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

Martina Pellicciaro, Guglielmo Lione, Luana Giordano[†], Paolo Gonthier[*]

Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino,

Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy

Martina Pellicciaro: martina.pellicciaro@unito.it, https://orcid.org/0000-0001-6778-723X

Guglielmo Lione: guglielmo.lione@unito.it, https://orcid.org/0000-0002-3777-0813

Luana Giordano: luana_giordano_cnt@regione.lombardia.it, https://orcid.org/0000-0003-1686-6338

Paolo Gonthier: paolo.gonthier@unito.it, https://orcid.org/0000-0002-7242-8239

[*]Corresponding author

E-mail address: paolo.gonthier@unito.it

[†] Current affiliation: Laboratory of Lombardy Plant Health Service, c/o Fondazione Minoprio, Viale

Raimondi 54, Vertemate con Minoprio, I-22070 (CO), Italy.

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*.

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64 **Declarations of interest:** none.

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1. Introduction

Root and butt rots caused by the fungal species complex *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (*s.l.*) stand among the most destructive diseases of conifers worldwide (Garbelotto and Gonthier, 2013). Financial losses associated with the three European species of *H. annosum s.l.*, i.e. *H. abietinum* Niemelä & Korhonen, *H. annosum sensu stricto* (*s.s.*), hereafter referred to as *H. annosum*, and *H. parviporum* Niemelä & Korhonen, were estimated a few decades ago at 790 million Euros per year due to a reduction in both wood quality and productivity (Woodward *et al.*, 1998). As previously recognized, losses are likely higher because this estimate did not take into account the reduction of resistance of forest stands associated with the presence of these fungi during storms, which may be locally relevant (Garbelotto and Gonthier, 2013; Woodward *et al.*, 1998).

In Europe, losses associated with *H. annosum s.l.* increased as a result of the introduction of the north American species *H. irregulare* Garbel. & Otrosina, which became invasive and is now distributed in coastal pine stands of central Italy (Gonthier *et al.*, 2004, 2007, 2012, 2014). Based on its current and potential impact, *H. irregulare* is a quarantine plant pest recommended for regulation under the European and Mediterranean Pant Protection Organization (EPPO) A2 list..

The distribution in Europe of the four *Heterobasidion* species was previously reviewed (Garbelotto and Gonthier, 2013). Some of the species display distinct host preference. In fact, while both the native *H. annosum* and the non-native *H. irregulare* are associated with pines (*Pinus* spp.), *H. abietinum* and *H. parviporum* preferentially attack *Abies alba* Mill. and *Picea abies* (L.) Karst., respectively (Garbelotto and Gonthier, 2013).

Regardless of the *Heterobasidion* species, spreading occurs by means of both basidiospores (primary infection) and mycelium (secondary infection) (Garbelotto and Gonthier, 2013). Primary infection takes place as a result of landing and germination of airborne basidiospores on freshly cut stump surfaces or, to a lesser extent, on wounds. Once the primary infection has occurred, mycelium spreads to the root system and eventually to neighbouring trees if root contacts are present (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

118 component of the bio fungicide Proradix® (SP Sourcon Padena GmbH, Tübingen, Germany).

119 According to the manufacturer of Proradix®, this bacterial strain is a naturally occurring PGPR, it is not

120 a genetically modified organism (GMO), and it is non-toxic and non-pathogenic to plants. Proradix®

121 was effective against silver scab caused by *Helminthosporium solani* Durieu & Mont. and stem canker

122 and black scurf caused by *Rhizoctonia solani* J.G. Kühn. Although Proradix® is currently targeting soil

123 borne pathogens, some evidences point to the ability of its active component to synthesise antifungal

124 compounds potentially effective against a range of fungal plant pathogens (Buddrus-Schiemann *et al.*,

125 2010; Compant *et al.*, 2005; Fröhlich *et al.*, 2012). Indeed, Roberti *et al.* (2012) reported antibiosis to

126 be the main mode of action of *P. protegens* (DSMZ 13134) in the control of zucchini foot and root rot

127 caused by the race 1 of *Fusarium solani* f.sp. *cucurbitae* W.C. Snyder & H.N. Hansen. Interestingly,

128 Proradix® proved to be efficient against *H. parviporum in vitro* and in a pilot field study on Norway

129 spruce stumps (Gžibovska, 2016; Rönnberg and Magazniece, 2018), making it a good candidate for

130 further assessments against all *Heterobasidion* spp. present in Europe.

