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

17

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34

35 **Abstract**

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

53

54

55 **Keywords**

56 Antibiosis

57 Cell-free filtrate

58 *Phlebiopsis gigantea*

59 *Proradix*[®]

60 *Rotstop*[®]

61 Urea

62

63

64 **Declarations of interest:** none.

65

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 Plant 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).

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

111 Despite extensive research on beneficial fungi and bacteria (Azeem *et al.*, 2019; Mesanza *et al.*,
112 2016, 2019; Nicolotti and Varese, 1996; Sz wajkowska-Michalek *et al.*, 2012; Terhonen *et al.*, 2016), a
113 comprehensive investigation on the effectiveness of a biocontrol agent against all *Heterobasidion*
114 species present in Europe, including the invasive species *H. irregulare*, is still lacking.

115 The relevance of plant growth-promoting rhizobacteria (PGPR) for the control of plant pathogens is
116 now recognized (Beneduzi *et al.*, 2012; Compant *et al.*, 2005; Mishra and Arora, 2018). Our study
117 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 (Tübingen, 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)

170 obtained from fresh wood of preferential host species, i.e. *Pinus pinea* L. for *H. annosum* and *H.*
171 *irregulare*, *A. alba* Mill. for *H. abietinum*, and *P. abies* (L.) H. Karst. for *H. parviporum*, to simulate the
172 growth substrate of *Heterobasidion* spp. in natural conditions. Sawdust was prepared by cutting fresh
173 stems using a circular saw and collecting the resulting sawdust in a polyethylene bag. Sawdust with a
174 maximum granulometry of 5 mm was added to ISP2 medium at a dose of 10 g L⁻¹ prior autoclaving for
175 20 min. at 121 °C. Fungal mycelial plug was taken from a 7 days old culture and inoculated towards
176 one edge of a 6-cm diameter Petri plate. *Pseudomonas protegens* (DSMZ 13134) (optical density at
177 600 nm [OD₆₀₀ = 0.4], approximately 10⁷ CFU mL⁻¹) was streaked (streak length 20 mm) at 15 mm
178 from the edge of the fungal plug on the opposite side of the Petri plate (treated plates). Control plates
179 were prepared by using only the *Heterobasidion* spp. genotypes. Treated and control plates were
180 incubated in the dark at both 25 and 10 °C until the fungal colonies in the control plates had grown to
181 fill the plate. The radius of *Heterobasidion* spp. colonies was measured (in mm) in treated (r_T) and
182 control (r_C) after 7 days in Petri plates incubated at 25 °C and after 11 days in Petri plates incubated
183 at 10 °C. The mycelial growth inhibition (MGI) of *Heterobasidion* spp. was assessed by calculating (in
184 %) the radial reduction observed in treated plates in relation to the corresponding control plates with
185 the following equation:

186
$$\text{MGI} = 100\% \cdot \frac{r_C - r_T}{r_C} \quad (\text{Equation 1})$$

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

189

190 2.2.2 Effects of volatile compounds on mycelial growth

191 The antagonistic potential of volatile compounds produced by *P. protegens* (DSMZ 13134) against
192 *Heterobasidion* spp. was explored using two-divided Petri plates. In treated plates, a 7-days-old
193 fungal mycelial plug and a bacterial suspension (OD₆₀₀ of 0.4) were spotted onto different halves of
194 the same 9 cm-diameter two-divided Petri plates. In control plates only *Heterobasidion* spp.

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

198 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 (OD₆₀₀ of 1.1) and 10 °C (OD₆₀₀ 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 (CFF₂₅ and CFF₁₀). 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

215 2.2.4 Inhibition of conidial germination by cell-free filtrate (CFF)

216 The CFF₂₅ and CFF₁₀ 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 shaken 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. CFF₂₅ and CFF₁₀) 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:

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

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, Tübingen, Germany) and CFF₂₅, 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 CFF₂₅ was
241 chosen instead of CFF₁₀ 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 CFF₂₅ 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 CFF₁₀ and
295 CFF₂₅. 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

307 **3. Results**

308 *3.1 Antifungal activity of P. protegens (DSMZ 13134) against Heterobasidion spp.*

309 *3.1.1 Dual culture assays*

310 *Pseudomonas protegens (DSMZ 13134)* inhibited mycelial growth regardless of *Heterobasidion*
311 species, culture medium and incubation temperature (Fig. 2.). Mycelial growth inhibition (MGI) varied
312 depending on *Heterobasidion* species and was significantly lower at 10 °C than that observed at 25
313 °C (Fig. 2.). On ISP2 medium after 7 days of incubation at 25 °C, MGI was highest for *H. abietinum*
314 and *H. parviporum* (83% and 81%, respectively), and significantly lower for *H. annosum* and *H.*
315 *irregulare* (75% and 76%, respectively) (Fig. 2a.). On ISP2 after 11 days at 10 °C, the MGI was
316 significantly higher for *H. abietinum* and *H. irregulare* (68% and 60%, respectively) compared to *H.*
317 *annosum* and *H. parviporum* (40% and 47%, respectively) (Fig. 2a.). On ISP2-s after 7 days of
318 incubation at 25 °C, MGI was highest for *H. abietinum*, *H. irregulare* and *H. parviporum* (73%, 68%,
319 69%, respectively), and significantly lower for *H. annosum* (59%) (Fig. 2b.). On the same medium
320 after 11 days of incubation at 10°C , again MGI was highest for *H. abietinum*, *H. irregulare* and *H.*
321 *parviporum* (48%, 57%, 54%, respectively) and significantly lower (29%) for *H. annosum* (Fig. 2b.).

