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Biological control of alien and native species of the forest pathogen *Heterobasidion annosum sensu lato* by using *Pseudomonas protegens*: efficacy, mechanisms of action, and side effects

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“E il mio maestro mi insegnò com'è difficile

trovare l'alba dentro l'imbrunire”

(Franco Battiato, Prospettiva Nevski)

Contents

List of original publications and manuscripts

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Pellicciaro, M., Lione, G., Giordano, L., Gonthier, P., 2021. Biocontrol potential of *Pseudomonas protegens* against *Heterobasidion* species attacking conifers in Europe. Biol. Control 157, 104583. https://doi.org/10.1016/j.biocontrol.2021.104583 (Reprint - Chapter I)

Pellicciaro, M., Lione, G., Ongaro, S., Gonthier, P., 2021. Comparative efficacy of state-of-the-art and new biological stump treatments in forests infested by the native and the alien invasive *Heterobasidion* species present in Europe. Pathogens 10, 1272. https://doi.org/10.3390/pathogens10101272 (Reprint - Chapter II)

Pellicciaro, M., Padoan, E., Lione, G., Celi, L., Gonthier, P., 2022. Pyoluteorin produced by the biocontrol agent *Pseudomonas protegens* is involved in the inhibition of *Heterobasidion* species present in Europe. Pathogens 11, 391. https://doi.org/10.3390/pathogens11040391 (Reprint - Chapter III)

Pellicciaro, M., Lione, G., Gonthier, P. Short-term impact of biological and chemical treatments against *Heterobasidion irregulare* on microbial communities of *Pinus pinea* stumps (Research paper to be submitted – Chapter IV)

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Abstract

Abstract

The *Heterobasidion annosum* species complex includes fungal pathogens well known as fearsome causal agents of root and butt rots in coniferous forests of the Northern Hemisphere. In Europe, the threat is posed by the three native European species (*H. abietinum*, *H. annosum sensu stricto*, *H. parviporum*) and by the invasive *H. irregulare* introduced from North America, each displaying distinct host preference. These pathogens may be controlled by treating freshly cut stumps with either chemical or biological treatments hampering airborne infections. Commercial formulations of urea and of the biological control agent *Phlebiopsis gigantea* (i.e. Rotstop®) may become no longer available or are not approved for use in Southern Europe, where the invasive *H. irregulare* is present, making the development and approval of new treatments timely. The main goals of this thesis were: i) assessing the effectiveness in controlled conditions and in the field, i.e. forests, of the biological control agent *Pseudomonas protegens* (strain DSMZ 13134; i.e. Proradix®) and of its cell-free filtrate (CFF) in comparison with the state-of-the-art treatments urea and Rotstop® against the four *Heterobasidion* species currently occurring in Europe; ii) exploring the modes of action of *P. protegens* (strain DSMZ 13134) against *Heterobasidion* species; and iii) evaluating the ecological impact of stump treatments based on *P. protegens* (strain DSMZ 13134) and its CFF on *Pinus pinea* stumps in a stand infested by the invasive *H. irregulare* on both bacterial and fungal communities inhabiting stump surfaces. As a matter of fact, a multidisciplinary approach involving microbiological, analytical chemistry and metagenomic techniques was used to shed light on the biological control potential of *P. protegens* (strain DSMZ 13134) and its CFF against *Heterobasidion* species. *Pseudomonas protegens* (strain DSMZ 13134) and its CFF proved to be effective as biological control agents against *Heterobasidion* species, although outcomes were not uniform across *Heterobasidion* species. On stumps of some host tree species, the effectiveness of *P. protegens* (strain DSMZ 13134) was weak in reducing the incidence or the area colonised by *Heterobasidion* spp. Conversely, in most cases the CFF of the bacterium performed similarly to the state-of-the-art treatments urea and Rotstop®. All lines of evidence indicate the antibiosis as the main mode of action of *P. protegens* (strain DSMZ 13134) against *Heterobasidion* spp. Among the secondary metabolites produced by the bacterium, we detected pyoluteorin at concentrations ranging from 0.01 to 10.21 mg/L and

2,4-diacetylphloroglucinol at concentrations not exceeding 0.5 mg/L. Pyoluteorin at a concentration of 3.77 mg/L was detected in the CFF, and data of *in vitro* experiments suggest this compound to be responsible, at least partially, of the inhibition of the four *Heterobasidion* species present in Europe. After four months from treatments, *P. protegens* (strain DSMZ 13134) and its CFF did not result in significant shifts of the structure of microbial communities inhabiting the surface of *P. pinea* stumps, while urea and Rotstop® did, at least in terms of fungal diversity and richness. However, correspondence analysis revealed that microbial community composition might be affected by treatments, including those based on *P. protegens* (strain DSMZ 13134).

In conclusion, the results presented in this thesis support the use of both *P. protegens* (strain DSMZ 13134) and its CFF against the four species of *H. annosum* species complex currently occurring in Europe. Overall, stump treatment with the CFF of *P. protegens* (strain DSMZ 13134) combining good effectiveness against *H. irregulare* and no adverse effects on microbial communities, at least on stumps of *P. pinea*, can be considered the best candidate treatment. However, comprehensive studies and long-term monitoring of the same stumps are desirable to exclude that adverse effect will occur later. Moreover, follow-up studies are needed to exclude any environmental hazard associated with the use of treatments on stumps of the other tree species included in the field trials of this thesis. This would be crucial to support a safe use of these stump treatments in practical forestry.

General introduction and aims

General introduction

Biological control

Biological control of plant pathogens is traditionally defined as the use of living organisms to suppress or reduce the population of a pathogen below a threshold of ecological and economic impact (Regnault-Roger *et al.,* 2012; Stenberg *et al.,* 2021). The large body of literature developed on this sustainable strategy to manage plant diseases has embraced over time new biological control agents involved through various mechanisms in the control of plant pathogens (Raymaekers *et al.,* 2020; Stenberg *et al.,* 2021). For example, although viruses are not regarded as living organisms, they are certainly considered as valid agents of biological control (Balla *et al.,* 2021; Stenberg *et al.,* 2021). Similarly, diverse non-living nature-based substances are commonly included in the area of biological control. These include semiochemicals, RNA interference, and protein applications, just to name a few. In the attempt to clarify and organize the large amount of terms spent on biological control, Stenberg and colleagues (2021) suggested the umbrella term bioprotection to cover both living and non-living biological control agents providing protection against plant pathogens. However, a clear boundary between living and non-living agents must be maintained for both scientific and regulatory reasons.

Biological control strategies have gained an increasing attention in relation to the urgent need of reducing the use of chemicals to control insect pests and diseases (Raymaekers *et al.,* 2020; Balla *et al.,* 2021; Teixidó *et al.,* 2022). Compared to conventional chemicals, biological control is regarded as a sustainable measure to control plant diseases, being affordable, environmentally safe, and without risks for humans (Raymaekers *et al.,* 2020; Balla *et al.,* 2021). The most important mechanisms of action of biological control agents include competition for nutrients and space, parasitism, production of secondary metabolites, and secretion of extracellular enzymes (Stenberg *et al.,* 2021; Balla *et al.,* 2021). However, as suggested by Köhl *et al.* (2019), life is certainly more complex and hence interactions occurring among the host plant, the target pathogen, the resident microbiota, and the biological control agent may not fit such scientific categories. An emblematic example of such complexity is the phenomenon of hypovirulence observed in

the chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr on European chestnut (*Castanea sativa* [Mill.]) (Grente, 1981).

Assembled consortia of microorganisms combining different modes of action have been proposed as biological control agents in the attempt to mimic the complexity of life (Köhl *et al.,* 2019; Balla *et al.,* 2021). The use of assembled consortia, instead of an individual biological control agent, can be an advantageous strategy, as the cooperative and synergistic interactions of different microorganisms can more easily maintain the plant pathogen population at non-hazardous level (Bhatia *et al.*, 2018; Köhl *et al.*, 2019; Balla *et al.,* 2021). An assembled consortium also appears as more stable and resilient towards environmental stress factors (Jain *et al.*, 2011; Bhatia *et al.*, 2018). In this context, biological control with strains belonging to one or more microbial communities of the microbiota (i.e. bulk and rhizosphere soil, phyllosphere and endophytic microbiota) of the target plant may represent a suitable approach (Balla *et al.,* 2021). For example, the research group of Andreas Ulrich in two paired studies performed a comparative analysis of ash leaf-colonizing bacterial and fungal communities in susceptible and tolerant ash trees (*Fraxinus excelsior* L.) in stands threatened by the invasive ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) in the attempt to find microorganisms with health-supporting potential (Becker *et al.*, 2020*;* Ulrich *et al.,* 2020). Although both bacterial and fungal community structure did not show significant differences depending on the health status, an *in vitro* screening of endophytes provided a set of promising microbial strains inhibiting the growth of *H. fraxineus* (Becker *et al.,* 2020; Ulrich *et al.,* 2020). New genomic technologies offer certainly an additional glimpse into these microbial communities, as well as a scientific basis for the identification of microbial strains displaying direct or indirect effects against plant pathogens. The efficacy of a single biological control agent against plant pathogens can also be improved with the application of so called helper strains (Massart *et al.*, 2015). Instead of selecting a microorganism for its biological control properties, the helper strain is identified, selected and used mixed to the biological control agent to facilitate or enhance its action in field conditions (Massart *et al.*, 2015). Helper strains may improve establishment or survival of the biological control agent on the host, or enhance the production by the biological control agent of secondary metabolites (Massart *et al.*, 2015; Niu *et al.*, 2020). Benefits of applying a mix of several microbial strains against plant pathogens have already been demonstrated

(Jain *et al.*, 2011; Niu *et al.*, 2020; Izquierdo-García *et al.*, 2021). However, mass production, storage, and registration of more than one microbial strain, add further difficulties and can make the production process costly (Bhatia *et al.*, 2018; Köhl *et al.*, 2019). Despite the high potential of this approach, its implementation is still at the very beginning (Czajkowski *et al.*, 2020).

Bacteria belonging to the genera *Bacillus* and *Pseudomonas* have been extensively studied for their biological control potential, as well as for their beneficial plant growth promoting traits (Mark *et al.*, 2006; Raaijmakers and Mazzola 2012; Mishra and Arora 2018). One of the best-studied modes of actions of *Pseudomonas* spp. hinges on their ability to produce secondary metabolites, including phenazines, pyrrolnitrin, rhizoxin, cyclic-lipopeptides, orfamides, 2,4-diacetylphloroglucinol, hydrogen cyanide, pyoluteorin with diverse biological properties as they may act as antimicrobial, antimitotic, herbicidal, anthelmintic, nematocidal, phytotoxic and, quorum-sensing signal molecules (Mishra and Arora 2018; Shahid *et al.*, 2018). The current literature is focused mainly on the production and biological properties of these secondary metabolites *in vitro* or under controlled laboratory conditions (Raaijmakers and Mazzola 2012). Whereas, less investigated and monitored is the spatiotemporal production of secondary metabolites *in situ* due to obvious constrains (Raaijmakers and Mazzola 2012). Raaijmakers and Mazzola (2012), in their review on secondary metabolites produced by bacteria, provided a list of papers where the *in situ* production of secondary metabolites by indigenous and introduced bacteria was demonstrated in different environments, such as soil, spermosphere, and rhizosphere. Reporter gene systems were used to monitor the transcriptional activity of specific genes involved in the expression of secondary metabolites (Raaijmakers and Mazzola 2012). Various analytical techniques, including thin layer chromatography, high-performance liquid chromatography coupled with mass spectrometry, merely confirmed that secondary metabolites production occurred (Raaijmakers and Mazzola 2012). In this context, the following question that comes up is whether the amounts of secondary metabolites detected are sufficient *in situ* to display their biological control activity extensively discussed in the literature. In addition, time and place of production are crucial to display their inhibitory or plant-growth promoting activity (Raaijmakers and Mazzola 2012). Such issues will deserve further investigation and will advance our knowledge on biological control.

Biological control of tree diseases

Trees and forests cover 31% of the land globally accounting for the 4.06 billion hectares (FAO, 2020). Forests are crucial for life, as they directly and indirectly make possible the life on Earth. Ecosystem services and benefits provided by forests range from carbon sequestration and support to biodiversity, to personal and cultural enrichment (Aznar-Sánchez *et al.*, 2018; Balla *et al.*, 2021). Trees and forests currently face a variety of threats, such as climate change and attacks by insect pests and pathogens, among others (Balla *et al*., 2021; Prospero *et al.*, 2021). Climate change per se is known as a trigger of emerging forest diseases (Prospero *et al*., 2021). However, biological control of tree diseases has received little attention compared to that of agricultural crops (Cazorla *et al.*, 2016; Prospero *et al.*, 2021). Biological control of tree diseases is challenging mainly because of the peculiarities of the host. Due to the perennial nature and greater longevity of trees compared to annual agricultural plants, the biological control agent may need to be active for a long time, even more than one growing season (Cazorla *et al*., 2016; Prospero *et al*., 2021). Another complication refers to the more complex anatomy and larger biomass of trees (Cazorla *et al.*, 2016). Tree pathogens are less managed and less manageable in general, as often pesticides are not authorized for use on forest and urban trees (Prospero *et al*., 2021). Often, management of forest pathogens is based on silvicultural practices (Prospero *et al*., 2021). However, some good examples of biological control implemented in practice against forest pathogens occur. The case of hypovirulence in *C. parasitica* was already mentioned in this thesis. *Cryphonectria parasitica* strains infected by the *Cryphonectria hypovirus* 1 can be artificially inoculated in chestnut stands to control the progress of the chestnut blight (Prospero *et al*., 2021). Biological control of the Dutch elm disease is another great example. The product Dutch Trig® based on *Verticillium albo-atrum* Reinke and Berthold (strain WCS850) injected into the vascular system of healthy elms (*Ulmus* spp.) protect trees from the infection by *Ophiostoma novo-ulmi* Brasier (Postma and Goossen-van de Geijn, 2016). This commercially available biological control product had successfully contributed to control Dutch elm disease for twenty-four years, as only 0.1% of the injected elms became infected through beetle transmission and 0.4% through root grafts (Postma and Goossen-van de Geijn, 2016). Root and butt rots of conifers caused by *Heterobasidion* spp. can be controlled by applying the basidiomycete *P. gigantea* (Fr.) Jülich. As the biological control

of these pathogens is the focus of thesis, this model system will be described extensively later.

Guidelines and recommendations for a successful development of biological control against plant pathogens are described in the review of Prospero and colleagues (2021), who specifically focused on tree and forest pathogens. Emphasis is given on the definition of the best strategy to deploy. According to the type of tree disease, pathogen and biological control agent, the targets can be decreasing i) the abundance of pathogen inoculum below a satisfactory threshold, ii) the probability of infection or iii) the rate of colonization of host tissues (Cook, 1993; Prospero *et al.,* 2021). Studies need to be performed initially *in vitro* to collect information on the biological control agent and its modes of action, as well as to its potential efficacy against the target pathogen (Prospero *et al.,* 2021). Trials in the field, i.e. forest are necessary to assess the performances of the biological control agent in real conditions (Prospero *et al.,* 2021). The performances of a biological control agent in the field may be enhanced by using several strategies, which are discussed in the next section.