131 In general, before field trials, the efficacy of potential biocontrol agents against *Heterobasidion* spp. is

132 assessed through experiments both *in vitro* and in controlled conditions on substrates such as wood

133 (Holdenrieder *et al.*, 1998). Experiments have been conducted using either billets (Sun *et al*., 2009a

134 and 2009b; Zaluma *et al*., 2019) or wood blocks (Nicolotti and Varese, 1996; Oliva *et al.,* 2015; Samils

135 *et al.,* 2008). In this study, we used wood samples reported to simulate freshly cut stump surfaces,

136 which are right the infection courts of *Heterobasidion* spp. and hence the target of treatments. These

137 wood samples, known as wood discs, although never employed for testing the efficacy of treatments

138 are widely used for investigating the epidemiology of *Heterobasidion* spp. (Gonthier *et al.*, 2001, 2007,

139 2012).

140 In this study, we tested the biocontrol potential of *P. protegens* (strain DSMZ 13134) towards the four

141 *Heterobasidion* spp. currently occurring in Europe. This was carried out by (i) determining the

142 inhibitory effect of both the bacterium itself and cell-free filtrate *in vitro*, (ii) assessing the role of

143 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:

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

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.

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195   genotypes were grown. The same growing media, incubation temperatures, *Heterobasidion* spp.

196   genotypes, number of replicates and MGI assessment method described in section 2.2.1 were used.

197

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

199   The CFF of *P. protegens* (DSMZ 13134) containing extracellular metabolites of the bacterium was

200   tested for its inhibitory activity against mycelial growth of *Heterobasidion* spp. at 25 °C. The incubation

201   temperature of 10 °C was omitted in this and in subsequent experiments because very little

202   *Heterobasidion* spp. mycelial growth was observed at this temperature in dual culture assays. The

203   CFF was prepared by culturing *P. protegens* (DSMZ 13134) in LB broth with constant shaking for 24 h

204   at both 25 °C (OD_{600} of 1.1) and 10 °C (OD_{600} of 0.3) to determine whether CFF prepared at different

205   temperatures may perform differently towards *Heterobasidion* spp. Cells were pelleted by

206   centrifugation at 4,000 rpm for 10 min, and the supernatant was filtered aseptically through a 0.22 μm

207   filter membrane to obtain CFF free from bacterial cells (CFF_{25} and CFF_{10}). CFF was incorporated into

208   the water agar medium (17 g L^{-1}) maintained at 50 °C to the final concentration of 80% (v/v). Petri

209   plates filled with 80% (v/v) LB broth instead of CFF served as controls. Mycelial plugs of

210   *Heterobasidion* spp. genotypes were individually placed at the centre of Petri plates. Petri plates were

211   then incubated until the fungal colonies in the control plates had grown to fill the plate. Experiments

212   were performed with ten replicates per each *Heterobasidion* genotype. MGI was determined as

213   described in section 2.2.1 after 7 days of incubation.

214

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

216   The CFF_{25} and CFF_{10} described in section 2.2.3 were also used to assess the inhibition potential of

217   extracellular metabolites of *P. protegens* (DSMZ 13134) on *Heterobasidion* spp. conidial germination.

218   The conidia of *Heterobasidion* spp. were obtained as follows. 500 μL of sterile water was loaded on

219   the surface of 7-10 days old fungal colonies previously incubated at 25 °C in the dark. The water was

220   gently shaked and collected by using a pipette. The concentration of conidia in the suspension was

221 assessed by using a Bürker chamber, and the conidial suspension was subsequently adjusted to 10^5

222 conidia mL^{-1}. The same growing media described in section 2.2.3 were used and poured as a thin

223 layer (2 mm) in Petri plate. 100 μL of conidial suspension was loaded in Petri dishes and uniformly

224 distributed using an L-shaped sterile Drigalski spatula. Three replicates per *Heterobasidion* spp.

225 genotypes were used for each treatment (i.e. CFF_{25} and CFF_{10}) and for controls. After 48 h incubation

226 at 25 °C, at least 100 conidia per Petri plate were inspected by direct observation on the underside of

227 plates through a microscope at 200X magnification. Conidia were scored as germinated when the

228 length of the germ tube exceeded the small-end diameter of the conidia. The conidial germination of

229 treated (g_T) and control (g_C) plates was calculated as the ratio (in %) between the number of conidia

230 germinated and the total number of conidia examined. Finally, the conidial germination inhibition

231 (CGI) of *Heterobasidion* spp. was assessed by calculating the germination reduction (in %) observed

232 in treated plates compared to control plates with the following equation:

233 $\text{CGI} = 100\% \cdot \dfrac{g_C - g_T}{g_C}$ (Equation 2)

