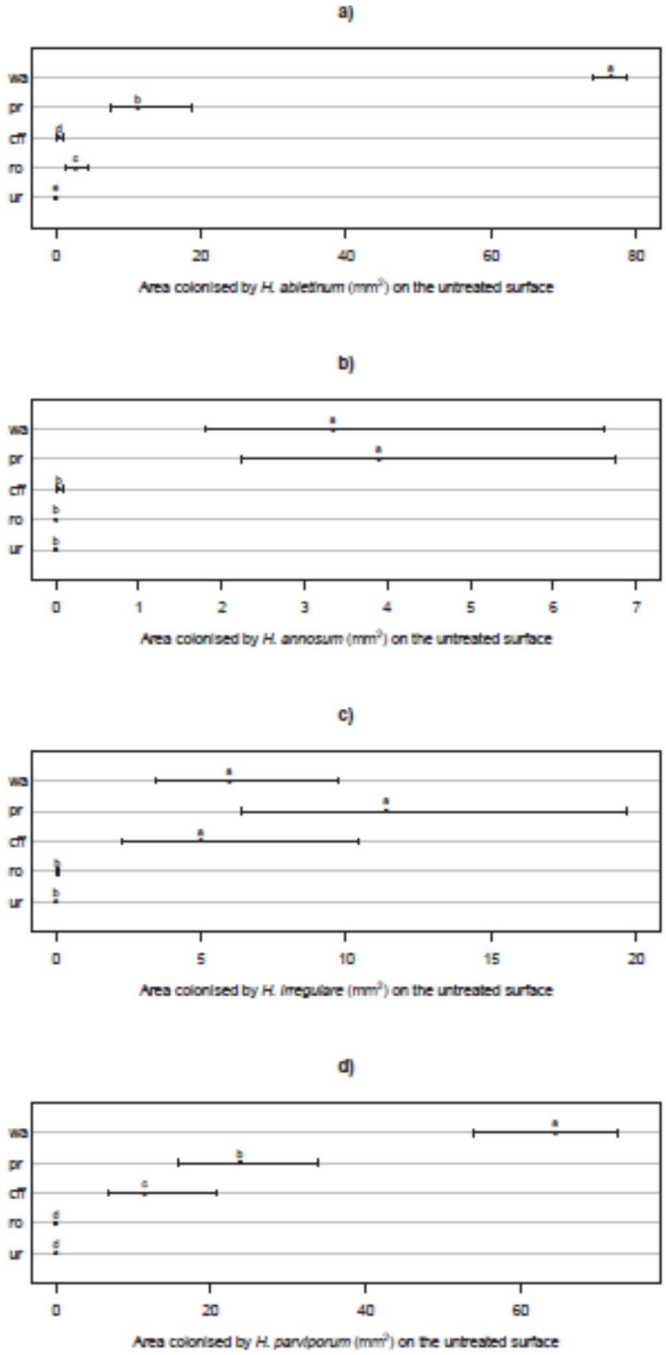
FIG. 4



818 FIG. 5


a)
wa
pr
cff
ro
ur
0
20
40
60
80
Area colonised by H. abietinum (mm²) on the untreated surface
b)
wa
pr
cff
ro
ur
0
1
2
3
4
5
6
7
Area colonised by H. annosum (mm²) on the untreated surface
c)
wa
pr
cff
ro
ur
0
5
10
15
20
Area colonised by H. irregulare (mm²) on the untreated surface
d)
wa
pr
cff
ro
ur
0
20
40
60
Area colonised by H. parviporum (mm²) on the untreated surface

819