322

323 *3.1.2 Effects of volatile compounds on mycelial growth*

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

330

331 *3.1.3 Inhibition of mycelial growth by cell-free filtrate (CFF)*

332 CFF₂₅ and CFF₁₀ effectively inhibited mycelial growth of *Heterobasidion* spp. after 7 days of
333 incubation (Table 2). CFF₂₅ resulted in 100% MGI for all tested fungal pathogens (Table 2 and Fig.
334 4.). Inhibitory effect of CFF₁₀ 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 CFF₂₅ and CFF₁₀ 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 CFF₁₀ than with CFF₂₅. After 48 h of incubation, CFF₂₅ resulted in at
342 least 98% CGI without significant differences among pathogens. CFF₁₀ 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) CFF₂₅ for *H. irregulare* (Fig. 5a-d.). CFF₂₅ 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*, CFF₂₅
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 CFF₂₅ was as effective as the state of the art treatments Rotstop® and urea (Fig. 5b). Against *H.*
356 *irregulare*, CFF₂₅ 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

358 compared to controls (Fig. 5d). Treatment with urea always resulted in 0 mm² 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; Rönnerberg
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

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 CFF₂₅ and CFF₁₀, 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 (CFF₂₅), 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.

482

483

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494 **References**

495 Annesi, T., Curcio, G., D'amico, L., Motta, E., 2005. Biological control of *Heterobasidion annosum* on
496 *Pinus pinea* by *Phlebiopsis gigantea*. For. Pathol. 35, 127-134. [https://doi.org/10.1111/j.1439-](https://doi.org/10.1111/j.1439-0329.2004.00394.x)
497 0329.2004.00394.x.

498

499 Asiegbu, F.O., Adomas, A., Stenlid, J., 2005. Conifer root and butt rot caused by *Heterobasidion*
500 *annosum* (Fr.) Bref. s.l. Mol. Plant Pathol. 6, 395-409. [https://doi.org/10.1111/j.1364-](https://doi.org/10.1111/j.1364-3703.2005.00295.x)
501 3703.2005.00295.x.

502

503 Azeem, M., Barba-Aliaga, M., Borg-Karlson, A.K., Terenius, O., Broberg, A., Rajarao, G.K., 2019.
504 *Heterobasidion*-growth inhibiting *Bacillus subtilis* A18 exhibits medium-and age-dependent production
505 of lipopeptides. Microbiol. Res. 223, 129-136. <https://doi.org/10.1016/j.micres.2019.04.006>.

506

507 Beneduzi, A., Ambrosini, A., Passaglia, L.M., 2012. Plant growth-promoting rhizobacteria (PGPR):
508 their potential as antagonists and biocontrol agents. Genet. Mol. 35, 1044-1051.
509 <https://doi.org/10.1590/S1415-47572012000600020>.

510

511 Buddrus-Schiemann, K., Schmid, M., Schreiner, K., Welzl, G., Hartmann, A., 2010. Root colonization
512 by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of
513 barley. Microb. Ecol. 60, 381-393. <https://doi.org/10.1007/s00248-010-9720-8>.

514

515 Carsey, T.M., Harden, J.J., 2014. Monte Carlo simulation and resampling methods for social science,
516 SAGE Publications.

517

518 Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., 2005. Use of plant growth-promoting
519 bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl.
520 Environ. Microbiol. 71, 4951-4959. <https://doi.org/10.1128/AEM.71.9.4951-4959.2005>.
521

522 Cornelison, C.T., Gabriel, K.T., Barlament, C., Crow, S.A., 2014. Inhibition of *Pseudogymnoascus*
523 *destructans* growth from conidia and mycelial extension by bacterially produced volatile organic
524 compounds. Mycopathologia. 177, 1-10. <https://doi.org/10.1007/s11046-013-9716-2>.
525

526 Crawley, M.J., 2013. The R Book, 2nd ed., John Wiley & Sons, Chichester.
527

528 Daskin, J.H., Bell, S.C., Schwarzkopf, L., Alford, R.A., 2014. Cool temperatures reduce antifungal
529 activity of symbiotic bacteria of threatened amphibians-implications for disease management and
530 patterns of decline. PLoS One. 9. <https://doi.org/10.1371/journal.pone.0100378>.
531

532 DiCiccio, T.J., Efron, B., 1996. Bootstrap confidence intervals. Stat. Sci. 11, 189-228.
533 <https://doi.org/10.1214/ss/1032280214>
534