234

235 *2.3 Comparative performances of P. protegens (DSMZ 13134) and state of the art treatments against*

236 *Heterobasidion spp. on wood discs*

237 Comparative experiments on wood discs were conducted using both Proradix® (SP Sourcon Padena

238 GmbH, Tübeningen, Germany) and CFF_{25}, and the two main treatments used against *Heterobasidion*

239 spp. in Europe, the biocontrol product Rotstop® (Verdera Oy, Espoo, Finland) and aqueous urea

240 (Fluka, Cologno Monzese, Italy) solution (30% w/v) (Gonthier and Thor, 2013). The CFF_{25} was

241 chosen instead of CFF_{10} due to its greater inhibitory effect against mycelial growth and conidial

242 germination of *Heterobasidion* spp. (see results).

243 Three genotypes for each species of *H. annosum s.l.* displaying highest growth rates in control plates

244 in dual culture assays were selected for this comparative experiment on wood discs (Table 1). Freshly

245 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

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

273   conidiophores as previously described.

274

275   *2.4 Statistical analyses*

276   Data on antifungal activity (i.e. dual culture assays, effects of volatile compounds on mycelial growth,

277   inhibition of mycelial growth and conidial germination by CFF, comparative experiments on wood

278   discs) of *P. protegens* (DSMZ 13134) were analysed by using conditional inference tree models

279   whose algorithm (Hothorn and Zeileis, 2015; Hothorn *et al.*, 2006) was set as described in Lione *et al.*

280   (2020). For the dual culture assay (see section 2.2.1) and for the assessment of the effects of volatile

281   compounds on mycelial growth (see section 2.2.2), the average MGI was calculated for each

282   *Heterobasidion* spp., temperature level (10 °C and 25 °C) and culture medium (ISP2 and ISP2-s),

283   along with the associated 95% Bias Corrected and accelerated (BCa) confidence interval ($CI_{95\%}$)

284   (DiCiccio and Efron, 1996). The above confidence interval was obtained through the bootstrap

285   iterative resampling method (Carsey and Harden, 2014) set as described in Lione *et al.* (2020).

286   Conditional inference tree models were fitted separately for the different growing media, comparing

287   the averages of MGI among the four *Heterobasidion* spp. for each temperature level, and between the

288   two temperature levels for each *Heterobasidion* spp. The same statistical analyses were performed

289   on data of inhibition of mycelial growth by CFF (see section 2.2.3), although in this case no models

290   were fitted to compare the averages of MGI among *Heterobasidion* spp. at 25 °C and between

291   temperature levels within species, because of the constant value displayed by MGI at 25 °C (see

292   results). Conditional inference tree models and BCa $CI_{95\%}$ were applied as described for sections

293   2.2.1 and 2.2.2 to analyse the inhibition of conidial germination by CFF (see section 2.2.4), replacing

294   the input variable MGI with CGI and the temperature levels with the two filtrates types CFF_{10} and

295   CFF_{25}. To compare the efficacy of the different treatments on wood discs, conditional inference tree

296   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)

332   CFF$_{25}$ and CFF$_{10}$ effectively inhibited mycelial growth of *Heterobasidion* spp. after 7 days of

333   incubation (Table 2). CFF$_{25}$ resulted in 100% MGI for all tested fungal pathogens (Table 2 and Fig.

334   4.). Inhibitory effect of CFF$_{10}$ was significantly higher for *H. abietinum* and *H. parviporum* (76% and

335   75%, respectively)*,* and lower for *H. annosum* and *H. irregulare* (70%, and 66%, respectively) (Table

336   2).

337

338   *3.1.4 Inhibition of conidial germination by cell-free filtrates (CFF)*

339   The addition of CFF$_{25}$ and CFF$_{10}$ in water agar medium had a drastic effect on conidial germination

340   compared to controls, regardless of *Heterobasidion* species (Table 3). Conidial germination inhibition

341   (CGI) was significantly lower with CFF$_{10}$ than with CFF$_{25}$. After 48 h of incubation, CFF$_{25}$ resulted in at

342   least 98% CGI without significant differences among pathogens. CFF$_{10}$ resulted in values of CGI

343   significantly higher for *H. irregulare* and *H. parviporum* (92% and 88%, respectively), compared to *H.*

344   *abietinum* and *H. annosum* (84% and 87%, respectively).