Hurdles in the commercialisation of biological control agents

A broad adoption of biological control depend on several factors, including the effectiveness, durability, ecological sustainability of the biological control agent, but also economic and practical incentives (He *et al*., 2021). In practice, such factors remain hurdles to the widespread use of biological control (He *et al*., 2021; Teixidó *et al*., 2022). Effectiveness of the biological control agent against the plant pathogen is clearly of outmost importance. An improved understanding of its modes of action can facilitate the optimization of its effectiveness. In addition, knowledge about its biology and peculiarities may allow predicting possible side effects, such as its persistence in the environment (Köhl *et al.,* 2019; He *et al.,* 2021). Attempts should be made to combine the biological control agent with other disease control measures, such as sanitary cuttings to reduce the inoculum sources, the use of more resistant trees or more diversified tree plantations under an integrated disease management approach (He *et al.,* 2021; Prospero *et al.,* 2021). Durability of the biological control agent is associated with intrinsic genetic and biological properties of both the target pathogen and the biological control agent (He *et al.*, 2021). Target

pathogens with high evolutionary potential are expected to rapidly adapt to changing conditions, and, as a consequence, to be more difficult to control. Similarly, mixed modes of action result in an advantage for the biological control agent, making it more durable compared to those with higher specificity. Biological control agents are generally considered as safe and sustainable with respect to humans and the environment (He *et al.*, 2021). However, candidate biological control agents should be evaluated carefully also for their impacts on ecosystems (He *et al.*, 2021). Attitude of users, i.e. farmers, forest growers and managers, can be powered not only by the technical factors discussed above, but also by economic and practical incentives (He *et al.*, 2021). Briefly, the biological control products should be easy to assess, ready to use, and economically advantageous compared to conventional control methods. Enormous is the difference existing between the huge amount of publications focused on screening new effective biological control agents and the scarce number of publications on key aspects of their commercialisation, such as mass-production, formulation, packaging and shelf life (Teixidó *et al*., 2022). Many potential biological control agents have not been taken forward at the stage of scaling up and or formulation trials (Teixidó *et al*., 2022). In part, this depends on the fact that biological control agents are living organisms and therefore particularly sensitive to conventional processes of production, formulations and storage (Teixidó *et al*., 2022). Moreover, such technological processes are usually carried out by private companies and rarely developed and implemented exclusively or in collaboration with scientific institutions (Teixidó *et al*., 2022). Hence, confidentially agreement usually covers information on all these aspects (Teixidó *et al*., 2022).

Finally, registration and cost development add further difficulties (Köhl *et al.*, 2019; Prospero *et al.*, 2021; Stenberg *et al.*, 2021). Current regulatory systems lack a common conceptual framework for biological control (Stenberg *et al.*, 2021). While effective and sustainable biological control agents are urgently required to balance out the decreasing number of allowed pesticides, as well as the lack of alternatives on the market, registration procedures in the EU mainly follow a precautionary principle (Köhl *et al*., 2019; Prospero *et al.*, 2021). Data requirements concerning the modes of action of biological control agents are set out by Commission Regulation (EU) No. 284/2013 in its Part B on microorganisms, including viruses. Attention is posed on the detailed evaluation of the principal modes of

action. Adverse effects of such mechanisms should be carefully evaluated on “the treated crop, operator exposure, viable residues, fate, and behaviour in the environment and at risk assessment of birds, mammals, aquatic organisms, bees, arthropods other than bees and earthworms and nitrogen and carbon mineralization in the soil” (Commission Regulation [EU], 2013; Köhl *et al*., 2019). Updated reviews on the subject (Köhl *et al*., 2019; Prospero *et al.,* 2021; Stenberg *et al.,* 2021) discussed in the present section critically reached the same conclusion, that is the need of a profound rethinking of the existing EU regulations on biological control if a widespread use of this approach in agricultural, urban and forest areas is deemed appropriate. Principles for the evaluation and decision making on biological control agents cannot be similar to those applied for risk assessment of pesticides, as the biological control agent is part of the environment and therefore humans and environment itself have already and still been exposed to it (Köhl *et al*., 2019; Prospero *et al.,* 2021).

The species complex *H. annosum sensu lato*

Distribution and taxonomy

Root and butt rots caused by the species of the fungal complex *H. annosum* (Fr.) Bref. *sensu lato* (s.l.) have long been described as some of the most destructive pathogens in coniferous forests of the Northern Hemisphere (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013). The yearly economic losses deriving from a reduction in both tree growth and wood quality were estimated a few decades ago at 790 million Euros per year (Woodward *et al.*, 1998). The fungal species complex has a worldwide distribution that currently reflects that of the main host tree species (Figure 1) (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013).

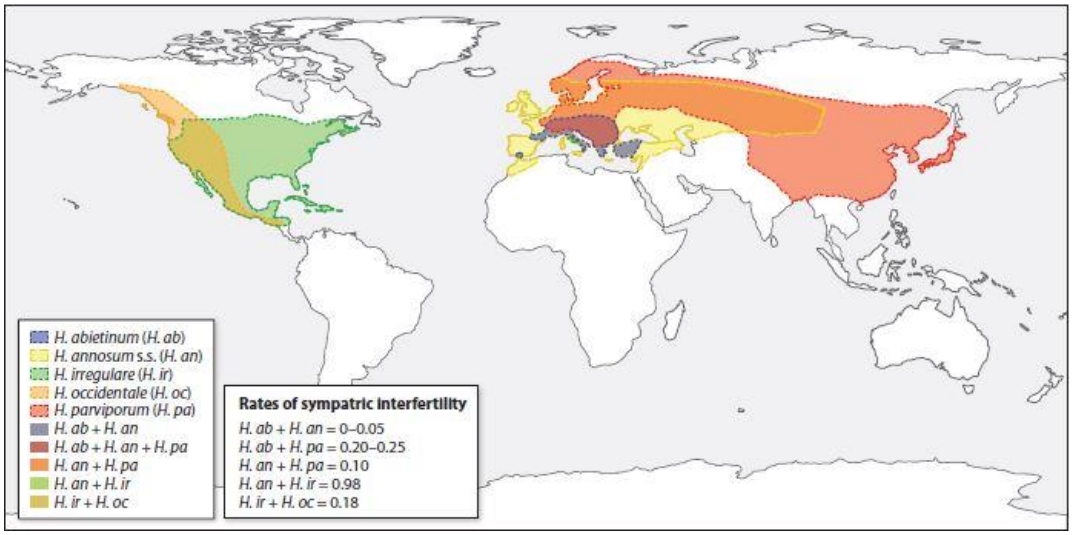

Figure 1. *Distribution area and rate of sympatric interfertility of species belonging to the* H. annosum *species complex (from Garbelotto and Gonthier, 2013).*

The *H. annosum* s.l. comprises five distinct species displaying a certain degree of host preference (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013). Three of these are Eurasian species described as *H. annosum sensu stricto* (s.s.), *H. abietinum* Niemelä & Korhonen, and *H. parviporum* Niemelä & Korhonen (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013). The North American species were described as *H. irregulare* (Underw.) Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel. (Garbelotto and Gonthier, 2013). *Heterobasidion annosum* s.s. displays a wide host range, although it is mainly associates as a root rot agent with pines (*Pinus* spp.) (Asiegbu *et al.*, 2005; Garbelotto and Gonthier,

2013). *Heterobasidion abietinum* is associated with silver fir (*Abies alba* Mill.) and other species of the genus *Abies*, while *H. parviporum* with Norway spruce (*Picea abies* [L.] Karst.) (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013). The North American *H. irregulare* attacks many coniferous species like pines, junipers (*Juniperus* spp.), and incense cedar (*Calocedrus decurrens* [Torr.] Florin). Host range of *H. occidentale* is wider as it encompasses the genera *Abies*, *Picea*, *Tsuga*, *Pseudotsuga*, and *Sequoiadendron* (Garbelotto and Gonthier, 2013; Poloni *et al.*, 2021).

Heterobasidion annosum s.s. is widespread all over Europe (Garbelotto and Gonthier, 2013). *Heterobasidion parviporum* distribution overlaps with that of Norway spruce since it is found from the Alps to the northern regions of Europe, and extends towards eastern Europe and Asia reaching China, Japan, and southern Siberia regions (Garbelotto and Gonthier, 2013). The distribution of *H. abietinum* was so far identified in central Europe and in the Mediterranean Basin from Spain in the west to Turkey and Caucasia in the east (Garbelotto and Gonthier, 2013). *Heterobasidion occidentale* is reported in western forests of North America from Alaska to southern Mexico (Garbelotto and Gonthier, 2013; Poloni *et al.*, 2021), while *H. irregulare* is more widely distributed in the United States, Mexico and part of Canada, from east to the west (Garbelotto and Gonthier, 2013; Poloni *et al.*, 2021). Intriguingly, *H. irregulare* is also present in an area of approximately 100 km along the Tyrrhenian coast of central Italy, near Rome (Italy), where it has been reported as invasive in Italian stone pine (*P. pinea* L.) stands (Gonthier *et al*., 2004, 2007, 2014; Garbelotto *et al.*, 2022). Introduction most likely occurred in association with the movement of infected wood by the US army during World War II (Gonthier *et al.*, 2004 and 2007). *Heterobasidion* species are among the most deeply studied tree pathogens worldwide as demonstrated by thousands scientific papers, reviews and books. This deep understanding makes these species ideal pathosystems to improve our knowledge on biological control programmes to be applied against forest pathogens.

Infection and spread

Heterobasidion annosum s.l. can produce perennial basidiocarps on stumps, roots, wood residues, and dead or diseased trees (Korhonen and Stenlid, 1998; Garbelotto and Gonthier, 2013). The main pathway for infection is represented by basidiospores that infect freshly exposed wood tissues, typically the freshly-cut stump surfaces creating during thinnings, or wounds from which mycelia (secondary infection) further spread via root contacts from infected stumps or trees to neighbouring healthy trees (Korhonen and Stenlid, 1998; Garbelotto and Gonthier, 2013). Stump susceptibility to airborne infections rarely exceeds one month, due to changes in the chemistry of nutrients available on the stump surface and to competition by other microorganisms (Garbelotto and Gonthier, 2013). The fungus grows downward to the root system at a rate of about 20 cm per month, although different growth rates have been reported depending on climate and host species (Garbelotto and Gonthier, 2013). It should be noted that the fungus is not able of growing freely in the soil, although it may survive for decades on wood residuals (e.g. portions of roots) buried in the soil (Garbelotto and Gonthier, 2013).

Control strategies

Forest management is the primary factor influencing *Heterobasidion* root and butt rots, at least indirectly (Redfern and Stenlid, 1998; Garbelotto and Gonthier, 2013). In natural and unmanaged forests where stumps may be rare or absent, *H. annosum* s.l. plays a more subordinate role than in managed forests (Redfern and Stenlid, 1998; Garbelotto and Gonthier, 2013). Conversely, in intensively managed forests, often characterised by monocultures, *H. annosum* s.l. can become destructive (Garbelotto and Gonthier, 2013). The rationale for attempting to control the disease hinges on the interruption of the life cycle of the pathogen on the stump, where its airborne spores land (Pratt *et al.*, 1999; Garbelotto and Gonthier, 2013). Treatment of the stump surface immediately after felling is so far the most effective and widespread control measure against *Heterobasidion* spp. (Asiegbu *et al.*, 2005; Garbelotto and Gonthier, 2013). Freshly-cut stump surfaces can be sprayed with either chemical or biological treatments to prevent basidiospores infection (Garbelotto and Gonthier, 2013; Gonthier and Thor, 2013; Poloni *et al.*, 2021). In Europe, urea as a chemical

treatment and the biological control agent *P. gigantea* have proved to be effective and are used in practical forestry (Garbelotto and Gonthier, 2013; Poloni *et al.*, 2021). Different formulations of *P. gigantea* are available on the market, among which the most common is the one registered as Rotstop® (Pratt *et al*., 1999; Gonthier and Thor, 2013). However, neither Rotstop® nor other stump treatments based on *P. gigantea* are currently approved for use in the majority of southern European countries. Whereas, the registration of the chemical treatment urea is close to the expiration date, currently fixed at August 2022 (EU Reg. 2021/745). Under this perspective, there is an urgent need to identify alternative stump treatments. This is of outmost importance for southern Europe, where no stump treatments will be available to control the three native *Heterobasidion* species and the non-native invasive *H. irregulare* currently occurring in this area.

Aims

The overall goal of this thesis was to investigate the effectiveness, the modes of action and the ecological impact of treatments based on the biological control agent *P. protegens* (strain DSMZ 13134) against native and invasive *Heterobasidion* species present in Europe. In detail, the studies conducted were aimed at:

Chapter I (paper I published in *Biological Control*) – exploring the biological control potential *in vitro* and on wood discs in controlled conditions of *P. protegens* (strain DSMZ 13134) towards the four *Heterobasidion* species currently occurring in Europe;

Chapter II (paper II published in *Pathogens*) – assessing the effectiveness of *P. protegens* (strain DSMZ 13134) and its cell-free filtrate (CFF) on stumps of *A. alba, P. abies, P. pinea* and *P. sylvestris* L. infested by each of the four *Heterobasidion* species present in Europe. In addition, we explored the effect of stump size on treatment effectiveness;

Chapter III (paper III published in *Pathogens*) – investigating the presence and concentration in the CFF of *P. protegens* (strain DSMZ 13134) used as stump treatment in the field experiments of Chapter II of some secondary metabolites known to be produced by the genus *Pseudomonas*. The antifungal activity of secondary metabolites found in such CFF was also assessed *in vitro* against *Heterobasidion* spp. In addition, we determined whether the variety and yield of such secondary metabolites could be enhanced by acting on culture conditions. Finally, our ultimate aim was to explore whether the production of these compounds by *P. protegens* (strain DSMZ 13134) is temperature-controlled during the interaction with *Heterobasidion* species;

Chapter IV (paper IV, manuscript) – evaluating by using a metagenomic approach the ecological impact of treatments applied on *P. pinea* stumps against *Heterobasidion irregulare* on bacterial and fungal communities inhabiting stump surfaces.

Chapter I

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

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

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HIGHLIGHTS

- *Pseudomonas protegens* was tested against all *Heterobasidion* spp. present in Europe.
- *P. protegens* was a strong antagonist of all *Heterobasidion* species *in vitro*.
- Antibiosis is the main mechanism of action.
- *P. protegens* is compared to urea and *P. gigantea* on wood discs.

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Urea

ABSTRACT

The biocontrol potential of a commercial product based on the bacterium *Pseudomonas protegens* (strain DSMZ 13134) and of its cell-free filtrate (CFF) was 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 that treatments 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*.

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

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

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

In this study, we tested the biocontrol potential of *P. protegens* (strain DSMZ 13134) towards the four *Heterobasidion* spp. currently occurring in Europe. This was carried out by (i) determining the inhibitory effect of both the bacterium itself and cell-free filtrate *in vitro*, (ii) assessing the role of diffusible and volatile compounds in the interaction between the bacterium and the fungal plant pathogens, and (iii) performing a comparative analysis of the efficacy of the bacterium (Proradix®), its cell-free filtrate and the state of the art treatments urea and *P. gigantea* (Rotstop®) on wood discs. Our hypothesis was that *P. protegens* (strain DSMZ 13134) could negatively affect *Heterobasidion* spp. through the mechanism of antibiosis.

2. Materials and methods

2.1. Microorganisms and culture conditions

Pseudomonas protegens (strain DSMZ 13134) was provided by SP Sourcon Padena GmbH (Tübeningen, Germany) and stored in Luria–Bertani (LB) broth amended with 30% glycerol at −80 °C. Fresh cultures were started from frozen stocks and refreshed in LB broth at 25 °C for 24 h with shaking before use. Five genotypes for each species of *H. annosum s.l.* occurring in Europe, i.e. *H. abietinum*, *H. annosum*, *H. irregulare* and *H. parviporum*, were randomly selected from the culture collection of the University of Turin (Table 1). Although some genotypes of *H. annosum* originated from the same area where the invasive and interfertile *H. irregulare* is present, none of them showed significant genetic admixing based on more than 500 AFLP markers (Gonthier and Garbelotto, 2011). Therefore, genotypes of *H. annosum* and *H. irregulare* selected for this study should be regarded as pure genotypes of either species, as previously suggested (Gonthier and Garbelotto, 2011). All genotypes of *H. annosum s.l.* derived from single spores collected on woody spore traps (i.e. wood discs) placed in the field and isolated in the laboratory as previously described (Gonthier et al., 2007). The fungal cultures were maintained on ISP2 medium (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g L^{-1} glucose and 20 g L^{-1} agar, pH 7.3) (Pridham et al., 1957) at 4 °C.