535 Efron, B., Tibshirani, R.J., 1994. An introduction to the bootstrap. CRC press. S original, from StatLib
536 and by Rob Tibshirani. R port by Friedrich Leisch. (2019). bootstrap: Functions for the Book "An
537 Introduction to the Bootstrap". R package version 2019.6. [https://CRAN.R-](https://CRAN.R-project.org/package=bootstrap)
538 [project.org/package=bootstrap](https://CRAN.R-project.org/package=bootstrap).
539

540 European Union Commission, Commission Implementing Regulation No 1160/2020 of 5 August 2020
541 amending Implementing Regulation (EU) No 540/2011. 2020.
542

543 Fernando, W.G.D., Nakkeeran, S., Zhang, Y. 2005. Biosynthesis of antibiotics by PGPR and its
544 relation in biocontrol of plant diseases, in: Siddiqui, Z.A. (Eds), PGPR: Biocontrol and Biofertilization.
545 Springer, Dordrecht, pp. 67-109. https://doi.org/10.1007/1-4020-4152-7_3.

546

547 Fröhlich, A., Buddrus-Schiemann, K., Durner, J., Hartmann, A., von Rad, U., 2012. Response of
548 barley to root colonization by *Pseudomonas* sp. DSMZ 13134 under laboratory, greenhouse, and field
549 conditions. J. Plant Interact., 7, 1-9, <https://doi.org/10.1080/17429145.2011.597002>.

550

551 Gabriel, K.T., Joseph Sexton, D., Cornelison, C.T., 2018. Biomimicry of volatile-based microbial
552 control for managing emerging fungal pathogens. J. Appl. Microbiol. 124, 1024-1031.
553 <https://doi.org/10.1111/jam.13667>.

554

555 Garbelotto, M., Gonthier, P., 2013. Biology, epidemiology, and control of *Heterobasidion* species
556 worldwide. Annu. Rev. Phytopathol, 51, 39–59. <https://doi.org/10.1146/annurev-phyto-082712-102225>.

557

558

559 Giordano, L., Gonthier, P., Lione, G., Capretti, P., Garbelotto, M., 2014. The saprobic and fruiting
560 abilities of the exotic forest pathogen *Heterobasidion irregulare* may explain its invasiveness. Biol.
561 Invasions. 16, 803-814. <https://doi.org/10.1007/s10530-013-0538-4>.

562

563 Gonthier, P., 2019. Frequency of stump infections by *Heterobasidion annosum* s.l. and benefits from
564 urea treatments vary with tree species and season in European Alpine forests. Forest Ecol Manag.
565 434, 76-86. <https://doi.org/10.1016/j.foreco.2018.12.011>.

566

567 Gonthier, P., Anselmi, N., Capretti, P., Bussotti, F., Feducci, M., Giordano, L., Honorati, T., Lione, G.,
568 Luchi, N., Michelozzi, M., Papparatti, B., Sillo, F., Vettraino, A.M., Garbelotto, M., 2014. An integrated

569 approach to control the introduced forest pathogen *Heterobasidion irregulare* in Europe. *Forestry*. 87,
570 471-481. <https://doi.org/10.1093/forestry/cpu015>.

571

572 Gonthier, P., Garbelotto, M., 2011. Amplified fragment length polymorphism and sequence analyses
573 reveal massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into
574 its introduced congener *H. irregulare*. *Mol. Ecol.* 20, 2756-2770. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-294X.2011.05121.x)
575 294X.2011.05121.x.

576

577 Gonthier, P., Garbelotto, M., Varese, G.C., Nicolotti, G., 2001. Relative abundance and potential
578 dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. *Can. J.*
579 *Bot.* 79, 1057-1065. <http://dx.doi.org/10.1139/b01-090>.

580

581 Gonthier, P., Lione, G., Giordano, L., Garbelotto, M., 2012. The American forest pathogen
582 *Heterobasidion irregulare* colonizes unexpected habitats after its introduction in Italy. *Ecol. Appl.* 22,
583 2135-2143. <https://doi.org/10.1890/12-0420.1>.

584

585 Gonthier, P., Nicolotti, G., Linzer, R., Guglielmo, F., Garbelotto, M., 2007. Invasion of European pine
586 stands by a North American forest pathogen and its hybridization with a native interfertile taxon. *Mol.*
587 *Ecol.* 16, 1389-1400. <https://doi.org/10.1111/j.1365-294X.2007.03250.x>.

588

589 Gonthier, P., Thor, M., 2013. Annosus root and butt rots, in: Gonthier, P., Nicolotti, G. (Eds.),
590 *Infectious Forest Diseases*. CAB International, Wallingford, pp.128-158.

591

592 Gonthier, P., Warner, R., Nicolotti, G., Mazzaglia, A., Garbelotto, M., 2004. Pathogen introduction as
593 a collateral effect of military activity. *Mycol. Res.* 108, 468-470.
594 <https://doi.org/10.1017/S0953756204240369>.