345

346   *3.2 Comparative performances of P. protegens (DSMZ 13134) and state of the art treatments against*

347   *Heterobasidion spp. on wood discs*

348   In general, treatments resulted in a significant reduction of colonised areas of wood discs by the

349   fungal pathogens compared to controls, with the exception of Proradix® for *H. annosum*, and of

350   Proradix® and *P. protegens* (DSMZ 13134) CFF$_{25}$ for *H. irregulare* (Fig. 5a-d.). CFF$_{25}$ performed

351   significantly better than Proradix® when used against all *Heterobasidion* species, with the exception of

352   *H. irregulare*, against which they performed similarly. In experiments against *H. abietinum*, CFF$_{25}$

353   reduced to a significantly greater extent than Rotstop® the area colonised by the pathogen compared

354   to controls, and was almost as effective as the best treatment urea (Fig. 5a). Against *H. annosum*,

355   CFF$_{25}$ was as effective as the state of the art treatments Rotstop® and urea (Fig. 5b). Against *H.*

356   *irregulare*, CFF$_{25}$ was not effective as state of the art treatments (Fig. 5c), and the same was true for

357   *H. parviporum*, despite in this case there was a significant and substantial reduction of colonised area

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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

17

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

409 ISP2 culture medium. In fact, while MGI of *Heterobasidion* spp. was greater at 25 °C than at 10 °C in

410 dual culture assays, the opposite was true when only the effect of volatile compounds was tested. As

411 discussed previously for antibiotics, also the production and effectiveness of antifungal volatile

412 compounds are influenced by several abiotic factors, including temperature (Gabriel *et al.,* 2018).

413 These observations stress the importance of considering environmental conditions when assessing

414 the effectiveness of biocontrol agents. Again, weaker performances of volatile compounds were

415 observed on medium supplemented with sawdust compared to the non-supplemented medium. The

416 reasons for that may be similar to those described previously for the dual culture assays.

417 The prominent role of antibiotics and diffusible antifungal compounds produced by *P. protegens*

418 (DSMZ 13134) in the interaction with *Heterobasidion* spp. is also demonstrated by the outcomes of

419 experiments using cell free filtrates (CFF), both CFF_{25} and CFF_{10}, and both on mycelial growth and

420 conidia germination. These findings prompt a comprehensive analysis of specific compounds

421 produced by *P. protegens* (DSMZ 13134) and involved in the inhibition of *Heterobasidion* spp. Several

422 strains of *Pseudomonas* spp. have been reported to produce a plethora of antifungal compounds,

423 such as hydrogen cyanide, pyoluteorin, phenazines, siderophores, cyclic lipopeptides, 2,4-

424 diacetylphloroglucinol, and pyrrolnitrin (Compant *et al.,* 2005, Mishra and Arora, 2018; Zhang *et al.,*

425 2010). Whether these compounds, or other antifungal compounds are produced by *P. protegens*

426 (DSMZ 13134), and whether some of these alone or in combination with each other may be

427 responsible for the inhibition of *Heterobasidion* spp. will deserve further investigation.

428 The inhibition of conidial germination by CFF, which is almost complete when CFF is produced at 25

429 °C (CFF_{25}), suggests a potential application of CFF on fresh stump surfaces during logging operations

430 in the attempt to prevent *Heterobasidion* spp. airborne infections. Although in nature conidia may not

431 be as abundant as spores (Garbelotto and Gonthier, 2013), such infectious propagules have been

432 widely used in artificial inoculation studies to mimic *Heterobasidion* basidiospore infection on logs, on

433 billets and on stumps (Annesi *et al.,* 2005; Sun *et al.,* 2009a, 2009b; Lehtijärvi *et al.*, 2011).

434   In this study we used conidial suspensions not only to test the effects of CFF on conidia germination,

435   but also to mimic natural spore deposition on treated or untreated wood discs simulating stumps. On

436   untreated control wood discs, the areas colonised by *H. abietinum* and *H. parviporum* on discs of *A.*

437   *alba* and *P. abies*, respectively, were much larger than those colonised by *H. annosum* and *H.*

438   *irregulare* on discs of *P. pinea*. This may be due to the wood colonisation ability of the fungal species,

439   to the host wood, or to the interaction between fungal and host wood species. While the elucidation of

440   factors associated with the colonisation of wood by *Heterobasidion* spp. was not listed in the aims of

441   this study, it is worth noting that on the same wood substrate (i.e. *P. pinea*), *H. irregulare* colonised a

442   larger area than *H. annosum* did, which is consistent with previous findings pointing to a greater

443   saprobic ability of the non-native *H. irregulare* compared to the native *H. annosum* on pine wood

444   (Giordano *et al*., 2014). In these comparative experiments on wood discs, treatments with *P.*

445   *protegens* (DSMZ 13134) CFF performed significantly better than Proradix[®] against most of the

446   *Heterobasidion* species, further confirming on wood that the antagonistic activity hinges on antibiotics

447   produced by the bacterium and present in the CFF rather than on other mechanisms.