2.2. Determination of antifungal activity of P. protegens (DSMZ 13134) against Heterobasidion spp.

2.2.1. Dual culture assays

Antifungal activity of *P. protegens* (DSMZ 13134) against *Heterobasidion* spp. was first assessed by 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

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

MUT[a] accession number	Isolation date	*Heterobasidion* species	Geographic origin
6198*	2016	*H. abietinum*	Chabodey, AO, Italy
6194*	2016	*H. abietinum*	Nus, AO, Italy
6195	2018	*H. abietinum*	Chiusa di Pesio, CN, Italy
6196	2018	*H. abietinum*	Chiusa di Pesio, CN, Italy
6197*	2018	*H. abietinum*	Chiusa Pesio, CN, Italy
1204	2005	*H. annosum*	Sabaudia, LT, Italy
3538*	2006	*H. annosum*	Ansedonia, GR, Italy
3656	2006	*H. annosum*	Sabaudia, LT, Italy
3543*	2006	*H. annosum*	Mesola, FE, Italy
6191*	2015	*H. annosum*	Saint-Denis, AO, Italy
5666	2006	*H. irregulare*	Nettuno, RM, Italy
3627	2005	*H. irregulare*	Sabaudia, LT, Italy
1197*	2005	*H. irregulare*	Sabaudia, LT, Italy
1151*	2005	*H. irregulare*	Sabaudia, LT, Italy
1193*	2005	*H. irregulare*	Castelfusano, RM, Italy
6192*	2016	*H. parviporum*	Chabodey, AO, Italy
5605*	2006	*H. parviporum*	Druogno, VB, Italy
5612*	2006	*H. parviporum*	Trasquera, VB, Italy
5615	1999	*H. parviporum*	Charvensod, AO, Italy
6193	2016	*H. parviporum*	Chabodey, AO, Italy

[a] MUT: Mycotheca Universitatis Taurinensis.

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:

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

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

2.2.2. Effects of volatile compounds on mycelial growth

The antagonistic potential of volatile compounds produced by *P. protegens* (DSMZ 13134) against *Heterobasidion* spp. was explored using two-divided Petri plates. In treated plates, a 7-days-old fungal mycelial plug and a bacterial suspension (OD_{600} of 0.4) were spotted onto different halves of the same 9 cm-diameter two-divided Petri plates. In control plates only *Heterobasidion* spp. genotypes were grown. The same growing media, incubation temperatures, *Heterobasidion* spp. genotypes, number of replicates and MGI assessment method described in Section 2.2.1 were used.

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

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

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

The CFF_{25} and CFF_{10} described in Section 2.2.3 were also used to assess the inhibition potential of extracellular metabolites of *P. protegens* (DSMZ 13134) on *Heterobasidion* spp. conidial germination. The conidia of *Heterobasidion* spp. were obtained as follows. 500 μL of sterile water was loaded on the surface of 7–10 days old fungal colonies previously incubated at 25 °C in the dark. The water was gently shaked and collected by using a pipette. The concentration of conidia in the suspension was assessed by using a Bürker chamber, and the conidial suspension was subsequently adjusted to 10^5 conidia mL^{-1}. The same growing media described in Section 2.2.3 were used and poured as a thin layer (2 mm) in Petri plate. 100 μL of conidial suspension was loaded in Petri dishes and uniformly distributed using an L-shaped sterile Drigalski spatula. Three replicates per *Heterobasidion* spp. genotypes were used for each treatment (i.e. CFF_{25} and CFF_{10}) and for controls. After 48 h incubation at 25 °C, at least 100 conidia per Petri plate were inspected by direct observation on the underside of plates through a microscope at 200X magnification. Conidia were scored as germinated when the length of the germ tube exceeded the small-end diameter of the conidia. The conidial germination of treated (g_T) and control (g_C) plates was calculated as the ratio (in %) between the number of conidia germinated and the total number of conidia examined. Finally, the conidial germination inhibition (CGI) of *Heterobasidion* spp. was assessed by calculating the germination reduction (in %) observed in treated plates compared to control plates with the following equation:

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

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

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

Three genotypes for each species of *H. annosum s.l.* displaying highest

growth rates in control plates in dual culture assays were selected for this comparative experiment on wood discs (Table 1). Freshly cut woody discs 9 to 12 cm in diameter and 1–2 cm thick were obtained from healthy branches of *P. pinea, A. alba,* and *P. abies* to mimic the substrate for natural infection, i.e. freshly cut stumps, of *H. annosum* or *H. irregulare, H. abietinum* and *H. parviporum,* respectively. One surface (S_T) of each disc was sprayed by using a sterilized Pirex® sprayer with either a treatment or with sterile water to be used as a control until the surface became uniformly wet, i.e. approximately 1 mm thick layer. Proradix® was prepared by using methods and doses (12 g L^{-1}) reported on the product label 12 h before the application on wood discs to foster the growth of bacteria. The CFF_{25} was prepared as described in Section 2.2.3, while Rotstop® was suspended in sterile water, according to the instructions provided by the manufacturer. The urea treatment was prepared by dissolving urea in sterile water. Four hours after treatment application, the S_T of discs was uniformly sprayed as described above with a conidial suspension of *Heterobasidion* spp. Conidial suspensions were prepared as described in Section 2.2.4 at the concentration of 10^4 conidia mL^{-1}. Wood discs were singly placed onto sterilized microscope slides in Petri plates (15 cm in diameter) containing pieces of sterile filter paper dampened with sterile water. Petri plates were incubated at room temperature in the dark while filter papers were dampened periodically with 1 mL of sterile water to provide an adequate relative humidity for fungal growth.

After 8 days of incubation, discs were reversed and incubated at room temperature for additional 5 days. Hence, the untreated surface (S_{UNT}) of discs was inspected after 13 days of incubation under a dissecting microscope (20X magnification) for the presence of *Heterobasidion* conidiophores as previously described (Gonthier et al., 2001, 2007). The area covered with *Heterobasidion* conidiophores was delimited with a marker (Fig. 1) and redrawn on a transparent sheet. The sheet was scanned at 1200 dpi and the area colonised by *Heterobasidion* spp. conidiophores was measured (in mm^2) for each disc by using a virtual planimeter. A total of 600 wood discs were analysed (10 repetitions per each combination of five treatments, four *Heterobasidion* species and three genotypes per species).

To avoid using branches already infected by *Heterobasidion* spp. prior to the establishment of the experiments, two discs from each branch were also incubated in Petri plates containing dampened filter papers as described above. They were also inspected for the presence of *Heterobasidion* spp. conidiophores as previously described.

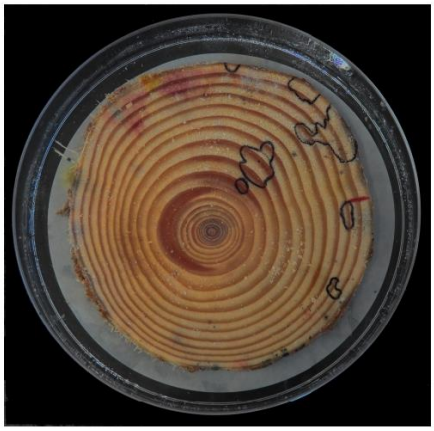

Fig. 1. Wood disc of *Abies alba* with areas colonised by *Heterobasidion abietinum* (marked areas).

2.4. Statistical analyses

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

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

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.*

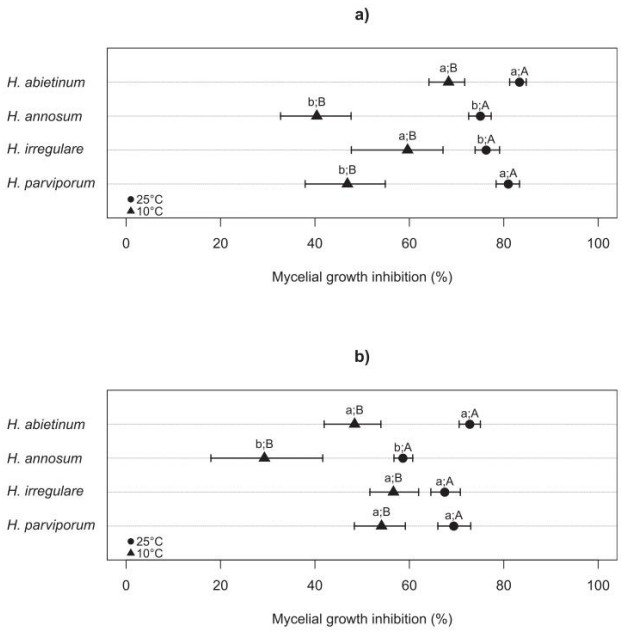

Fig. 2. Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI) resulting from the dual culture assays with *Pseudomonas protegens* (DSMZ 13134) on ISP2 (panel a) and ISP2-s (panel b) culture media at 10 °C and 25 °C. For each combination of culture medium, temperature 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.

irregulare and *H. parviporum* (73%, 68%, 69%, respectively), and significantly lower for *H. annosum* (59%) (Fig. 2b.). On the same medium after 11 days of incubation at 10 °C, again MGI was highest for *H. abietinum, H. irregulare* and *H. parviporum* (48%, 57%, 54%, respectively) and significantly lower (29%) for *H. annosum* (Fig. 2b.).

3.1.2. Effects of volatile compounds on mycelial growth

On ISP2, MGI varied depending on *Heterobasidion* species and was significantly lower at 25 °C than at 10 °C for *H. abietinum* and *H. parviporum* (Fig. 3a.). At both incubation temperatures, *H. annosum* was the least inhibited species (4 and 12% of MGI at 25 °C and 10 °C, respectively) (Fig. 3a.).

On ISP2-s, MGI was significantly higher at 25 °C than at 10 °C (Fig. 3b.). After 7 days at 25 °C, the MGI was highest for *H. annosum* and *H. parviporum* (24 and 21%, respectively), compared to the other two species.

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

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

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

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

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

In general, treatments resulted in a significant reduction of colonised areas of wood discs by the fungal pathogens compared to controls, with the exception of Proradix® for *H. annosum*, and of Proradix® and *P. protegens* (DSMZ 13134) CFF_{25} for *H. irregulare* (Fig. 5a-d.). CFF_{25} performed significantly better than Proradix® when used against all *Heterobasidion* species, with the exception of *H. irregulare*, against which they performed similarly. In experiments against *H. abietinum*, CFF_{25} reduced to a significantly greater extent than Rotstop® the area colonised by the pathogen compared to controls, and was almost as effective as the best treatment urea (Fig. 5a). Against *H. annosum*, CFF_{25} was as effective as the state of the art treatments Rotstop® and urea (Fig. 5b). Against *H. irregulare*, CFF_{25} was not effective as state of the art treatments (Fig. 5c), and the same was true for *H. parviporum*, despite in this case there was a significant and substantial reduction of colonised area compared to controls (Fig. 5d). Treatment with urea always resulted in 0 mm^2 of area colonised by all the tested *Heterobasidion* species.

4. Discussion

This study is the first dealing with the effects of *P. protegens* (DSMZ 13134) against all *Heterobasidion* species present in Europe. Previous pioneering reports referred to pilot studies conducted against a single

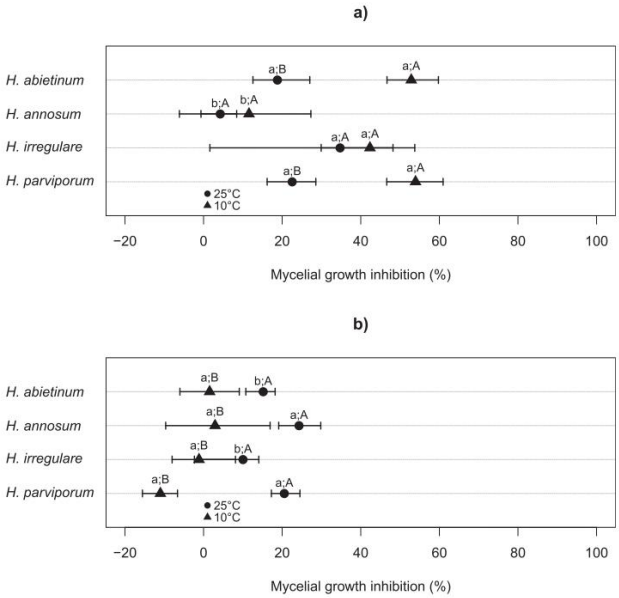

Fig. 3. Comparison of the average values of *Heterobasidion* spp. mycelial growth inhibition (MGI) resulting from the trials testing the effects of volatile compounds of *Pseudomonas protegens* (DSMZ 13134) on mycelial growth on ISP2 (panel a) and ISP2-s (panel b) culture media at 10 °C and 25 °C. For 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.

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

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)

Heterobasidion species, i.e. *H. parviporum* (Gžibovska, 2016; Rönnberg and Magazniece, 2018).

In dual culture experiments, *P. protegens* (DSMZ 13134) proved to be a strong antagonist of all the tested *Heterobasidion* species regardless of culture medium and incubation temperature. Nevertheless, the antifungal activity of the bacterium was significantly affected by the incubation temperature. The higher antagonistic activity at 25 °C than at 10 °C than may be due to environmental conditions closest to the optimum for the bacterium. It should be noted that the optimum growth temperature of *P. protegens* (DSMZ 13134) was recorded at 26 °C (Giovanni Amenta, personal communication).

Biological control involves different mechanisms including competition for nutrients and space, induction of plant defensive mechanisms, and antibiosis (Compant, 2005; Mérillon and Ramawat, 2012). The clear inhibition zone in dual culture assays precluding contact between the bacterium and the fungal genotypes, suggests that antibiosis through the production of antifungal compounds could be the main mechanism involved in the inhibition of *Heterobasidion* spp. by *P. protegens* (DSMZ 13134), as previously documented for other plant pathogens (Roberti et al., 2012). The production of antibiotics by bacteria has been reported to be affected by several abiotic factors, including temperature (Raaijmakers et al., 2002; Shanahan et al., 1992). Further, the production of antibiotics may be regulated by multiple genes that could possess different temperature thresholds (Daskin et al., 2014). In our case, the observed reduction in mycelial growth inhibition (MGI) of *Heterobasidion* spp. may be caused by different antifungal compounds, whose production by *P. protegens* (DSMZ 13134) and/or activity is mediated by temperature.

It has been previously suggested that the culture medium supplemented with sawdust is the closest medium to the woody natural substrate (Mgbeahuruike et al., 2011), providing a realistic approach to understand the fungal behaviour in woody substrates in the field (Woods et al., 2005). Overall, *P. protegens* (DSMZ 13134) showed a lower inhibition activity in culture medium supplemented with sawdust compared to the non-supplemented medium. This may be due either to the stimulation of *Heterobasidion* spp. operated by sawdust in the dual culture assay or by the inhibition of the bacterium or the suppression of production of antibiotics, or by all the above hypotheses since these are not mutually exclusive. It should be noted that while *Heterobasidion* spp. are fungi strictly associated with wood (Garbelotto and Gonthier, 2013); *P. protegens* is a PGPR associated with soil (Ramette et al., 2011), hence it is not surprising that dual culture assays on medium supplemented with sawdust resulted in a lower inhibition activity of the bacterium compared to the non-supplemented medium.

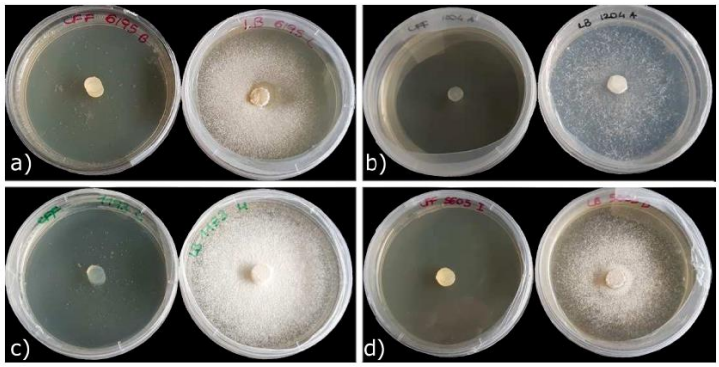

Fig. 4. Inhibition of mycelial growth of *Heterobasidion* spp. caused by CFF_{25} of *Pseudomonas protegens* (DSMZ 13134). The mycelial growth of a single genotype of *H. abietinum* (a), *H. annosum* (b), *H. irregulare* (c), and *H. parviporum* (d) on 80% CFF_{25} culture medium (left side) and on 80% LB culture medium (right side) after 7 days of incubation at 25 °C.