595

596 Gžibovska, Z., 2016. Evaluation of *Phlebiopsis gigantea* and *Pseudomonas* spp. for biocontrol of
597 *Heterobasidion* spp. in Norway spruce. Master thesis, Swedish University of Agricultural Sciences, 39
598 pp.

599

600 Holdenrieder, O., Greig, B.J.W., 1998. Biological methods of control, in: Woodward, S., Stenlid, J.,
601 Karjalainen, R., Huttermann, A. (Eds), *Heterobasidion annosum*: Biology, Ecology, Impact and
602 Control. CAB International, Wallingford, pp. 235-258. [https://doi.org/10.1046/j.1365-](https://doi.org/10.1046/j.1365-3059.1999.0366b.x)
603 [3059.1999.0366b.x](https://doi.org/10.1046/j.1365-3059.1999.0366b.x).

604

605 Hosmer, D.W., Lemeshow, S., 1989. Applied Logistic Regression, Johns Wiley & Sons, New York.

606

607 Hothorn, T., Hornik, K., Zeileis, A., 2006. Unbiased recursive partitioning: A conditional inference
608 framework. *J. Comput. Graph. Stat.* 15, 651-674. <https://doi.org/10.1198/106186006X133933>.

609

610 Hothorn, T., Zeileis, A., 2015. partykit: A modular toolkit for recursive partytioning in R. *J. Mach.*
611 *Learn. Res.* 16, 3905-3909. <http://jmlr.org/papers/v16/hothorn15a.html>.

612

613 Johansson, M., Prat, J.E., Asiegbu, F.O., 2002. Treatment of Norway spruce and Scots pine stumps
614 with urea against the root and butt rot fungus *Heterobasidion annosum*: possible modes of action.
615 *For. Ecol. Manage.* 157, 87-100. [https://doi.org/10.1016/S0378-1127\(00\)00661-7](https://doi.org/10.1016/S0378-1127(00)00661-7).

616

617 Lehtijärvi, A., Doğmuş-Lehtijärvi, H.T., Aday, A.G., Oskay, F., 2011. The efficacy of selected biological
618 and chemical control agents against *Heterobasidion abietinum* on *Abies cilicica*. *For. Pathol.* 41, 470-
619 476. <https://doi.org/10.1111/j.1439-0329.2010.00705.x>.

620

621 Lione, G., Giordano, L., Turina, M., Gonthier, P., 2020. Hail-induced infections of the chestnut blight
622 pathogen *Cryphonectria parasitica* depend on wound size and may lead to severe diebacks.
623 Phytopathology. 110, 1280-1293. <https://doi.org/10.1094/PHYTO-01-20-0006-R>.
624

625 Mérillon, J.M., Ramawat, K.G., 2012. Plant defence: Biological control, Eds Springer Netherlands.
626 <https://doi.org/10.1007/978-94-007-1933-0>.
627

628 Mesanza, N., Crawford, B.D., Coulson, T.J., Iturrutxa, E., Patten, C.L., 2019. Colonization of *Pinus*
629 *radiata* D. Don Seedling Roots by Biocontrol Bacteria *Erwinia billingiae* and *Bacillus simplex*. Forests,
630 10, 552. <https://doi.org/10.3390/f10070552>.
631

632 Mesanza, N., Iturrutxa, E., Patten, C.L., 2016. Native rhizobacteria as biocontrol agents of
633 *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*. Biol. Control. 101, 8-16.
634 <https://doi.org/10.1016/j.biocontrol.2016.06.003>.
635

636 Mgbearhuruike, A.C., Sun, H., Fransson, P., Kasanen, R., Daniel, G., Karlsson, M., Asiegbu, F.O.,
637 2011. Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer
638 pathogen *Heterobasidion annosum*. Biol. Control. 57, 118-129.
639 <https://doi.org/10.1016/j.biocontrol.2011.01.007>.
640

641 Mishra, J., Arora, N.K., 2018. Secondary metabolites of fluorescent pseudomonads in biocontrol of
642 phytopathogens for sustainable agriculture. Appl. Soil. Ecol. 125, 35-45.
643 <https://doi.org/10.1016/j.apsoil.2017.12.004>.
644

645 Nicolotti, G., Varese, G.C., 1996. Screening of antagonistic fungi against air-borne infection by
646 *Heterobasidion annosum* on Norway spruce. *Forest Ecol. Manag.* 88, 249-257.
647 [https://doi.org/10.1016/S0378-1127\(96\)03844-3](https://doi.org/10.1016/S0378-1127(96)03844-3).
648

649 Oliva, J., Zhao, A., Zarei, S., Sedlák, P., Stenlid, J., 2015. Effect of temperature on the interaction
650 between *Phlebiopsis gigantea* and the root-rot forest pathogen *Heterobasidion* spp. *Forest Ecol.*
651 *Manag.* 340, 22-30. <https://doi.org/10.1016/j.foreco.2014.12.021>.
652