448   *P. protegens* (DSMZ 13134) CFF significantly reduced the areas colonised by *H. annosum* and *H.*

449   *abietinum* to the level of state of the art treatments. Against this latter species, *P. protegens* (DSMZ

450   13134) CFF performed even significantly better, although not substantially, than Rotstop[®]. Although

451   *P. protegens* (DSMZ 13134) CFF did not performed as well as state of the art treatments against *H.*

452   *parviporum*, the reduction of the colonised area occupied by the fungus compared to control was

453   significant and substantial.

454   While both *P. protegens* (DSMZ 13134) treatments or at least CFF proved to be effective against

455   native European *Heterobasidion* spp., neither CFF nor Proradix[®] showed significant effects against

456   the non-native *H. irregulare* on wood discs. This observation is in contrast with the efficacy of

457   treatments against *H. irregulare* recorded in *in vitro* experiments, suggesting a possible role played by

458   the wood disc in the interaction between the biological control agent and the fungal pathogen. We

459   cannot exclude that the observed response could be related to the chemical-physical properties of the

460 *P. pinea* wood used in this experiment. However, this hypothesis should be tested through

461 appropriate experiments.

462 Comparative experiments on wood discs provided few additional side information: 1- the suitability of

463 using wood discs not only for studying the epidemiology of *Heterobasidion* spp. but also for testing the

464 efficacy of treatments against these fungal plant pathogens; 2- the overall efficacy of state of the art

465 treatments Rotstop® and, especially, urea. While this last treatment was already proved to be effective

466 against native European *Heterobasidion* spp. on stumps of several tree species (Gonthier, 2019),

467 data presented in this paper are the first pointing to the efficacy of urea against the non-native *H.*

468 *irregulare*.

469 The efficacy of treatments is commonly evaluated in terms of average performance, but the variability

470 of data displayed on wood discs by both Proradix® and *P. protegens* (DSMZ 13134) CFF against all

471 the *Heterobasidion* species may imply that their application in the forest could lead to variable and

472 perhaps uncertain results. However, this hypothesis needs to be tested by *ad hoc* experiments on

473 stumps in the forest.

474 In conclusion, *P. protegens* (DSMZ 13134) and especially the CFF of the bacterium showed for the

475 first time a potential against all the native European *Heterobasidion* species, by performing mostly as

476 well as state of the art chemical or biological treatments. Based on experiments on wood discs in

477 controlled conditions, the tested treatments seem poorly effective against the non-native *H. irregulare*.

478 Nevertheless, as the results point to the antibiosis as the main mode of action, the efficacy of

479 treatments may be significantly improved by identifying the active molecules and/or by optimizing their

480 production or application, making *P. protegens* (DSMZ 13134) a suitable candidate for the biological

481 control of root and butt rots caused by *Heterobasidion* spp.

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732

Tables

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

[a] MUT: Mycotheca Universitatis Taurinensis

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)

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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

Figure captions

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

Fig. 2. Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI)

resulting from the dual culture assays with *Pseudomonas protegens* (DSMZ 13134) on ISP2 (panel a)

and ISP2-s (panel b) culture media at 10 °C and 25 °C. For each combination of culture medium,

temperature and *Heterobasidion* species, the average values of MGI are reported (circular and

triangular dots) along with the associated 95% Bias Corrected and accelerated (BCa) confidence

interval ($CI_{95\%}$) (whiskers). Significant differences ($P < 0.05$) of MGI values between *Heterobasidion*

species at each temperature level are identified by lowercase letters, while between temperature

levels for each *Heterobasidion* species by uppercase letters.

Fig. 3. Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI)

resulting from the trials testing the effects of volatile compounds of *Pseudomonas protegens* (DSMZ

13134)on mycelial growth on ISP2 (panel a) and ISP2-s (panel b) culture media at 10 °C and 25 °C.

For each combination of culture medium, temperature and *Heterobasidion* species, the average

values of MGI are reported (circular and triangular dots) along with the associated 95% Bias

Corrected and accelerated (BCa) confidence interval ($CI_{95\%}$) (whiskers). Significant differences ($P <$

0.05) of MGI values between *Heterobasidion* species at each temperature level are identified by

lowercase letters, while between temperature levels for each *Heterobasidion* species by uppercase

letters.