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

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)

Regardless of the temperature of incubation and the culture medium, mycelial growth inhibition (MGI) determined by *P. protegens* (DSMZ 13134) in dual culture assays was constantly lower for *H. annosum* than for the other *Heterobasidion* species, suggesting that the outcomes of the interaction are not uniform across *Heterobasidion* species, but rather they may be taxon-specific.

Previous studies demonstrated the antifungal nature of volatile compounds produced by bacteria, including *Pseudomonas* spp., suggesting a potential role of these compounds in the management of fungal plant pathogens (Cornelison et al., 2014; Fernando et al., 2005; Gabriel et al., 2018; Yuan et al., 2012). In the current study, *P. protegens* (DSMZ 13134) showed a much weaker ability to inhibit *Heterobasidion* species through volatile compounds compared to the combined exposure of both diffusible and volatile compounds in dual culture assays. This finding supports the hypothesis that the antifungal compounds responsible for the inhibition of *Heterobasidion* spp. are more likely diffusible than volatile. It is worth noting that an opposite effect of incubation temperatures was observed on ISP2 culture medium. In fact, while MGI of *Heterobasidion* spp. was greater at 25 °C than at 10 °C in dual culture assays, the opposite was true when only the effect of volatile compounds was tested. As discussed previously for antibiotics, also the production and effectiveness of antifungal volatile compounds are influenced by several abiotic factors, including temperature (Gabriel et al., 2018). These observations stress the importance of considering environmental conditions when assessing the effectiveness of biocontrol agents. Again, weaker performances of volatile compounds were observed on medium supplemented with sawdust compared to the non-supplemented medium. The reasons for that may be similar to those described previously for the dual culture assays.

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

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

In this study we used conidial suspensions not only to test the effects of CFF on conidia germination, but also to mimic natural spore deposition on treated or untreated wood discs simulating stumps. On untreated control wood discs, the areas colonised by *H. abietinum* and *H. parviporum* on discs of *A. alba* and *P. abies*, respectively, were much larger than those colonised by *H. annosum* and *H. irregulare* on discs of *P. pinea*. This may be due to the wood colonisation ability of the fungal species, to the host wood, or to the interaction between fungal and host wood species. While the elucidation of factors associated with the colonisation of wood by *Heterobasidion* spp. was not listed in the aims of this study, it is worth noting that on the same wood substrate (i.e.

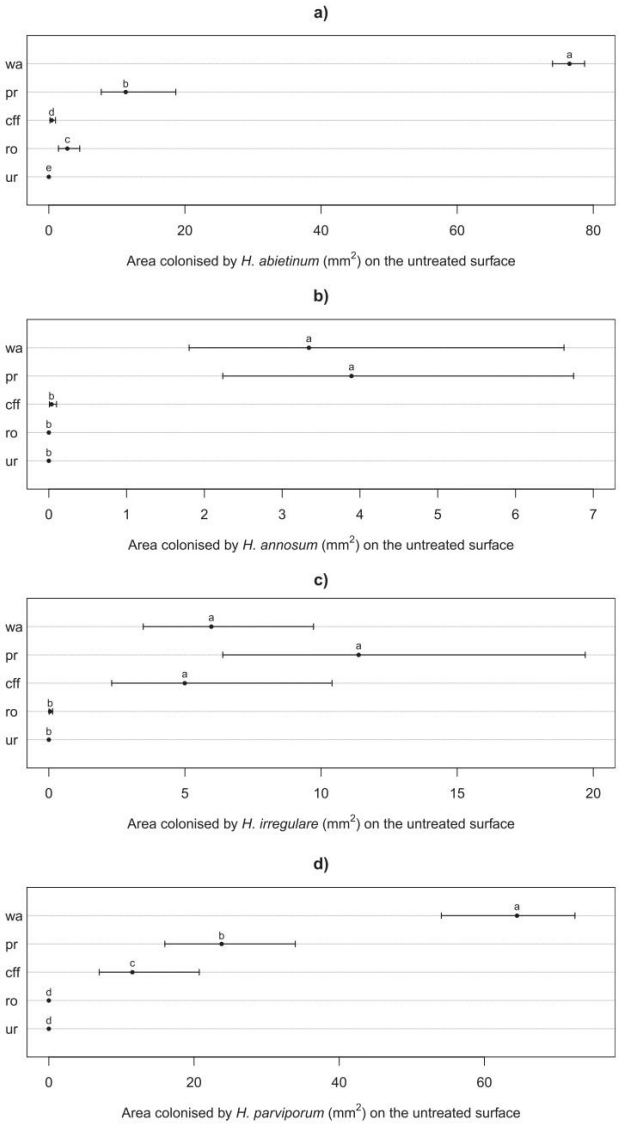

Fig. 5. Comparative performances of *Pseudomonas protegens* (DSMZ 13134) and state of the art treatments against *Heterobasidion abietinum* (panel a), *H. annosum* (panel b), *H. irregulare* (panel c) and *H. parviporum* (panel d) on wood discs. For each treatment (wa – water; pr – Proradix®; cff – CFF_{25};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

P. pinea), *H. irregulare* colonised a larger area than *H. annosum* did, which is consistent with previous findings pointing to a greater saprobic ability of the non-native *H. irregulare* compared to the native *H. annosum* on pine wood (Giordano et al., 2014). In these comparative experiments on wood discs, treatments with *P. protegens* (DSMZ 13134) CFF performed significantly better than Proradix® against most of the *Heterobasidion* species, further confirming on wood that the antagonistic activity hinges on antibiotics produced by the bacterium and present in the CFF rather than on other mechanisms.

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

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

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

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

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

CRediT authorship contribution statement

Martina Pellicciaro: Methodology, Investigation, Data curation, Writing - original draft. **Guglielmo Lione:** Formal analysis, Data curation, Visualization. **Luana Giordano:** Conceptualization, Methodology, Investigation. **Paolo Gonthier:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter II

Comparative efficacy of state-of-the-art and new biological stump treatments in forests infested by the native and the alien invasive *Heterobasidion* species present in Europe

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Comparative Efficacy of State-of-the-Art and New Biological Stump Treatments in Forests Infested by the Native and the Alien Invasive *Heterobasidion* Species Present in Europe

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Abstract: The *Heterobasidion annosum* species complex includes major fungal pathogens of conifers worldwide. State-of-the-art preventative stump treatments with urea or with commercial formulations of the fungal biological control agent *Phlebiopsis gigantea* (i.e., Rotstop®) may become no longer available or are not approved for use in many areas of Europe infested by the three native *Heterobasidion* species and by the North American invasive *H. irregulare*, making the development of new treatments timely. The efficacy of Proradix® (based on *Pseudomonas protegens* strain DSMZ 13134), the cell-free filtrate (CFF) of the same bacterium, a strain of *P. gigantea* (MUT 6212) collected in the invasion area of *H. irregulare* in Italy, Rotstop®, and urea was comparatively investigated on a total of 542 stumps of *Abies alba*, *Picea abies*, *Pinus pinea*, and *P. sylvestris* in forest stands infested by the host-associated *Heterobasidion* species. Additionally, 139 logs of *P. pinea* were also treated. Results support the good performances of Rotstop®, and especially of urea against the native *Heterobasidion* species on stumps of their preferential hosts and, for the first time, towards the invasive North American *H. irregulare* on stumps of *P. pinea*. In some experiments, the effectiveness of Proradix® and of the strain of *P. gigantea* was weak, whereas the CFF of *P. protegens* strain DSMZ 13134 performed as a valid alternative to urea and Rotstop®. The mechanism of action of this treatment hinges on antibiosis; therefore, further improvements could be possible by identifying the active molecules and/or by optimizing their production. Generally, the performance of the tested treatments is not correlated with the stump size.

Keywords: biological control; cell-free filtrate; forest pathogens; *Phlebiopsis gigantea*; Proradix®; *Pseudomonas protegens*; Rotstop®; urea

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1. Introduction

Heterobasidion annosum (Fr.) Bref. *sensu lato* (*s.l.*) is a complex of species comprising fungal plant pathogens causing root rots, butt rots and wood decay in coniferous forests of the Northern Hemisphere [1,2]. Annual economic losses caused by *H. annosum s.l.* in Europe were estimated at EUR 790 million [1] and occur in association with the decrease in wood production and the reduction in wood quality, which are reported as directly correlated to the incidence of the pathogens [3]. In Europe, *H. annosum s.l.* includes the native species *H. abietinum* Niemelä & Korhonen, *H. annosum sensu strictu (s.s.)* and *H. parviporum* Niemelä & Korhonen, mainly associated with *Abies alba* Mill., *Pinus* spp., and *Picea abies* (L.) Karst., respectively [2]. In addition, Italian stone pine (*Pinus pinea* L.) is located along the western coastline of central Italy is currently challenged by the invasive North American species *H. irregulare* Garbel. & Otrosina [4,5]. The risk posed by *H. irregulare* to European forestry is high [6]; therefore, the pathogen is recommended for regulation under the European and Mediterranean Plant Protection Organization (EPPO) A2 list.

Heterobasidion spp. infect their hosts through basidiospores or mycelium. Primary infection occurs when airborne basidiospores released from fruiting bodies land on the surface of freshly cut stumps and germinate, hence producing mycelia able to colonize the stump tissues. Once the root system of the stump is colonized, the mycelium of *Heterobasidion* spp. spreads to the neighboring heathy trees by means of root contacts and grafts [2]. This second method of spreading is acknowledged as secondary infection. Hence, stumps created during thinnings and cuttings play a crucial role as starters for new infection foci.

Silvicultural, chemical and biological strategies have been designed and tested to control *Heterobasidion* spp. infections and spreading [2]. Most of the silvicultural methods are based on providing spacing among trees in new plantations, on planning thinnings and cuttings during periods of low sporulation of the pathogens, and on the mechanical removal of stumps of diseased trees, including their root systems [7–11].

Although effective in reducing the risk of new infections, such strategies imply remarkable technical challenges and high costs for their execution; hence, they may be either unfeasible, or scarcely practicable [12–14]. Conversely, chemical or biological treatments applied on freshly cut stumps act as preventative measures targeting primary infection and are recognized as more sustainable than most of the other strategies [2,13].

A large body of literature has been published on the efficacy of urea [10,15–17]. The mechanism of action of urea has been reported as indirect. The urea solution raises the pH of the stump up to a level that inhibits basidiospore survival and germination [18]. Urea is the only chemical treatment used in practical forestry in Europe. However, the current European regulations might pose some constraints for the future use of urea, unless an extension of its authorization as a pesticide is issued after the deadline of August 2022 [19].

In the attempt to reduce the use of chemicals in forestry, several studies have focused on the selection of microorganisms that may act as biocontrol agents and inhibit *Heterobasidion* spp. The most successful results were obtained in the field with *Phlebiopsis gigantea* (Fr.) Jülich, a wood decay basidiomycete outcompeting *Heterobasidion* spp. thanks to its rapid colonization of the stump surface [12,16,17,20–28].

Phlebiopsis gigantea strains patented as Rotstop® are widely used in practical forestry both in Europe and North America [13,29], although neither such treatment, nor other products based on *P. gigantea*, are registered for use in southern European countries. Most of the results currently available on the efficacy of *P. gigantea* originate from experiments conducted against *H. parviporum* on *P. abies*, although information about the performance of *P. gigantea* against other *Heterobasidion* spp. on stumps of other tree species, i.e., *A. alba*, *P. pinea* and *P. sylvestris*, are still scarce. Moreover, few studies dealt with the comparative assessment of the efficacy of *P. gigantea* and urea on stumps [16,24,28,30].

As a result of preliminary studies, the bacterial biocontrol agent *Pseudomonas protegens* (strain DSMZ 13134), patented as Proradix®, proved its efficacy against *Heterobasidion* spp. [31–33]. The production of antibiotics and diffusible antifungal compounds allows *P. protegens* (strain DSMZ 13134) to inhibit the growth of *Heterobasidion* spp., as suggested by the outcomes of trials carried out by using the cell-free filtrate (CFF) of *P. protegens* (strain DSMZ 13134) [33]. Although promising, treatments based on *P. protegens* (strain DSMZ 13134) still need to be tested against all *Heterobasidion* species on stumps of their preferential hosts. In fact, so far, available data about *P. protegens* are limited to a small-scale pilot study on stumps of *P. abies*.Stump diameter influences the colonization process of *Heterobasidion* spp. [34,35]. It has been suggested that large areas of heartwood might create more opportunities for *H. parviporum* infection in *P. abies* [11]. However, in several tree species, sapwood is much more extensively infected by *Heterobasidion* spp. than heartwood [36], which is consistent with the observation that the fungus can establish even in small (<2 cm) stumps [37]. Although the effects of stump size on primary infections by *Heterobasidion* spp. have been investigated, the relationship between the treatment efficacy and the stump size has received little attention despite its potential importance in practical forestry [30].Comparative studies contrasting the effectiveness of chemical and biological

treatments on freshly cut stumps are advocated to design effective and sustainable control measures against *Heterobasidion* spp. on different host species present in Europe [28], taking into account that the level of efficacy of treatments may vary depending on climate and on local environmental conditions [38,39]. This issue is of the utmost importance not only in relation to the alien species *H. irregulare* threatening *P. pinea* across its invasion area in central Italy, but also for southern European countries, where treatments against *Heterobasidion* spp. are either not registered, or will be soon revoked. The aims of this study were to compare the efficacy of Proradix®, the CFF of *P. protegens* strain DSMZ 13134, the strain of *P. gigantea* MUT 6212 collected on *P. pinea* in the *H. irregulare* invasion area in central Italy and screened for its biocontrol potential [40], the biological control agent Rotstop®, and the chemical control agent urea as stump treatments in forests of *A. alba*, *P. abies*, *P. pinea*, and *P. sylvestris* each infested by their own host-associated *Heterobasidion* species (i.e., *H. abietinum*, *H. parviporum*, *H. irregulare*, *H. annosum s.s.*, respectively). Our experiments were carried out in two of the main ecoregions where the above cited hosts of *Heterobasidion* spp. grow, namely, the Alps (for *A. alba*, *P. abies*, and *P. sylvestris*) and the Mediterranean area (for *P. pinea*) [41,42]. To further investigate the efficacy of treatments against *H. irregulare*, an experiment was also set up in the field using *P. pinea* logs. An additional and final goal was to explore the effect of stump size on treatment efficacy.

2. Results

2.1. Comparative Efficacy of Treatments in Stumps of Abies alba, Picea abies, Pinus pinea and P. sylvestris

Stumps of *A. alba* treated and sampled were as follows: 24 control stumps sprayed with sterile water, 22 stumps treated with Proradix®, 23 with CFF, 23 with *P. gigantea* MUT 6212, 22 with Rotstop® and 21 with urea. Isolates obtained from control stumps of *A. alba* were all typed as *H. abietinum*. Incidence and areas colonized by *Heterobasidion* spp. on wood discs sampled from stumps are shown in Figure 1. In the Figure 1 barplots, each bar refers to a cluster of treatments and shows the related overall incidence of *Heterobasidion* spp. Treatments included within the same cluster are not associated with significant differences ($p > 0.05$), whereas treatments belonging to different clusters did result in significant differences ($p < 0.05$) of the incidence of *Heterobasidion* spp. Abbreviations listed under the bars indicate the treatments included within the corresponding cluster. In the dotcharts of the same figure, point marks show the average areas colonized by *Heterobasidion* spp. for each treatment. In both barplots and dotcharts, different letters indicate significant differences of the associated values ($p < 0.05$), and error bars refer to the 95% confidence intervals. The incidence of the pathogen was significantly ($p < 0.05$) higher in *A. alba* control stumps (95.8%) than in stumps treated with biological products (63.3%), that clustered together and separately from stumps treated with urea (9.5%) (Figure 1a). All treatments resulted in a significant reduction in area colonized by the pathogen compared to controls (Figure 1b). Urea provided the best performance among treatments (0.3% of area colonized by the pathogen), whereas stumps treated with *P. gigantea* MUT 6212 and Rotstop®resulted in a less severe infection of stumps (10.7% and 11.0%, respectively) compared to Proradix® and CFF (15.4% and 24.2%, respectively).