653 Pratt, J.E., Gibbs, J.N., Webber, J.F., 1999. Registration of *Phlebiopsis gigantea* as a forest biocontrol
654 agent in the UK: recent experience. *Biocontrol Sci. Technol.* 9, 113-118.
655 <https://doi.org/10.1080/09583159929974>.
656

657 Pridham, T.G., Anderson, P., Foley, C., Lindenfelser, L.A., Hesseltine, C.W., Benedict, R.G., 1957. A
658 selection of media for maintenance and taxonomic study of *Streptomyces*. *Antib. Ann.* 1957, 947-953.
659

660 R Core Team 2019. R: A language and environment for statistical computing. R Foundation for
661 Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
662

663 Raaijmakers, J.M., Vlami, M., De Souza, J.T., 2002. Antibiotic production by bacterial biocontrol
664 agents. *Anton. Leeuw. Int. J. G.* 81, 537-547. <https://doi.org/10.1023/A:1020501420831>.
665

666 Ramette, A., Frapolli, M., Fischer-Le Saux, M., Gruffaz, C., Meyer, J. M., Défago, G., Sutra, L.,
667 Moënne-Loccoz, Y., 2011. *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria
668 producing the biocontrol compounds 2, 4-diacetylphloroglucinol and pyoluteorin. *Syst. Appl. Microbiol.*
669 34, 180-188. <https://doi.org/10.1016/j.syapm.2010.10.005>.
670

671 Roberti, R., Veronesi, A., Flamigni, F., 2012. Evaluation of microbial products for the control of
672 zucchini foot and root rot caused by *Fusarium solani* f. sp. cucurbitae race 1. *Phytopathol. Mediterr.*
673 51, 317-331. https://doi.org/10.14601/Phytopathol_Mediterr-9680.
674

675 Rönnerberg, J., Magazniece, Z., 2018. Potential “new” protective agents for biocontrol of *Heterobasidion*
676 spp. on Norway spruce. LIFE+ ELMIAS Ash and Elm, and IUFRO WP 7.02.01 Root and Stem Rots
677 Conference (LIFE-IUFRO). Program & Book of Abstracts, p. 27.
678

679 Rönnerberg, J., Sidorov, E., Petrylaitė, E., 2006. Efficacy of different concentrations of Rotstop® and
680 Rotstop® S and imperfect coverage of Rotstop® S against *Heterobasidion* spp. spore infections on
681 Norway spruce stumps. *For. Pathol.* 36, 422-433. <https://doi.org/10.1111/j.1439-0329.2006.00476.x>.
682

683 Samils, N., Olson, Å., Stenlid, J., 2008. The capacity in *Heterobasidion annosum s.l.* to resist
684 overgrowth by the biocontrol agent *Phlebiopsis gigantea* is a heritable trait. *Biol. Control.* 45, 419-426.
685 <https://doi.org/10.1016/j.biocontrol.2008.03.010>.
686

687 Shanahan, P., O'Sullivan, D.J., Simpson, P., Glennon, J.D., O'Gara, F., 1992. Isolation of 2,4-
688 diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters
689 influencing its production. *Appl. Environ. Microbiol.* 58, 353-358.
690

691 Sun, H., Korhonen, K., Hantula, J., Asiegbu, F.O., Kasanen, R., 2009a. Use of a breeding approach
692 for improving biocontrol efficacy of *Phlebiopsis gigantea* strains against *Heterobasidion* infection of
693 Norway spruce stumps. *FEMS Microbiol. Ecol.* 69, 266-273. [https://doi.org/10.1111/j.1574-](https://doi.org/10.1111/j.1574-6941.2009.00711.x)
694 6941.2009.00711.x.
695

696 Sun, H., Korhonen, K., Hantula, J., Kasanen, R., 2009b. Variation in properties of *Phlebiopsis*
697 *gigantea* related to biocontrol against infection by *Heterobasidion* spp. in Norway spruce stumps. For.
698 Pathol. 39, 133-144. <https://doi.org/10.1111/j.1439-0329.2008.00574.x>.
699

700 Sz wajkowska-Michalek, L., Kwasna, H., Lakomy, P., Perkowski, J., 2012. Inhibition of *Armillaria* and
701 *Heterobasidion* growth by *Penicillium adametzii* isolated from *Pinus sylvestris* forest soil. For. Pathol.
702 42, 454-466. <https://doi.org/10.1111/j.1439-0329.2012.00780.x>.
703

704 Terhonen, E., Sipari, N., Asiegbu, F.O., 2016. Inhibition of phytopathogens by fungal root endophytes
705 of Norway spruce. Biol. Control. 99, 53-63. <https://doi.org/10.1016/j.biocontrol.2016.04.006>.
706

707 Vasaitis, R., Stenlid, J., Thomsen, I.M., Barklund, P., Dahlberg, A., 2008. Stump removal to control
708 root rot in forest stands. A literature study. Silva Fenn. 42, 457-483.
709

710 Woods, C.M., Woodward, S., Redfern, D.B., 2005. *In vitro* interactions in artificial and wood-based
711 media between fungi colonizing stumps of Sitka spruce. For. Pathol. 35, 213-229.
712 <https://doi.org/10.1111/j.1439-0329.2005.00403.x>.
713