Fig. 4. Inhibition of mycelial growth of *Heterobasidion* spp. caused by CFF_{25} of *Pseudomonas*

protegens (DSMZ 13134). The mycelial growth of a single genotype of *H. abietinum* (a), *H. annosum*

794   (b), *H. irregulare* (c), and *H. parviporum* (d) on 80% CFF_{25} culture medium (left side) and on 80% LB

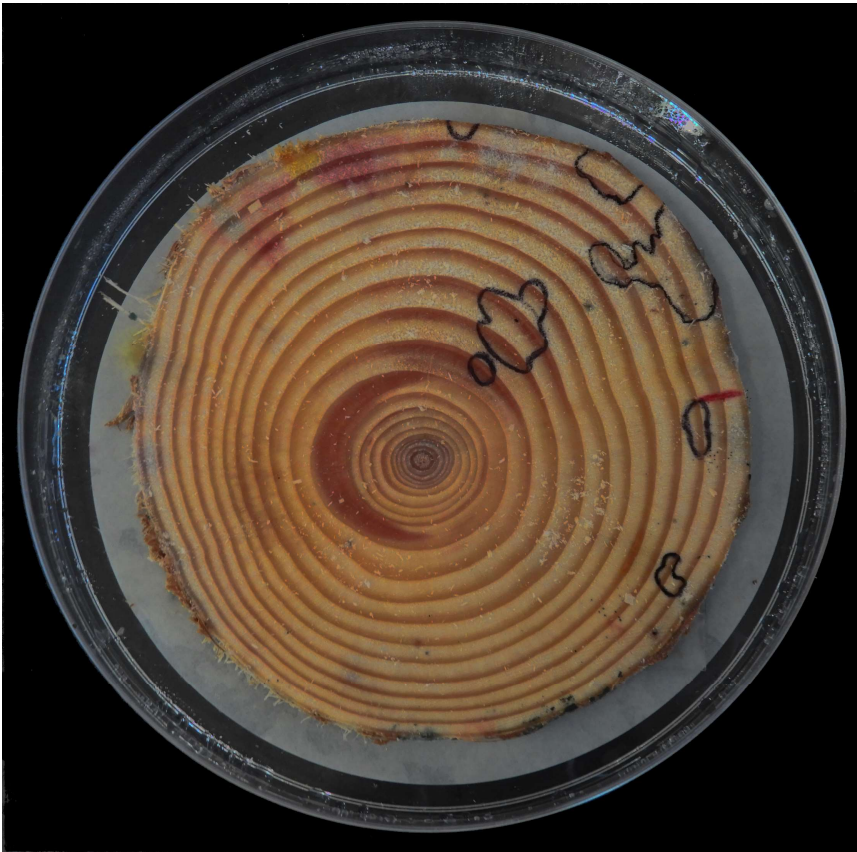795   culture medium (right side) after 7 days of incubation at 25 °C.

796

797   **Fig. 5.** Comparative performances of *Pseudomonas* sp. DSMZ 13134 and state of the art treatments

798   against *Heterobasidion abietinum* (panel a), *H. annosum* (panel b), *H. irregulare* (panel c) and *H.*

799   *parviporum* (panel d) on wood discs. For each treatment (wa - water; pr - Proradix®; cff - CFF_{25}; ro -

800   Rotstop®; and ur - urea) the average area colonised by *Heterobasidion* spp. (mm^2) on the untreated

801   surface is shown (circular dots) along with the associated 95% Bias Corrected and accelerated (BCa)

802   confidence interval ($CI_{95\%}$) (whiskers). Different letters indicate a significant difference ($P < 0.05$)