Stumps of *P. abies* treated and sampled were as follows: 21 control stumps sprayed with sterile water, 20 stumps treated with Proradix®, 21 with CFF, 21 with *P. gigantea* MUT 6212, 21 with Rotstop® and 21 with urea. Isolates obtained from control stumps of *P. abies* were all typed as *H. parviporum*. The incidence of the pathogen was significantly higher in control stumps and in stumps treated with Proradix® (60.0%) than in the other treatments (25.8%) (Figure 1c). In *P. abies* stumps, all treatments resulted in areas colonized by the pathogen significantly smaller than in controls (Figure 1d).

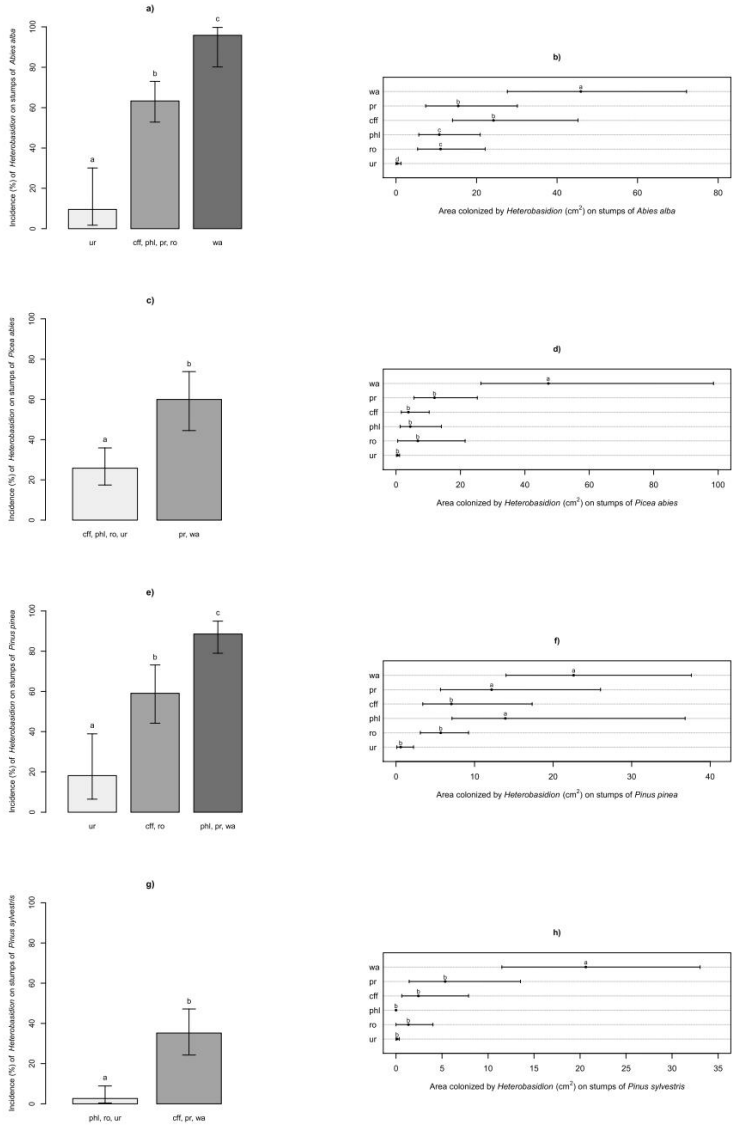

Figure 1. Barplots of the incidence (%—left panels) and dotcharts of the areas colonized by *Heterobasidion* spp. (cm^2—right panels) on treated stumps of *Abies alba* (panels (**a**,**b**)), *Picea abies* (panels (**c**,**d**)), *Pinus pinea* (panels (**e**,**f**)), *P. sylvestris* (panels (**g**,**h**)). Treatment abbreviations: wa—water; pr—Proradix®; cff—cell-free filtrate; phl—*Phlebiopsis gigantea* MUT 6212; ro—Rotstop®; and ur—urea. For details, refer to the text.

Stumps of *P. pinea* treated and sampled were as follows: 25 control stumps sprayed with sterile water, 23 stumps treated with Proradix®, 22 with CFF, 22 with *P. gigantea* MUT 6212, 22 with Rotstop® and 22 with urea. Isolates obtained from control stumps of *P. pinea* were all typed as *H. irregulare*. In *P. pinea* stumps, the incidence of the pathogen was significantly higher in control stumps and in stumps treated with Proradix® and *P. gigantea* MUT 6212 (88.6%) than in stumps treated with CFF and Rotstop®(59.1%) (Figure 1e). Urea performed significantly better than the other treatments (18.2% incidence of the pathogen). All treatments reduced the area colonized by the pathogen compared to control stumps (Figure 1f). However, such a reduction was significant only for CFF, Rotstop® and urea (7.1%, 5.7%, and 0.6%, respectively) (Figure 1f).

Stumps of *P. sylvestris* treated and sampled were as follows: 24 control stumps sprayed with sterile water, 23 stumps treated with Proradix®, 24 with CFF, 25 with *P. gigantea* MUT 6212, 24 with Rotstop® and 26 with urea. Isolates obtained from control stumps of *P. sylvestris* were all typed as *H. annosum s.s.* The incidence of the pathogen was significantly higher for control stumps, stumps treated with Proradix®and CFF (35.2%) than for stumps treated with *P. gigantea* MUT 6212, Rotstop®and urea, which showed an incidence of 2.6% (Figure 1g). The area colonized by the pathogen in treated stumps was significantly lower than that in control stumps (20.6%) for all treatments (Figure 1h). Treatment with *P. gigantea* MUT 6212 and with urea resulted in 0% and 0.1% areas colonized by the pathogen, respectively. Stumps treated with Proradix®, CFF and Rotstop® resulted in 5.3%, 2.4% and 1.3% areas colonized by the pathogen, respectively.

2.2. Efficacy of Treatments on Logs of Pinus pinea

Logs of *P. pinea* treated and sampled were as follows: 22 control logs sprayed with sterile water, 24 logs treated with Proradix®, 24 with CFF, 23 with *P. gigantea* MUT 6212, 24 with Rotstop® and 22 with urea. Isolates obtained from control logs of *P. pinea* were all typed as *H. irregulare*. Incidence and areas colonized by the pathogen on wood discs gathered from logs are shown in Figure 2. Barplots and dotcharts of Figure 2 were built as shown in Figure 1. The incidence of the pathogen in control logs and in logs treated with Proradix® was significantly higher (84.7%) than that in logs treated with the other treatments (26.8%) (Figure 2a). Significant reductions in the area colonized by the pathogen compared to control logs (9.5% of area colonized) were observed for all treatments (Figure 2b). In terms of the area colonized by the pathogen, CFF, *P. gigantea* MUT 6212 and Rotstop® performed significantly better than Proradix® and urea (0.8%, 0.4 and 0.2% vs. 1.7% and 1.0% of area colonized, respectively).

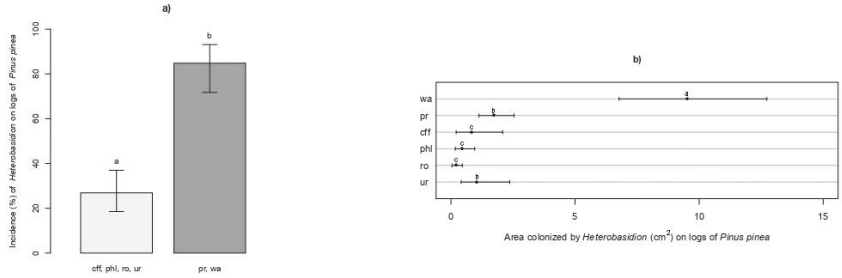

Figure 2. Barplots of the incidence (%—panel (**a**)) and dotcharts of the areas colonized by *Heterobasidion* spp. (cm^2—panel (**b**)) on treated logs of *Pinus pinea*. Treatment abbreviations: wa—water; pr—Proradix®; cff—cell-free filtrate; phl—*Phlebiopsis gigantea* MUT 6212; ro—Rotstop®; and ur—urea. For details, refer to the text.

2.3. Correlation between Stump Size and Efficacy of Treatments

The correlations between stump size and the area colonized by *Heterobasidion* spp. on wood discs sampled from stumps are shown in Table 1. For *P. abies*, *P. pinea*, and *P. sylvestris*, there were no significant correlations, either in control or in treated stumps.

For *A. alba*, a significant correlation between stump size and the area colonized by the pathogen was observed in control stumps (R = 0.474; $p = 0.019$) and in stumps treated with Proradix® (R = 0.447; $p = 0.037$). The other treatments did not display significant correlations between the two variables.

Table 1. Pearson's correlation coefficient between stump size and the area colonized by *Heterobasidion* spp. on stumps of *Abies alba*, *Picea abies*, *Pinus pinea* and *P. sylvestris* treated with water (wa), Proradix®(pr), cell-free filtrate (cff), *Phlebiopsis gigantea* MUT 6212 (phl), Rotstop®(ro), and urea (ur). The symbol * marks significant correlation ($p < 0.05$).

Host Tree Species	Stump Treatments					
	wa	pr	cff	phl	ro	ur
A. alba	0.474 * ($p = 0.019$)	0.447 * ($p = 0.037$)	0.016 ($p = 0.941$)	0.007 ($p = 0.975$)	0.313 ($p = 0.155$)	−0.315 ($p = 0.163$)
P. abies	0.220 ($p = 0.313$)	0.250 ($p = 0.261$)	−0.250 ($p = 0.249$)	−0.177 ($p = 0.406$)	−0.065 ($p = 0.766$)	0.017 ($p = 0.939$)
P. pinea	0.055 ($p = 0.794$)	−0.036 ($p = 0.870$)	0.004 ($p = 0.985$)	0.047 ($p = 0.834$)	−0.248 ($p = 0.264$)	0.017 ($p = 0.941$)
P. sylvestris	−0.230 ($p = 0.278$)	−0.205 ($p = 0.347$)	−0.323 ($p = 0.122$)	NA (p = NA)	−0.067 ($p = 0.757$)	−0.167 ($p = 0.414$)

3. Discussion

In this study, four biological stump treatments (Proradix®, CFF, *P. gigantea* MUT 6212 and Rotstop®) and one chemical treatment (urea) were tested against naturally occurring primary infections operated by all *Heterobasidion* species present in Europe, including the invasive *H. irregulare*, on their preferential hosts. Since the pioneering work of Rishbeth [43–46], who first elucidated the infection biology of the fungal pathogen and the potentiality of stump treatments for disease control, several studies have reported the effectiveness of stump treatments against primary infections of *Heterobasidion* spp. (see [10,20,22,24,28,30,47] and literature therein), but only a few of them compared the efficacy of biological and chemical control agents, namely, urea, in the same experiments [15,16,48]. In addition, biological control with bacteria, although promising and effective in vitro against forest pathogens [33,49–51], have never been tested in the field thus far, with the exception of a pilot study conducted on a few *P. abies* stumps [31,32]. Overall, with the exception of Proradix® and *P. gigantea* MUT 6212 on *P. pinea* stumps, all biological and chemical treatments tested were effective in significantly reducing the area colonized by *Heterobasidion* spp. on stumps of the tested tree species compared to controls. Furthermore, the efficacy of treatments was not correlated with stump size, with Proradix® on *A. alba* stumps being the only exception.

The experiments were carried out in periods at high risk for primary infections of *Heterobasidion* spp. in the Alps (i.e., in summer and autumn) and in coastal regions of central Italy (i.e., in winter), based on previously published information on the availability of airborne inoculum or on the frequency of stump infections [9,10,52]. This allowed relying on natural airborne infections for the experiments, without the need for artificial inoculations of the pathogens. Indeed, results of the experiments suggest that the risk of stump infection by *H. annosum s.l.* may be relevant where treatments are not ordinarily carried out on freshly cut stumps. It was previously suggested that stump treatments are economically profitable when stump infection frequency in untreated stumps reaches or exceeds the threshold of 20% to 30% [53,54]. In the present study, the incidence of the pathogen in control stumps ranged from 50% to 95.8% depending on tree species, thus recommending stump treatments in such situations.

Study sites were pre-selected based on the availability of airborne inoculum of the *Heterobasidion* species commonly associated with the most abundant tree species in the forest stands. Indeed, all isolates obtained from control stumps in each stand were identified as belonging the *Heterobasidion* species predicted. Therefore, results of our treatment experiments on *A. alba*, *P. abies*, *P. pinea* and *P. sylvestris* stumps should be regarded as targeting—and hence, valid against—*H. abietinum*, *H. parviporum*, *H. irregulare* and *H. annosum s.s.*, respectively. The above hosts grew in sites located either in the Alps (*A. alba*, *P. abies*, and *P. sylvestris*) or in the Mediterranean area (*P. pinea*). It is worth noting that most of the Italian forest stands harboring the tree species listed above are located in such ecoregions [41,42], although the distribution areas of *A. alba, P. abies*, *P. sylvestris*, and *P. pinea* across Europe overlap a wide range of forest types, habitats and ecosystems, whose environmental conditions (e.g., climate, soils, geographic position) and management practices may be highly variable [55–58]. Although the study sites we selected may be considered representative of the ecological and silvicultural conditions of two distinct and very different ecoregions, the Alpine and the Mediterranean, further studies replicating the same experiments in other ecoregions and areas of Europe (e.g., northern and eastern Europe) are needed to corroborate our core results, which are detailed below.

Overall, considering the results both in terms of the reduction in incidence of the pathogen and of the reduction in area colonized by the pathogen compared to controls, urea was the most effective treatment. This chemical treatment ranked either alone (on *A. alba* and *P. pinea*), or with other treatments (on *P. abies* and *P. sylvestris*) at the lower bound of incidence of *Heterobasidion* spp. Urea performed better than the other treatments in terms of areas colonized by the pathogen, although significance was observed only in the case of *A. alba*. The good performance of urea corroborated previous results obtained using wood discs of the same tree species in controlled conditions [33]. Urea has already been tested against *Heterobasidion* spp. with good results on stumps of *A. alba*, *P. abies* and *P. sylvestris* [10,16,39], and also against *H. annosum s.s.* on stumps of *P. pinea*; however, this is the first report of the efficacy of urea against *H. irregulare* on stumps of such host species.

Rotstop® was also shown to be rather effective against *Heterobasidion* spp. on stumps of several coniferous tree species, as expected based on a large body of literature and of its widespread use in practical forestry. However, our results should not be regarded as merely confirmatory. In fact, for the first time, this paper provides evidence about the efficacy of Rotstop® on *A. alba* stumps. Furthermore, whereas previous experiments conducted with Rotstop® on *P. pinea* stumps targeted *H. annosum s.s.* [20], this is first report of the efficacy of this treatment against *H. irregulare* on the same host. Adding some additional pieces of evidence on their efficacy, the results obtained with the commercial treatments against *Heterobasidion* spp. urea and Rotstop® could serve as references to appraise the performance of the newly tested treatments based on *P. protegens* strain DSMZ 13134 (i.e., Proradix® and CFF), and on *P. gigantea* MUT 6212 isolated from *P. pinea* in the *H. irregulare* invasion area. With the exception of *P. pinea*, the tested strain of *P. gigantea* performed as well as Rotstop® and, at least on some tree species, as well as urea. Surprisingly, results of *P. gigantea* MUT 6212 on *P. pinea* stumps, which we expected to be well adapted to the host in the Mediterranean region, were undistinguishable from those of untreated control stumps. The reason for this weak efficacy is unknown. Notable is the performance of this treatment especially on *P. sylvestris*. Although the efficacy of Rotstop® on *P. sylvestris* is in agreement with previous research conducted on the same host tree species in Latvia by Kenigsvalde et al. [23], treatment of *P. sylvestris* stumps with the *P. gigantea* MUT 6212 isolated from *P. pinea* was fully effective in preventing pathogen infections. Therefore, we can only speculate that either *P. gigantea* MUT 6212 is better adapted to *P. sylvestris* than to *P. pinea*, or it performs better when inoculated in summer and under Alpine environmental conditions. Although the two hypotheses are clearly not mutually exclusive, this study was designed to compare the performances of treatments on the same tree species and not across species; hence, the above inferences should be regarded as speculation.