714 Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A., 1998. Preface, in: *Heterobasidion*
715 *annosum*: Biology, Ecology, Impact and Control, CAB International, Wallingford, pp. xi–xii.
716 <https://doi.org/10.1046/j.1365-3059.1999.0366b.x>.
717

718 Yuan, J., Raza, W., Shen, Q., Huang, Q., 2012. Antifungal activity of *Bacillus amyloliquefaciens* NJN-
719 6 volatile compounds against *Fusarium oxysporum* f. sp. cubense. Appl. Environ. Microbiol. 78, 5942-
720 5944. <https://doi.org/10.1128/AEM.01357-12>.
721

722 Zaluma, A., Bruna, L., Klavina, D., Burnevica, N., Kenigvalde, K., Lazdins, A., Gaitnieks, T., 2019.
723 Growth of *Phlebiopsis gigantea* in wood of seven conifer species. For. Pathol. 49.
724 <https://doi.org/10.1111/efp.12555>.

725

726 Zeileis, A., Leisch, F., Hornik, K., Kleiber, C., 2002. strucchange: An R package for testing for
727 structural change in linear regression models. J. Stat. Softw. 7, 1-38.
728 <https://doi.org/10.18637/jss.v007.i02>

729

730 Zhang, J., Wang, W., Lu, X., Xu, Y., Zhang, X., 2010. The stability and degradation of a new
731 biological pesticide, pyoluteorin. Pest. Manag. Sci. 66, 248-252. <https://doi.org/10.1002/ps.1856>.

732

733 **Tables**

734

735 **Table 1**

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

736 ^a MUT: Mycotheca Universitatis Taurinensis

737

738

739 **Table 2**

740

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

741

742

743 **Table 3**

744

CFF	<i>Heterobasidion</i> species	CGI (%)
CFF ₂₅	<i>H. abietinum</i>	99.08a;A (98.46-99.59)
	<i>H. annosum</i>	98.40a;A (97.74-98.99)
	<i>H. irregulare</i>	98.93a;A (98.24-99.44)
	<i>H. parviporum</i>	99.01a;A (98.57-99.37)
CFF ₁₀	<i>H. abietinum</i>	83.74b;B (82.45-85.31)
	<i>H. annosum</i>	86.71b;B (82.30-91.53)
	<i>H. irregulare</i>	91.87a;B (88.07-94.78)
	<i>H. parviporum</i>	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 (CFF₂₅) and 10 °C (CFF₁₀). 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 (CFF₂₅) and 10 °C (CFF₁₀). 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 CFF₂₅ of *Pseudomonas*
793 *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% CFF₂₅ culture medium (left side) and on 80% LB
795 culture medium (right side) after 7 days of incubation at 25 °C.

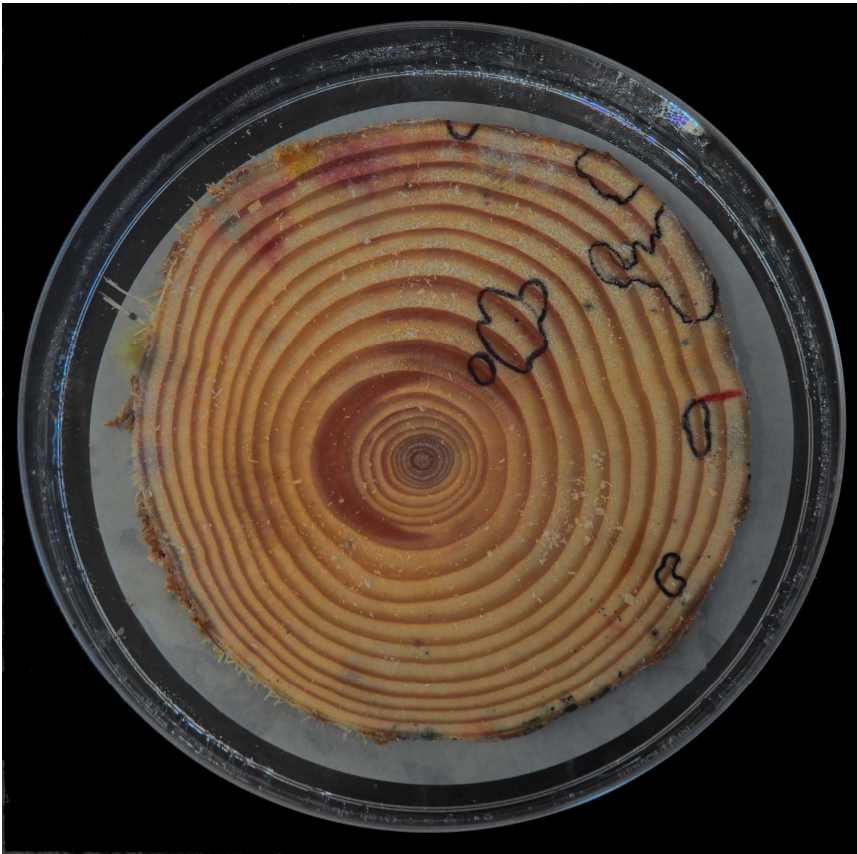
796

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 - CFF₂₅; ro -
800 Rotstop®; and ur - urea) the average area colonised by *Heterobasidion* spp. (mm²) 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.