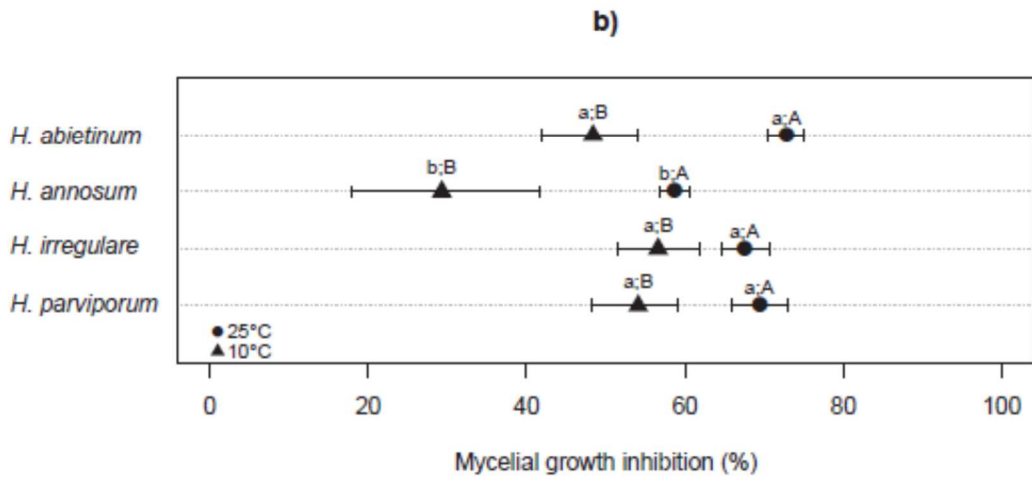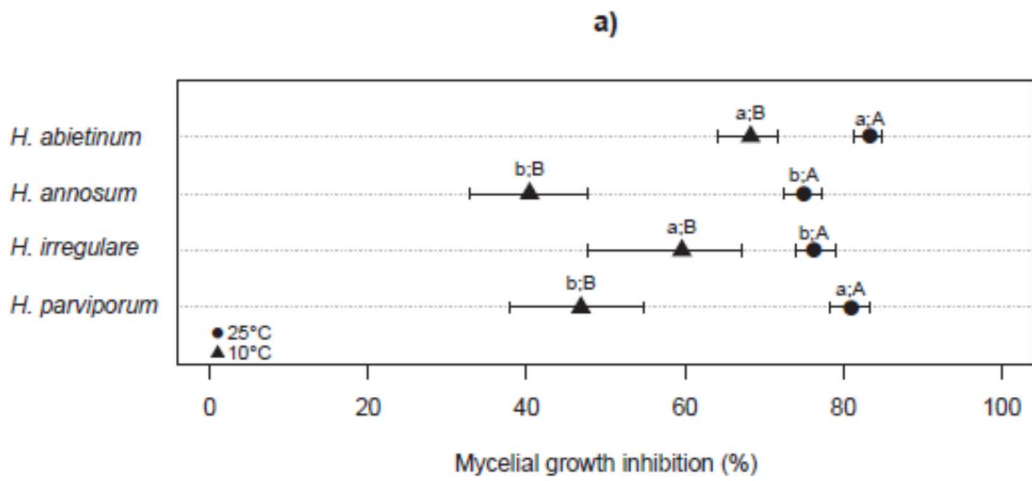803   among the average values associated with each treatment.
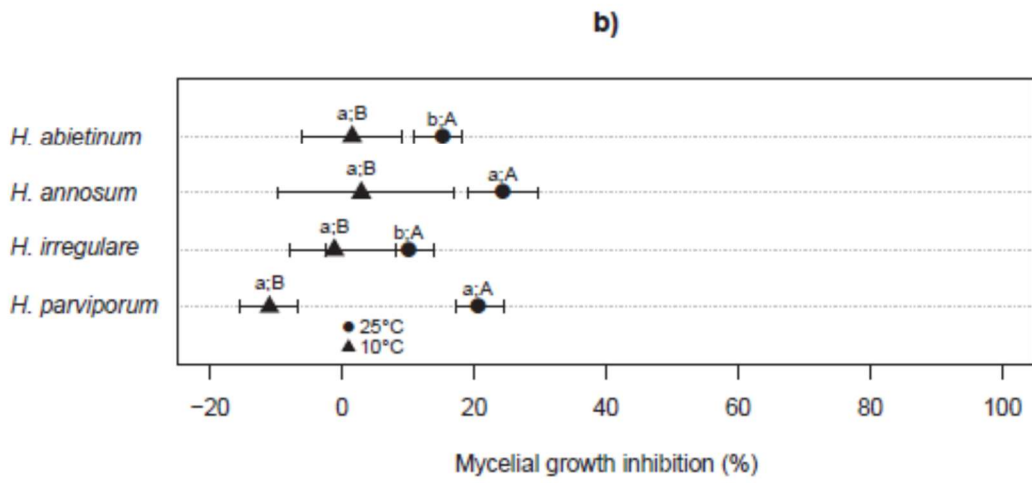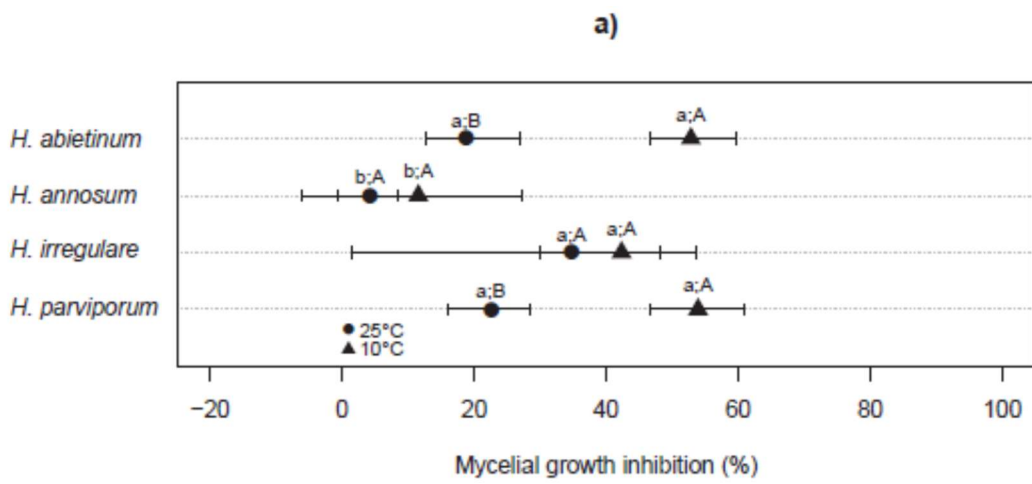
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806   FIG. 1