With the exception of applications on *A. alba* stumps, Proradix® failed in reducing the incidence of the pathogen compared to controls, although a significant reduction in the area colonized by the pathogens was observed on the stumps of most tree species. On the other hand, the CFF of *P. protegens* strain DSMZ 13134 performed as well as Rotstop® in reducing the incidence of the pathogen compared to controls, with the only exception being treatments on *P. sylvestris*. CFF of *P. protegens* strain DSMZ 13134 was comparable to the most effective state-of-the-art treatments (urea and Rotstop ®) in terms of reducing the areas colonized by the pathogen compared to control stumps, with the exception of *A. alba*. Overall, and with a few exceptions, CFF of *P. protegens* strain DSMZ 13134 performed better than Proradix®, supporting previous observations conducted on wood discs under controlled conditions and pointing to a clear role played by antibiosis in the interaction of *P. protegens* strain DSMZ 13134 with *Heterobasidion* spp. [33]. Wood-inhabiting bacteria and fungi may interact in a variety of ways, such as competing for low-molecular-weight compounds released by extracellular fungal enzymes, bacterial mycophagy or the production of toxic bacterial or fungal secondary metabolites [59], but very little is known about the above interactions [60,61]. Oligomers released during lignin degradation by basidiomycetes are appropriate substrates for most wood-inhabiting bacteria that can take advantage of the degradation activity of fungi [59]. In this way, fungi can be systematically deprived of a large part of their growth substrates [59]. By examining the results obtained on *P. abies*, *P. pinea* and *P. sylvestris* stumps, Proradix® was often able to reduce the spreading of the pathogen within stumps (i.e., area colonized by the mycelium), but not its occurrence on stumps (i.e., incidence). Therefore, *Heterobasidion* spp. might colonize wood more rapidly in comparison to *P. protegens* strain DSMZ 13134, by infecting stumps and starting its wood decay process, while the bacterium remains latently present. It should be noted that *P. protegens* strain DSMZ 13134 is a plant-growth-promoting rhizobacteria (PGPR) [62], and may be scarcely adapted to wood. Later, *P. protegens* strain DSMZ 13134 may release secondary metabolites in response to stress signals, thereby affecting *Heterobasidion* spp. Whether or not this scenario is realistic requires further investigations on the patterns of wood colonization by *P. protegens* strain DSMZ 13134 and on its interaction with *Heterobasidion* spp. Conversely, metabolites contained in the CFF of *P. protegens* strain DSMZ 13134 make this treatment ready to go against *Heterobasidion* spp. However, further research aimed at identifying the most active metabolites present in CFF is desirable, because it could lead to significant improvements in the performance of this biological treatment.

This paper is the first focusing on the effectiveness of biological and chemical stump treatments against the invasive *H. irregulare* on *P. pinea*, its main known host in Europe. In the framework of the recently released national regulatory control system for *H. irregulare* [63], data presented in this paper could guide National Plant Protection Organisations in the choice of the most appropriate product for containment. Urea, Rotstop®, as well as CFF of *P. protegens* strain DSMZ 13134 are appropriate for this purpose, and hence can be used interchangeably, pending regulatory approvals. Surprisingly, CFF of *P. protegens* strain DSMZ 13134 performed as well as state-of-the-art treatments when applied on stumps of *P. pinea*, although this was not true when treatments were simulated on wood discs of *P. pinea* in controlled conditions [33]. Physical conditions differentiating wood discs and stumps, especially in terms of moisture, may have accounted for this. This finding confirms the need for field tests for the screening of biological control agents, as previously suggested [24].

Experiments carried out on *P. pinea* logs resulted in the good performance of treatments based on *P. gigantea* and on the CFF of *P. protegens* strain DSMZ 13134, and in a lower efficacy of urea and Proradix®. A relatively low efficacy of urea on logs could be explained by the mechanisms of action of urea against *Heterobasidion* spp. In fact, the hydrolysis of urea leads to an increase in pH that prevents spore germination on the living tissues of the stump surface [18]; however, such tissues are expected to live shorter in logs than in stumps. Results of the experiment conducted on logs could contribute to the drafting of

practical recommendations aimed at preventing infection and the subsequent fruiting of *H. irregulare* on wood residues, should residues be kept on-site for naturalistic purposes. It should be noted that several forests in the outbreak area of *H. irregulare* in Italy are either parks or a Site of Community Importance (SCI) that need to be managed appropriately.

No correlation was observed between stump size and the area colonized by *H. annosum s.l.* in treated stumps of *P. abies*, *P. pinea* and *P. sylvestris*. This finding supports the hypothesis that, generally, the performance of the treatments does not depend upon the stump size. However, a significant correlation between the two variables was found in *A. alba* in both untreated control stumps and stumps treated with Proradix®. We cannot exclude that such correlation could have been favored by a longer incubation period of the pathogen on *A. alba* compared to the other tree species (8 months vs. 4 months), making a greater colonization of the fungus on stumps more likely, especially on control stumps and on stumps treated with poorly effective products.

Testing and comparing the efficacy of different stump treatments in the field is pivotal to fine-tune the management of *Heterobasidion* spp. in forest stands. Nonetheless, the availability of chemical or biological products with proven efficacy is not the only prerequisite needed to decide whether stump treatments could be profitably carried out, or not. In fact, a large body of literature suggests that the risk of stump infection should be assessed before applying treatments (see [53,64,65] and the literature therein). Such risks may be highly dependent on climate and on seasonality, influencing the propagule deposition patterns of *Heterobasidion* spp. [9]. Hence, climate change may play an important role in the future by affecting the risk of stump infection by *Heterobasidion* spp. [66,67]. However, our experimental design was not conceived to implement decision-making processes about the opportunity of conducting stump treatments based on the risk of infection.

In conclusion, this paper provides new evidence supporting the good performances of Rotstop®, and especially that of urea, against the three native *Heterobasidion* species on stumps of their preferential hosts and, for the first time, against the North American *H. irregulare* on stumps of *P. pinea*, which is currently a key host of this invasive pathogen in Europe. Although urea may not be longer available as a pesticide against *H. annosum s.l.* and Rotstop® is not approved for use in southern Europe, the CFF of *P. protegens* strain DSMZ 13134 appears a valid alternative to these two state-of-the-art treatments. This is relevant because this treatment, differently from the others, is based on antibiotics contained in a crude cultural cell broth, and could be further improved in terms of efficacy by identifying the active molecules and/or by optimizing their production or application, as previously suggested [33]. In more general terms, the possibility of using different treatments with comparable efficacy may lead to a higher acceptance by citizens of phytosanitary treatments in forests and could also minimize the ecological impact that a large-scale application of a single stump treatment may have.

4. Materials and Methods

4.1. Study Sites and Treatments

Stump treatment experiments were conducted in three forest stands in the north-west of Italy dominated by *A. alba*, *P. abies* and *P. sylvestris*, respectively, and in one *P. pinea* stand in central Italy (Table 2). Those forest stands are included in areas known to be infested by *H. annosum s.l.* [3,5]. The study sites in north-west of Italy were typical naturally regenerated, uneven-aged and mixed stands, harboring the relevant tree species in sizeable patches. The study site of central Italy (La Gallinara Park) was an even-aged plantation included in SCIs, preserving coastal Mediterranean habitats. The four study sites were pre-selected based on information on the *Heterobasidion* airspora pointing to the presence, in each stand, of the host-associated *Heterobasidion* species determined in previous studies by using the wood disc exposure method combined with the taxon-specific molecular typing of single spore isolates [4,5,10]. In each site, freshly cut stumps were created during selective thinnings carried out in the frame of the ordinary forest management. Thinnings were conducted on *P. pinea* at La Gallinara Park in January 2020, and on *A. alba*, *P. abies* and *P. sylvestris* in the

study sites of north-west of Italy from June to September 2020 (Table 2). Before treatments, all freshly cut stumps were visually inspected for symptoms of wood decay caused by *Heterobasidion* spp., and only asymptomatic stumps were included in the experiments. Thinnings conducted at La Gallinara Park could only include a limited number of trees as prescribed by SCI-related forest regulations, logs deriving from branches of recently felled trees were also used as proxies to simulate *P. pinea* stumps. Logs were approximately 40–50 cm long, with a diameter of 9–33 cm and did not show any visible symptom of decay. Logs were placed upright in the *P. pinea* stand. The diameter of each stump and log was measured along two perpendicular directions on the upper cutting surface.

The efficacy of the following treatments was tested: Proradix® (SP Sourcon Padena GmbH, Tübeningen, Germany), the CFF of *P. protegens* strain DSMZ 13134, a conidial suspension of *P. gigantea* MUT 6212 isolated from fruiting bodies on *P. pinea* at La Gallinara Park (Rome, Italy), the biocontrol product Rotstop® (Verdera Oy, Espoo, Finland), and aqueous urea (Fluka, Cologno Monzese, Italy) solution (30% w/v). *P. protegens* strain DSMZ 13134 was provided by SP Sourcon Padena GmbH (Tübeningen, Germany) and stored in Luria–Bertani (LB) broth amended with 30% glycerol at −80 °C. Fresh cultures were initiated from frozen stocks and refreshed in LB broth at 25 °C for 24 h with shaking before use. CFF preparation was set up based on evidence showing that the antagonistic effect of *P. protegens* (strain DSMZ 13134) against *Heterobasidion* is maximum if CFF is obtained from a pure culture of *P. protegens* (strain DSMZ 13134) at 25 °C [33]. Hence, the CFF was prepared by culturing *P. protegens* (strain DSMZ 13134) in LB broth with constant shaking for 24 h at 25 °C (OD_{600} of 1.1). Cells were pelleted by centrifugation at 4000 rpm for 10 min, and the supernatant was filtered aseptically through a 0.22 µm filter membrane to obtain CFF, free from bacterial cells. The strain of *P. gigantea* MUT 6212 was selected based on its good performances against *Heterobasidion* spp. in in vitro tests [40]. The conidial suspension of *P. gigantea* MUT 6212 was obtained by loading 500 µL of sterile water on the surface of 7-day-old fungal colonies in 9 cm diameter Petri dishes previously incubated at 25 °C in the dark. The water was gently shaken and collected by using a pipette. The concentration of conidia in the suspension was assessed by using a Bürker chamber, and the conidial suspension was subsequently adjusted to 10^4 conidia mL^{-1}. The remaining stump treatments were prepared as described previously [33]. Control stumps received sterile water instead of treatments; we will refer to water as one of the six treatments.

Treatments were carried out manually by spraying the suspensions or solutions onto the surface of freshly cut stumps or logs within 1 hour after their cutting, until the surface became uniformly wet, i.e., an approximately 1 mm thick layer of suspension or solution. At least twenty replicate stumps were used for each treatment in each study site. A total of 135 stumps of *A. alba*, 125 of *P. abies*, 136 of *P. pinea*, and 146 of *P. sylvestris* were included in the experiments. A total of 139 logs of *P. pinea* were used for the experiments, at least 20 for each treatment. For both stumps and logs, treatments were conducted according to a completely randomized design.

Table 2. Main features of the study sites hosting the treatment experiments against *Heterobasidion* spp.

Location	Latitude, Longitude	Elevation (m a.s.l.)	Host Tree Species	Number of Stumps	Stump Diameter, Min–Max (cm)	Mean Stump Diameter ± SD (cm)	Period of Thinning/Treatments	Period of Sampling
La Salle (AO)	45.75667, 7.07907	1001	*A. alba*	135	10–70	24.2 ± 12.7	September 2020	May 2021
Nus (AO)	45.78494, 7.45994	1495	*P. abies*	125	9.5–110	24.7 ± 13.9	June–July 2020	October–November 2020
La Gallinara Park (RM)	41.53156, 12.56187	3	*P. pinea*	136	17–70	39.1 ± 11.0	January 2020	May 2020
Nus (AO)	45.77761, 7.44911	1495	*P. sylvestris*	146	6.5–76	23.9 ± 12.7	June–July 2020	October–November 2020

4.2. Samplings and Laboratory Analyses

Stumps and logs were sampled after 16 weeks from treatments, with the exception of stumps of *A. alba*, which were sampled after 32 weeks due to technical constraints (e.g., a snowy winter). Two 3–5 cm thick half-discs were cut from the top of each stump or log. The upper wood disc was discarded, while the second disc was taken to the laboratory for further analyses; the sampling was performed from one half of the stump or log surface. To avoid the computation of possible infections originating from roots, samples displaying visible symptoms of decay were discarded. The half-discs of stumps or logs were debarked, washed with tap water, and incubated for 10–14 days in plastic bags at room temperature with an optimal relative humidity for fungal growth [10]. Subsequently, the upper surface of the half discs was inspected under a dissecting microscope (20× magnification) for the presence of typical *Heterobasidion* conidiophores, as previously described [4,68]. The area covered with *Heterobasidion* conidiophores was delimited with a marker and measured by using a transparent 1 cm grid. Measurements were expressed as cm^2 of surface covered with *Heterobasidion* conidiophores.

To determine the species of *Heterobasidion* colonizing the disc surface, diagnostic assays were conducted on five randomly selected discs of control stumps or logs. Isolations were made under a dissecting microscope (20× magnification) with a needle by scraping the surface of conidiophores randomly chosen from the largest infection areas of the control disc. DNA extraction and species typing were conducted as previously described [4,68].

4.3. Statistical Analyses

The incidence of *Heterobasidion* spp. for each host species and treatment was calculated in percentage as the ratio between the number of discs colonized by the pathogen and the total number of discs. The exact 95% confidence intervals associated with the incidence values were calculated as reported in Blaker [69]. The effects of the treatments on the incidence of *Heterobasidion* spp. were assessed separately for each host species. In the case of *P. pinea*, the analysis of data was conducted separately for stumps and logs. The treatments were compared by contrasting the incidence of *Heterobasidion* spp. with conditional inference tree models based on unbiased recursive partitioning algorithms [70,71]. The algorithms clustered the treatments based on the following criteria: (1) treatments exerting comparable effects on the incidence of *Heterobasidion* spp. (i.e., treatments resulting in incidence values not significantly different, $p > 0.05$) were grouped within the same cluster; (2) treatments resulting in different effects on the incidence of *Heterobasidion* spp. (i.e., treatments associated with incidence values significantly different, $p < 0.05$) were split in different clusters. Each cluster of treatments was characterized by an overall incidence value of *Heterobasidion* spp., with such values representing a proxy of the expected efficacy of the treatments.

The average area colonized by *Heterobasidion* spp. on the half-surface of stumps and logs was compared among treatments for each host species, separating the analysis of stumps and logs in the case of *P. pinea*. For the above average areas, the 95% bias-corrected and accelerated (BCa) confidence intervals were calculated, as described in DiCiccio and Efron [72]. The above confidence intervals were obtained through the bootstrap iterative resampling method [73], as described in Lione et al. [74]. The comparisons between the average areas colonized by *Heterobasidion* spp. were carried out by running the algorithms fitting the unbiased recursive partitioning conditional inference tree models [70,71]. Algorithms were run on the identity function of the area colonized by *Heterobasidion* spp. as an outcome variable, and on the treatment as an input variable.