804

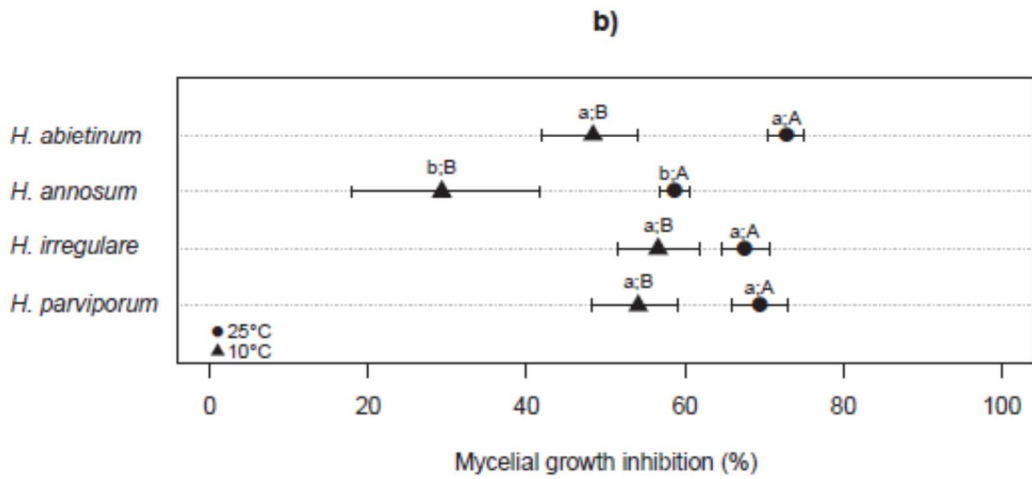
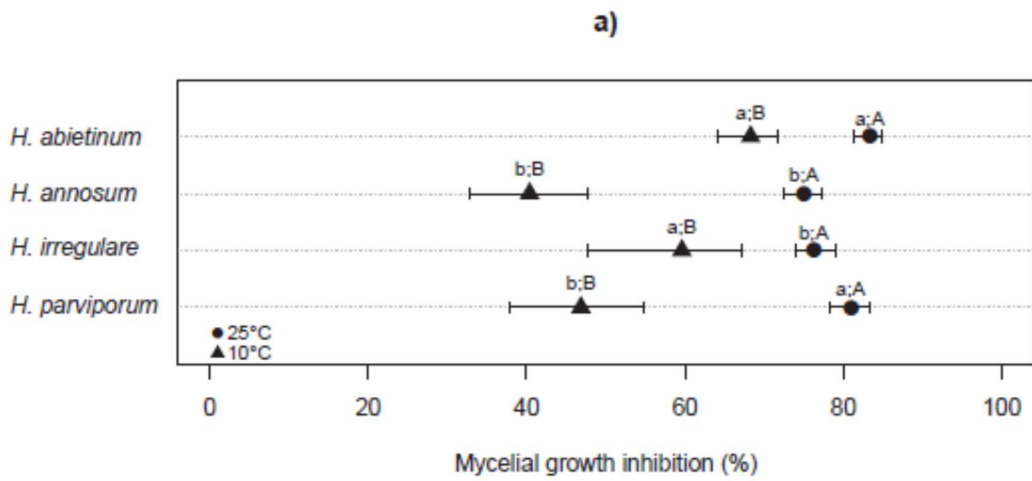
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806 FIG. 1