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809   FIG. 2

a)

b)

810

811

812   FIG. 3

a)

b)
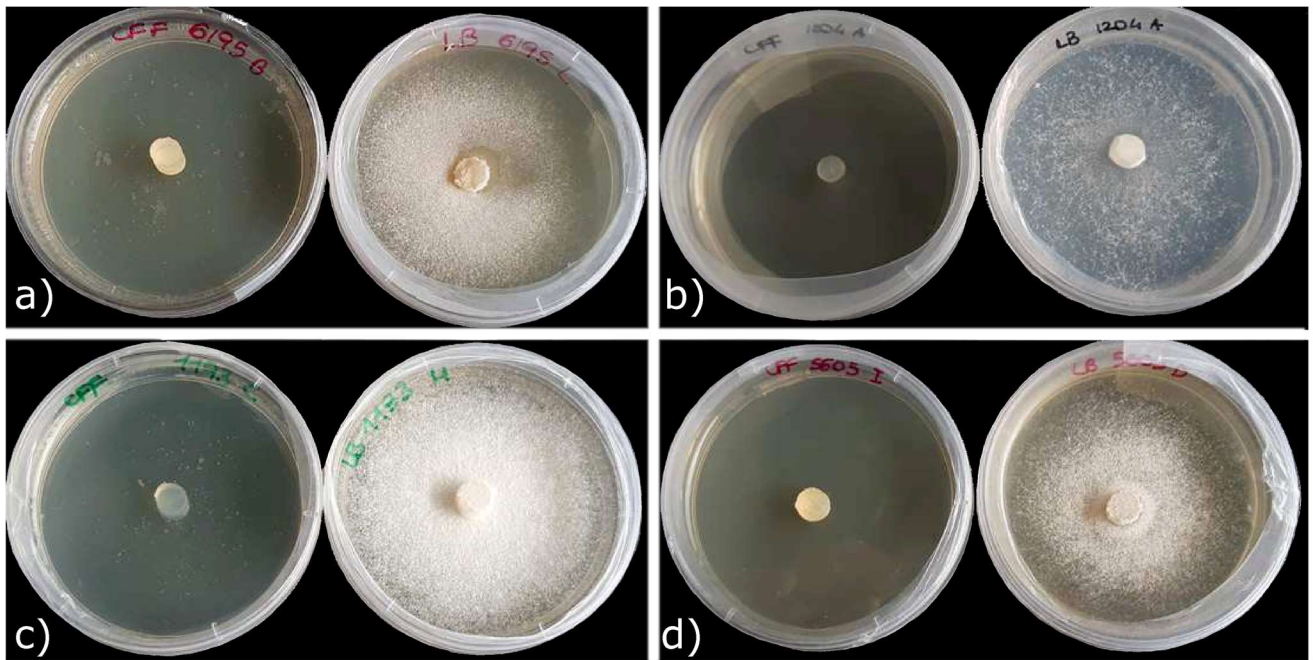

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815   FIG. 4

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817

818   FIG. 5

a)

Area colonised by *H. abietinum* (mm²) on the untreated surface

b)

Area colonised by *H. annosum* (mm²) on the untreated surface

c)

Area colonised by *H. irregulare* (mm²) on the untreated surface

d)

Area colonised by *H. parviporum* (mm²) on the untreated surface

819