The correlation between the area colonized by *Heterobasidion* spp. on the half-surface of the stump and the stump diameter was assessed by testing the significance of the Pearson's correlation coefficient (R) [75]. The coefficient R was calculated for each treatment and host species.

Statistical analyses were conducted with R version 3.6.0 [76] and with the associated packages bootstrap [77], partykit [70], and binGroup [78]. The significance threshold was set to 0.05 for all tests.

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Chapter III

Pyoluteorin produced by the biocontrol agent *Pseudomonas protegens* is involved in the inhibition of *Heterobasidion* species present in Europe

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Pyoluteorin Produced by the Biocontrol Agent *Pseudomonas protegens* Is Involved in the Inhibition of *Heterobasidion* Species Present in Europe

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Abstract: *Pseudomonas protegens* (strain DSMZ 13134) is a biocontrol agent with promising antagonistic activity hinging on antibiosis against the fungal forest pathogens *Heterobasidion* spp. Here, by using High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS), we assessed whether monocultures of *P. protegens* (strain DSMZ 13134) produce the three major determinants of biocontrol activity known for the genus *Pseudomonas*: 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN). At the tested culture conditions, we observed the production of PLT at concentrations ranging from 0.01 to 10.21 mg/L and 2,4-DAPG at a concentration not exceeding 0.5 mg/L. Variations of culture conditions involving culture medium, incubation temperature, and incubation period had no consistent influence on PLT production by the bacterium. Assays using culture medium amended with PLT at the same concentration of that present in cell-free filtrate of the bacterium, i.e., 3.77 mg/L, previously documented as effective against *Heterobasidion* spp., showed a remarkable activity of PLT against genotypes of all the four *Heterobasidion* species present in Europe, including the non-native invasive *H. irregulare*. However, such antifungal activity decreased over time, and this may be a constraint for using this molecule as a pesticide against *Heterobasidion* spp. When the bacterium was co-cultured in liquid medium with genotypes of the different *Heterobasidion* species, an increased production of PLT was observed at 4 °C, suggesting the bacterium may perform better as a PLT producer in field applications under similar environmental conditions, i.e., at low temperatures. Our results demonstrated the role of PLT in the inhibition of *Heterobasidion* spp., although all lines of evidence suggest that antibiosis does not rely on a single constitutively produced metabolite, but rather on a plethora of secondary metabolites. Findings presented in this study will help to optimize treatments based on *Pseudomonas protegens* (strain DSMZ 13134) against *Heterobasidion* spp.

Keywords: biological control; antibiosis; secondary metabolites; co-culture; 2,4-diacetylphloroglucinol; pyrrolnitrin; HPLC-MS; Proradix®; cell-free filtrate; forest pathogen

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1. Introduction

A variety of Plant Growth-Promoting Rhizobacteria (PGPR) exhibit antagonistic effects against plant pathogens via nutrient competition, induced resistance, priming, and antibiosis [1–3]. *Pseudomonas* spp. have been widely studied for their biocontrol potential [1,2,4]. They show antagonistic effects against a wide range of fungal plant pathogens, including ascomycetes, basidiomycetes, and mitosporic fungi [1–3]. The genus *Pseudomonas* is reported to inhibit pathogens by antibiosis through the secretion of secondary metabolites [1,2,4–6]. Different *Pseudomonas* species secrete a plethora of secondary metabolites with antibiotic activity, including pyoluteorin (PLT), pyrrolnitrin (PRN), 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines, cyclic lipopeptides, and volatile com-

pounds [1–3,6]. Based on genomic information, other undetected secondary metabolites could also be secreted [4].

Diseases caused by the fungal pathogens *Heterobasidion* spp. include destructive root and butt rots in coniferous forests of the Northern Hemisphere [7]. Members of the species complex *H. annosum* (Fr.) Bref. *sensu lato* (*s.l.*) are native to Europe., including *H. abietinum* Niemelä & Korhonen, *H. annosum sensu stricto* (*s.s.*) (Fr.) Bref., hereafter referred to as *H. annosum*, and *H. parviporum* Niemelä & Korhonen which are mainly associated with *Abies alba* Mill., *Pinus* spp., and *Picea abies* (L.) Karst., respectively [7]. In central Italy, stone pine (*P. pinea* L.) stands along the west coastline are threatened by the invasive North American *H. irregulare* Garbel. & Otrosina [8]. Following a pest risk analysis [9], this non-native *Heterobasidion* species is currently recommended for regulation under the European and Mediterranean Plant Protection Organization (EPPO) A2 list. Disease management implies treating freshly cut stumps with either chemical or biological products to prevent stump infection by spores and subsequent spreading of the fungi to neighboring healthy trees through root contacts [7].

Our study focuses on the PGPR *Pseudomonas protegens* (strain DSMZ 13134), which is the active component of the bio fungicide Proradix® (SP Sourcon Padena GmbH, Tübingen, Germany) currently commercialized against black scurf caused by *Rhizoctonia solani* J.G. Kühn and silver scab caused by *Helmintosporium solani* Durieu & Mont. on potatoes and other tubers. Previous laboratory and field studies had shown the ability of this PGPR to display significant antagonistic effects towards the fungal forest pathogens *Heterobasidion* spp., making it a candidate biocontrol agent against the *Heterobasidion* species [10–13]. Moreover, the cell-free filtrate (CFF) of the same bacterium displayed the ability to inhibit both mycelial growth (100% inhibition) and conidial germination (99% inhibition) of the pathogen in vitro [12] and performed even better than the bio fungicide Proradix® in experiments conducted in controlled conditions and in the forest on stumps of several host tree species [12,13]. It has been shown that antibiosis is likely the main mechanism of action of *P. protegens* (strain DSMZ 13134) against *Heterobasidion* spp. [12,13], as well as towards other fungal pathogens [14]. The secondary metabolites with antifungal activity present in the CFF are the putative determinants of both Proradix® and CFF efficacy, but those compounds and their mechanisms of action remain completely unknown. Knowing the mechanisms of action of a biocontrol agent can help with the optimization of the disease control, but it is also required for registration where risks for humans and the environment, including risks for resistance development, have also to be indicated [4,15]. Regulation in the EU makes a distinction between biocontrol agents able to produce secondary metabolites with antimicrobial activity in situ and such compounds in product without living cells of the biocontrol agent [4].

This study investigated the formation and the concentration of three of the major known determinants of biocontrol activity of *Pseudomonas* spp., namely 2,4-DAPG, PLT and PRN [1–3,6,16] in the CFF of *P. protegens* (strain DSMZ 13134) used as both an amendment in medium inhibiting *Heterobasidion* spp. in vitro and a stump treatment in a previous field study [12,13]. The antifungal activity of the selected secondary metabolites present in the CFF, and its contribution to the antifungal activity of the raw CFF, were evaluated in vitro at different times against the four *Heterobasidion* spp. currently occurring in Europe, including the non-native invasive *H. irregulare*. In addition, we assessed whether the variety and yield of such metabolites could be enhanced by acting on culture conditions (culture medium, incubation temperature, and incubation period). Our ultimate aim was to explore whether the production of these compounds by *P. protegens* (strain DSMZ 13134) is temperature-controlled during the interaction with *Heterobasidion* species. This could help to predict the production of metabolites when the bacterium (i.e., Proradix®) is applied on stump surfaces in infested sites under different environmental conditions.

2. Results

2.1. Presence and Concentration of Selected Secondary Metabolites in the CFF of P. protegens (Strain DSMZ 13134)

To determine whether the selected secondary metabolites are produced by *P. protegens* (strain DSMZ 13134), we generated a monoculture of the bacterium in Luria–Bertani (LB) broth for 24 h at 25 °C. The CFF of the monoculture was obtained by filtering the bacterial culture aseptically through a 0.22 µm filter membrane. Chemical analysis of CFF through High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS) allowed us to determine the presence of PLT at the concentration of 3.77 mg/L (Table 1). Neither 2,4-DAPG nor PRN were detected in the CFF of *P. protegens* (strain DSMZ 13134) (Table 1).

Table 1. Concentration of 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN) in monocultures of *P. protegens* (strain DSMZ 13134). Monocultures were grown in Luria–Bertani, King B, and a modified King B broth at 4 °C, 25 °C, and 30 °C and incubating them for 24 h and 7 days.

Monocultures of *P. protegens* (Strain DSMZ 13134)			Mean Concentration ± SD [1] (mg/L)		
Culture Medium	**Incubation Temperature (°C)**	**Incubation Period**	**2,4-DAPG**	**PLT**	**PRN**
Luria–Bertani broth	4	24 h	0.17 ± 0.00	0.01 ± 0.00	<LOD [2]
		7 days	<LOD	3.63 ± 0.38	<LOD
	25	24 h	<LOD	3.77 ± 0.30	<LOD
		7 days	<LOD	0.66 ± 0.00	<LOD
	30	24 h	0.26 ± 0.00	0.81 ± 0.38	<LOD
		7 days	<LOD	0.35 ± 0.24	<LOD
King B broth	4	24 h	<LOQ [3]	0.32 ± 0.00	<LOD
		7 days	<LOQ	8.03 ± 0.00	<LOD
	25	24 h	<LOQ	6.12 ± 0.42	<LOD
		7 days	<LOQ	2.34 ± 0.01	<LOD
	30	24 h	<LOQ	0.37 ± 0.20	<LOD
		7 days	<LOQ	0.49 ± 0.50	<LOD
Modified King B broth	4	24 h	<LOD	0.27 ± 0.00	<LOD
		7 days	<LOD	<LOD	<LOD
	25	24 h	<LOD	<LOD	<LOD
		7 days	<LOD	1.10 ± 0.02	<LOD
	30	24 h	<LOD	4.52 ± 0.49	<LOD
		7 days	<LOD	10.21 ± 3.18	<LOD

[1] The values represent the mean and standard error of the mean for three replicates, each represents one monoculture of *P. protegens* (strain DSMZ 13134). Values were rounded to two significant digits. [2] <LOD means under the limit of detection, being 0.1 mg/L for every compound. [3] <LOQ means under the limit of quantification, being approximately 0.5 mg/L for each compound.

2.2. Effects of PLT on Mycelial Growth of Heterobasidion spp.

PLT at the concentration of 3.77 mg/L (consistent with that present in CFF) was assayed for mycelial growth inhibition (MGI in %) of *Heterobasidion* genotypes. The average MGI of *Heterobasidion* spp. ranged from 20.4% (12.6–27.5% 95% CI) to 41.5% (32.8–48.7% 95% CI) at 4 days, and from 2.3% (0.9–3.9% 95% CI) to 28.7% (25.1–33.3% 95% CI) at 7 days (Figure 1). At 4 days, *H. annosum* and *H. irregulare* attained average values of MGI over 40% and 30%, respectively, which were significantly higher than those observed for *H. abietinum* and *H. parviporum*, both at around 20% ($p < 0.05$) (Figure 1). *H. annosum* was the most inhibited species at 7 days with an average MGI around 30%, a value twofold higher than that attained by *H. abietinum* ($p < 0.05$) (Figure 1). At the same time point, *H. irregulare* and *H. parviporum* were significantly less inhibited than the other *Heterobasidion* species

($p < 0.05$), attaining average values of MGI around 2 and 7%, respectively (Figure 1). From the first to the second time point, a reduction in the average MGI was observed for all *Heterobasidion* species achieving approximately −6% for *H. abietinum* ($p > 0.05$), −13% for *H. annosum* and *H. parviporum* ($p < 0.05$), and −30% for *H. irregulare* ($p < 0.05$) (Figure 1).

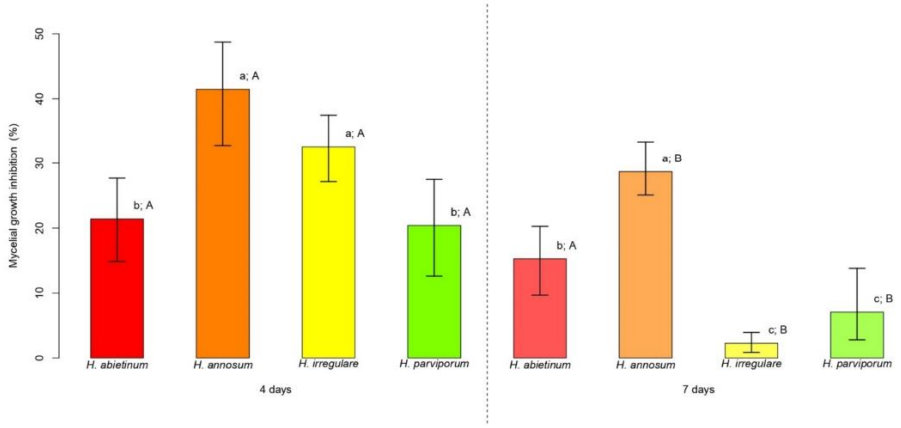

Figure 1. Mycelial growth inhibition (MGI) comparisons. For each time point (4 and 7 days) bars indicate the average MGI displayed by the four species of *Heterobasidion*. Error bars refer to the lower and upper bounds of the 95% confidence interval. Above each bar, lowercase letters refer to the comparisons of average MGI values among *Heterobasidion* species for each time point, while upper case letters refer to the comparison of the two time points for the same species. Different letters indicate significant differences ($p < 0.05$).

2.3. Effects of Culture Conditions on Secondary Metabolites Production by Monocultures of P. protegens (Strain DSMZ 13134)

To evaluate how cultural parameters may affect secondary metabolites production in *P. protegens* (strain DSMZ 13134), we tested a panel of monocultures of the bacterium growing at different conditions. *Pseudomonas protegens* (strain DSMZ 13134) produced PLT at concentrations ranging from 0.01 to 10.21 mg/L depending on cultural conditions (Table 1). Quantification of 2,4-DAPG was possible only in monocultures grown in LB for 24 h at 4 and 30 °C, whereas in the other culture conditions, 2,4-DAPG was under the limit of quantification (LOQ, 0.5 mg/L) or it was not detected (Table 1). Concentrations of PRN were always under the detection limit (LOD) (Table 1).

Statistical analysis carried out with unbiased recursive partitioning tree model showed that the concentration of PLT produced by monocultures of *P. protegens* (strain DSMZ 13134) was not significantly associated with either culture medium, incubation temperature, or incubation period ($p > 0.05$) (Table 2). The average concentration of PLT was 2.39 mg/L (1.68–3.32 mg/L 95% CI) (Figure 2).

Table 2. Association between concentration of pyoluteorin (PLT) and culture conditions of *P. protegens* (strain DSMZ 13134). The *c* statistics and its *p*-value are reported for each of the factors tested for their association with the response variable. Multiple values of *c* marked by a numeric index in subscript refer to subsequent splits of the tree model. Significant *c* values ($p < 0.05$) are indicated by the symbol *.

Response Variable	Incubation Temperature	Culture Medium	Incubation Period	Co-Culture Type
Concentration of PLT produced by monocultures of *P. protegens* (strain DSMZ 13134)	$c = 0.542$ $p = 0.987$	$c = 2.132$ $p = 0.718$	$c = 1.985$ $p = 0.405$	- -
Concentration of PLT produced by co-cultures of *P. protegens* (strain DSMZ 13134) and *Heterobasidion* spp.	$c_1 = 33.35$ * $p_1 = 1.142 \times 10^{-7}$ $c_2 = 10.078$ * $p_2 = 2.999 \times 10^{-3}$	- - - -	- - - -	$c_1 = 0.626$ $p_1 = 0.988$ $c_2 = 4.754$ $p_2 = 0.345$

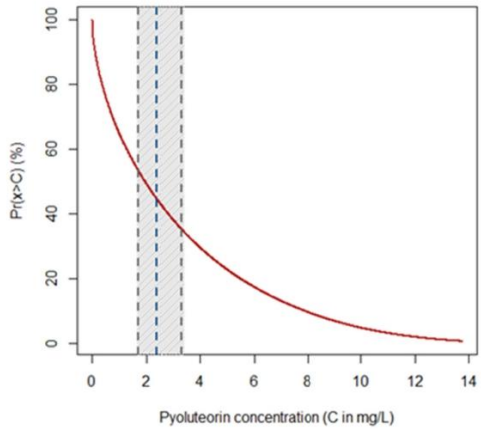

Figure 2. Probability of retrieving pyoluteorin (PLT) from monocultures of *P. protegens* (strain DSMZ 13134) with concentrations over a given threshold. The graph shows the thresholds C of PLT concentration (in mg/L) on the x-axis, while the y-axis reports the probability ($Pr(x > C)$ in %) that *P. protegens* (strain DSMZ 13134) may release higher concentrations of the compound. The red curve quantifies the probability, while the blue dashed lines represent the average expected concentration of PLT along with the lower and upper bounds (gray dashed lines) of its 95% confidence interval (gray strip).