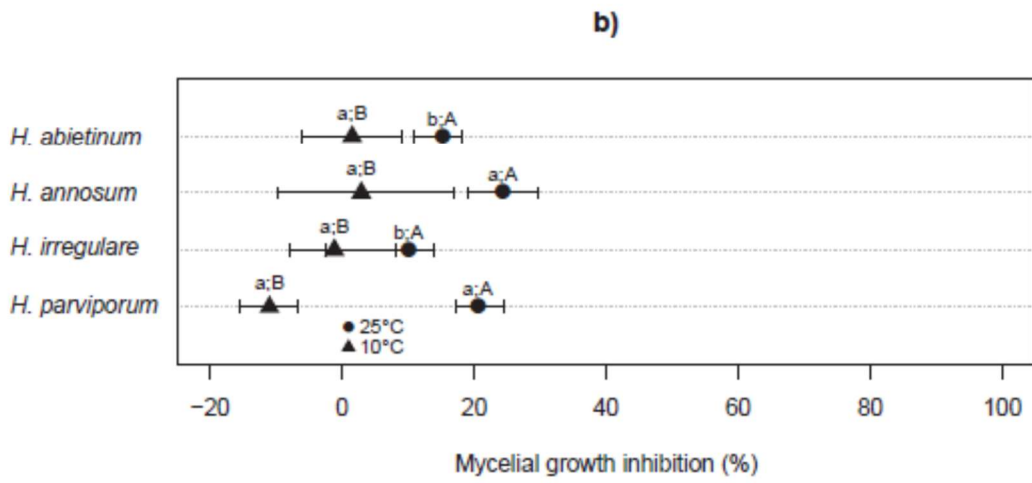
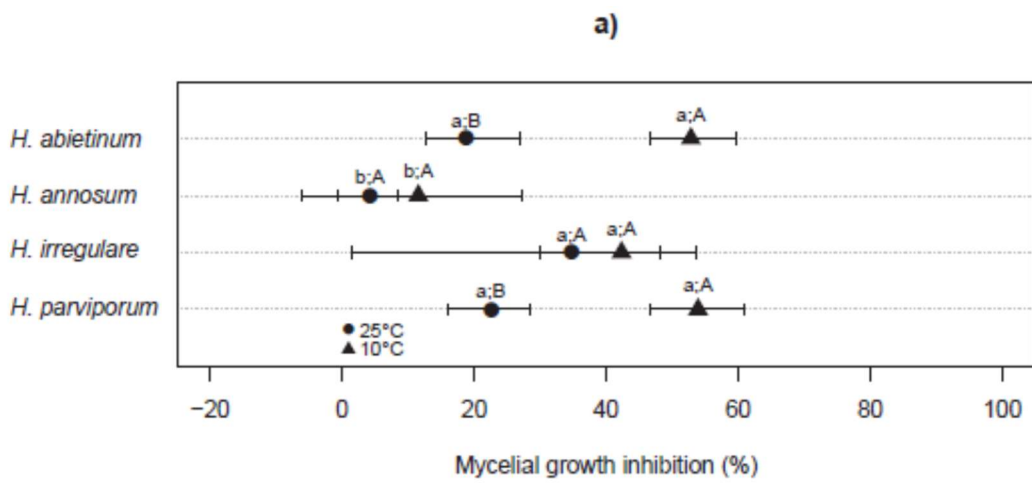
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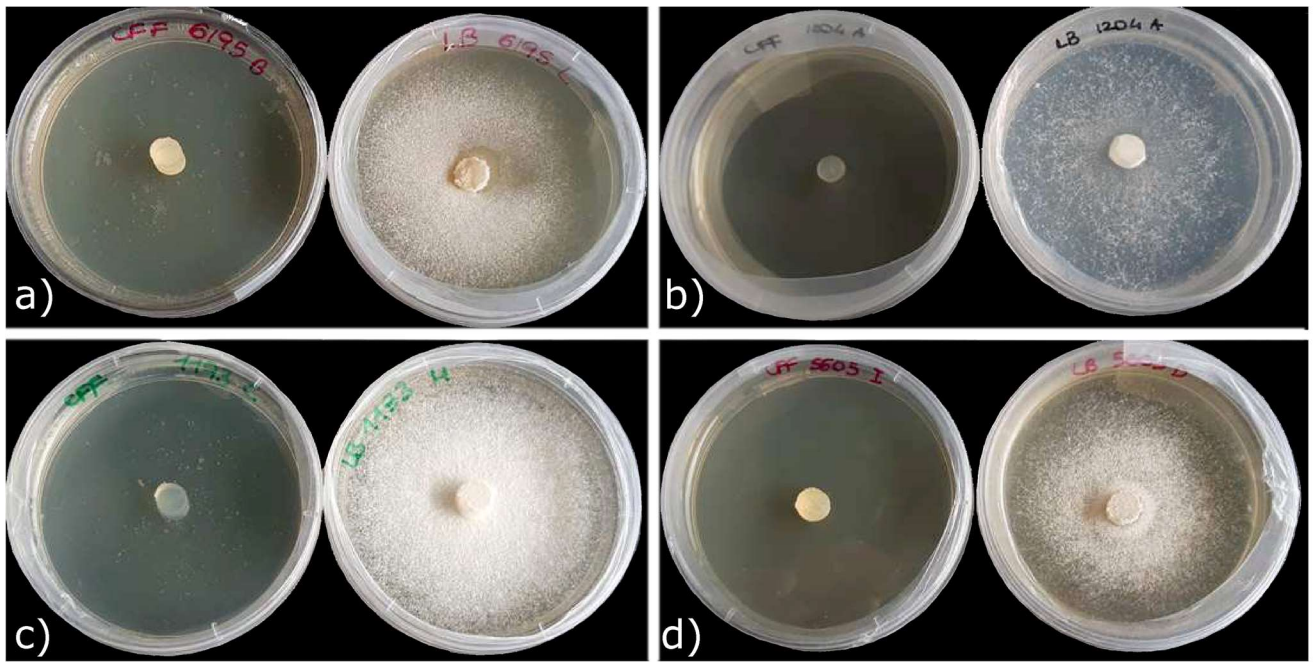
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813

814

815 FIG. 4



816

817