Among the distribution types included in the Pearson system of generalized frequency curves, type I attained the lowest AIC (68.0), while all others displayed AIC values over 200. The equation of the corresponding upper tail probability distribution function obtained was:

$$\Pr(x > C) = \int_{C}^{+\infty} \frac{1}{|s|} \frac{\Gamma(a+b)}{\Gamma(a)\Gamma(b)} \left(\frac{x-\lambda}{s}\right)^{a-1} \left(1 - \frac{x-\lambda}{s}\right)^{b-1} dx \quad (1)$$

where the probability $Pr(x > C)$ that monocultures of *P. protegens* (strain DSMZ 13134) release PLT with a concentration x higher than a given threshold C is provided by the integral of the Pearson curve of type I (Figure 2). In the above equation, Γ is the Euler Gamma function, while the curve parameters obtained through maximum likelihood estimation

were a = 0.589, b = 2.881, $\lambda = -3.990 \cdot 10^{-13}$, and s = 18.158. The curve shows that PLT is produced by monocultures of *P. protegens* (strain DSMZ 13134) (i.e., the concentration of the metabolite is higher than 0) with a probability approaching 100% (Figure 2). The same curve indicates that the probability of retrieving PLT with a concentration of up to 3.77 mg/L (i.e., mean concentration measured in the CFF) is approximately 70% (Figure 2).

2.4. Effects of Co-Cultures of P. protegens (Strain DSMZ 13134) with Heterobasidion spp. on Secondary Metabolites Production by the Bacterium

We explored whether the production of secondary metabolites by *P. protegens* (strain DSMZ 13134) was enhanced or reduced during the interaction with *Heterobasidion* species at different temperatures. The ability to produce PLT was confirmed in co-culture experiments (Table 3; Figure 3). *Pseudomonas protegens* (strain DSMZ 13134) produced PLT at concentrations ranging from 0.07 to 9.90 mg/L in extracts of all co-cultures except one with *H. abietinum* at 30 °C (Table 3). At all tested temperatures, *P. protegens* (strain DSMZ 13134) in co-culture produced 2,4-DAPG under the LOQ (Table 3). Again, despite some variations in culture conditions, *P. protegens* (strain DSMZ 13134) failed to produce a detectable concentration of PRN (Table 3).

Table 3. Concentration of 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN) produced in co-cultures of *P. protegens* (strain DSMZ 13134) and one genotype of different *Heterobasidion* species. Co-cultures were grown in King B broth at 4 °C, 25 °C, and 30 °C, and incubating them for 7 days.

Co-Cultures of *P. protegens* (Strain DSMZ 13134) and *Heterobasidion* spp.		**Mean Concentration ± SD [1] (mg/L)**		
Incubation Temperature (°C)	***Heterobasidion* Species**	**2,4-DAPG**	**PLT**	**PRN**
4	*H. abietinum*	<LOQ [2]	8.05 ± 0.01	<LOD [3]
	H. annosum	<LOQ	9.14 ± 0.01	<LOD
	H. irregulare	<LOQ	9.90 ± 0.02	<LOD
	H. parviporum	<LOQ	8.28 ± 0.15	<LOD
25	*H. abietinum*	<LOQ	2.52 ± 0.02	<LOD
	H. annosum	<LOQ	0.84 ± 0.00	<LOD
	H. irregulare	<LOQ	3.31 ± 0.13	<LOD
	H. parviporum	<LOQ	<LOQ	<LOD
30	*H. abietinum*	<LOQ	<LOD	<LOD
	H. annosum	<LOQ	0.16 ± 0.18	<LOD
	H. irregulare	<LOQ	0.07 ± 0.12	<LOD
	H. parviporum	<LOQ	0.51 ± 0.30	<LOD

[1] The values represent the mean and standard error of the mean for three replicates, each represents one co-culture of *P. protegens* (strain DSMZ 13134) and *Heterobasidion* spp. Values were rounded to two significant digits. [2] <LOQ means under the limit of quantification, with quantification limit being approximately 0.5 mg/L for each compound. [3] <LOD means under the limit of detection, with detection limit being approximately 0.1 mg/L for each compound.

The outcomes of the unbiased recursive partitioning tree model pointed out that only the incubation temperature exerted a significant effect on the production of PLT from *P. protegens* (strain DSMZ 13134) in co-culture with *Heterobasidion* spp. ($p < 0.05$) (Table 2). Conversely, the co-culture type did not display any significant association with the response variable ($p > 0.05$) (Table 2). Decreasing incubation temperatures were associated with raising concentrations of PLT ranging from an average of 0.18 mg/L at 30 °C, to 1.74 mg/L at 25 °C, up to 8.84 mg/L at 4 °C ($p < 0.05$) (Figure 3).

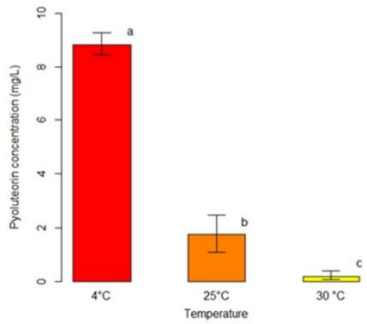

Figure 3. Comparison among average concentrations of pyoluteorin (PLT) produced by *P. protegens* (strain DSMZ 13134) in co-culture with *Heterobasidion* spp. at different temperatures. For each temperature, bars indicate the average concentration of PLT (mg/L). Error bars refer to the lower and upper bounds of the 95% confidence interval. Different letters above the bars indicate significant differences ($p < 0.05$).

3. Discussion

Plant Growth-Promoting Rhizobacteria are ubiquitous in nature, and they may have a range of agricultural, environmental, and industrial applications. The genus *Pseudomonas* is highly appreciated for its plant growth promoting traits and because of its ability to produce a range of secondary metabolites with antibiotic activity [2,3,5,6]. Among the investigated secondary metabolites, only PLT was present in the CFF of *P. protegens* (strain DSMZ 13134). Pyoluteorin is a polyketide metabolite produced by fluorescent *Pseudomonas* known for its fungicidal, bactericidal, and herbicidal activities [2,17]. The concentration of PLT in the CFF was measured as high as 3.77 mg/L. This concentration is consistent with those found in the well-studied rhizosphere bacterium *P. protegens* Pf-5 [18,19].

PLT at the same concentration present in the CFF, i.e., 3.77 mg/L, was assayed against the mycelial growth of *Heterobasidion* spp. to determine the individual contribution of PLT to the overall efficacy of the raw CFF, which was previously shown to be 100% [12]. The probability distribution function obtained from the Pearson system showed that the concentration of PLT present in the CFF is slightly over the average, but there is a large probability (70%) that *P. protegens* (strain DSMZ 13134) can release up to this concentration. Conversely, testing higher concentrations might be not sound because only in 30% of cases the bacterium is expected to produce PLT over the threshold of 3.77 mg/L. In this study, PLT proved to have antifungal activity against all the tested *Heterobasidion* species. Yet, its efficacy decreased significantly after 7 days of incubation against all *Heterobasidion* species except *H. abietinum*. The decline in the effectiveness may be due to PLT stability, as suggested by a previous experimental study on the degradation of PLT showing that temperature, solution pH and UV irradiation had a strong influence on its degradation [20]. Interestingly, PLT was found to be unstable in acid and in alkaline solutions, and under UV irradiation, making its use as a pesticide difficult without any modification to improve the stability [20]. In vitro culture conditions might have increased PLT instability, thus reducing its efficacy against the fungal pathogens over time. Significant reductions of the efficacy of PLT in a few days may be a constraint for using this molecule as a pesticide against *Heterobasidion* spp. Because stumps remain susceptible to infection by these pathogens for a few weeks [7].

In a previous work, the same *Heterobasidion* genotypes used in this study were completely inhibited when grown on media containing the CFF, without differences in sensitivity among different species and over time [12]. Our results clearly document that PLT is not the sole compound responsible for *Heterobasidion* inhibition, as PLT alone can explain

at best 41.5% of the total inhibition caused by the CFF. Consequently, all lines of evidence suggest that antibiosis does not rely on a single constitutively produced metabolite, but rather on a plethora of secondary metabolites.

The optimization of culture conditions to enhance secondary metabolites production has gained attention for its potential to implement the success of biocontrol-based products and to maximize disease control [3,4,15]. Alterations of culture conditions may have a pronounced influence on yield enhancement and de novo induction of secondary metabolites [21,22]. In the present study, *P. protegens* (strain DSMZ 13134) was efficient in producing PLT, but this production was not affected by any of the tested cultural parameters (culture medium, incubation temperature and incubation period). Although it has been reported that *P. protegens* strains can be considered as a new bacterial group able to produce 2,4-DAPG, PLT and PRN [23], it appears that our *P. protegens* strain is not capable of producing high levels of 2,4-DAPG or PRN.

Since these metabolites are usually produced and released by microorganisms in small quantities [4,16,24], although their production is strongly dependent on nutrient availability [4,15,16,25], even substantial variation of culture conditions may not be able to increase their production. The modified King B (KBM) broth, containing a larger amount of glycerol, should have indeed enhanced PLT production over 2,4-DAPG production [26–28]. However, this was not observed in our experiment: in fact, the substrate did not affect PLT production in our bacterial strain. Pyoluteorin and 2,4-DAPG production is interrelated in *P. protegens* and other *Pseudomonas* species, despite the independent biochemical and genetic determinants for their biosynthesis [29–31]. Brodhagen et al. [29] have demonstrated that PLT production is induced by positive autoregulation in *P. fluorescens* Pf-5. In addition to its autoregulatory role, PLT repressed 2,4-DAPG production [29]. Such complex interactions may have occurred in our experiments as well.

The probability distribution curve obtained from the Pearson system can be regarded as the PLT production fingerprint of *P. protegens* (strain DSMZ 13134) growing in monocultures. The curve is more informative than the simple average of the PLT concentration since it allows one to quantify the probability of retrieving up to, or more than, a given amount of the compound. More importantly, the curve clearly shows that the probability of obtaining PLT from monocultures of *P. protegens* (strain DSMZ 13134) is almost 100%, despite the fact that the concentrations can be variable and with a high probability density centered on low values. This may have a practical significance, for instance, to compare production curves of PLT or other secondary metabolites under different conditions. In fact, from an applied perspective, optimization for the production of the preferred secondary metabolites during the fermentation process of the target biocontrol agent appears as a valuable strategy toward the enhancement of the biocontrol agent itself [4,15]. This strategy is focusing on the development of a formulation that contains secondary metabolites together with living cells of the producing biocontrol agent so that the performance in field is expected to be the result of the combined effects of secondary metabolites and the potential production of additional secondary metabolites in situ [4]. This appears as a valuable alternative to the commercialization of secondary metabolites without the biocontrol agent. In fact, strictly speaking, in the absence of living cells such secondary metabolites have to be considered chemicals in the EU [4], implying an extensive characterization for risk assessment, thus increasing substantially the costs for registration [4].

In the attempt to improve our understanding of the strategies adopted by *P. protegens* (strain DSMZ 13134) in response to *Heterobasidion* spp., we assessed the effects of the interaction between the bacterium and the four *Heterobasidion* species currently occurring in Europe on the production of 2,4-DAPG, PLT and PRN at different temperatures (4 °C, 25 °C and 30 °C). Such temperatures were chosen to simulate the widest range of environmental conditions at which stump treatments are performed and hence antagonist–pathogen interaction may occur. In fact, co-culture experiments simulate natural scenarios where bacteria and fungi co-inhabit and interact in a same confined environment, in this model system a stump surface, thereby exerting intense microbial competition and interspecies

crosstalk [32–34]. The outcomes of our co-culture experiment confirmed that *P. protegens* (strain DSMZ 13134) is capable of producing PLT. The concentration of 2,4-DAPG was again found under 0.5 mg/L, whereas no PRN production was observed. The incubation temperature of 4 °C is conducive to the production of PLT in co-culture, indicating that the antagonist–pathogen interaction at low temperatures might lead to an increased production of this antifungal compound. Among abiotic factors, temperature has been reported to affect antibiotic production by bacterial biocontrol agents [35–39]. However, there is not a general defined temperature at which the production of secondary metabolites by *Pseudomonas* strains is optimized because different strains have their own requirements [35–37,39]. Since we did not observe a similar trend in monocultures of *P. protegens* (strain DSMZ 13134), the increased production of PLT at low temperatures might be due to the interaction between the bacterium and the pathogens. The microbial co-culture is used as an experimental tool to increase the yield and variety of secondary metabolites; thus, microbial competition is deliberately provoked to activate silent metabolic pathways and/or to up-regulate gene expression [32–34]. Hence, we can only speculate that Proradix® may perform better under similar environmental conditions, i.e., at low temperatures, thanks to its higher production of the antifungal compound PLT. An increased production of PLT on the stump surface may also be expected if the product is kept at low temperatures before use. It would be interesting to verify if a remarkably increased production of PLT, or other antifungal compounds, occurs when the bacterium grows in co-culture with saprobic, non-pathogenic fungi. It should be noted that among the possible approaches to improve biocontrol, two different scenarios based on assembled consortia of microorganisms are predicted [4]. On one side, the selection of helper strains applied to support the biocontrol agent in its establishment, survival and antagonistic activity, e.g., the production of antibiotic compounds [4]. On the other side, the application of biocontrol products consisting of different biocontrol strains combining different modes of action [4].

4. Materials and Methods

4.1. Microorganisms and Culture Conditions

Pseudomonas protegens (strain DSMZ 13134) was provided by SP Sourcon Padena GmbH (Tübeningen, Germany) and stored in Luria–Bertani (LB) broth containing 30% glycerol at −80 °C. Fresh cultures were started from frozen stocks for each experiment by inoculating 100 µL into LB broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 L of H_2O [pH 7.2]) and incubating at 25 °C for 24 h while shaking.

Fungal genotypes of each species of *H. annosum s.l.* occurring in Europe, i.e., *H. abietinum*, *H. annosum*, *H. irregulare*, and *H. parviporum*, were selected among those tested against *P. protegens* (strain DSMZ 13134) in a previous study [12] (Table 4). All genotypes of *H. annosum s.l.* were preserved in the culture collection of the University of Turin and maintained at 4 °C in pure culture on malt extract agar (MEA) (30 g of malt extract, 3 g of enzymatic digest of soybean meal, 15 g of agar-agar, 1 L of H_2O [pH 5.6]). Inoculum for the experiments was grown by transferring a MEA plug from these maintenance cultures to fresh MEA and incubating plates at 25 °C for 7 days.

Other culture media used in this study were King B (KB) broth (20 g of proteose peptone, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 10 mL of glycerol, 1 L of H_2O [pH 7.6]) [40] and a KBM broth (20 g of proteose peptone, 2.5 g of K_2HPO_4, 6 g of $MgSO_4 \cdot 7H_2O$, 15 mL of glycerol, 1 L of H_2O [pH 7.6]).

Table 4. *Heterobasidion* genotypes used in this study. Asterisks after the accession numbers indicate genotypes selected for co-culture experiments of *P. protegens* (strain DSMZ 13134) and *Heterobasidion* spp.

MUT [1] Accession Number	*Heterobasidion* Species	Isolation Date	Geographic Origin
6194 *	*H. abietinum*	2016	Nus, AO, Italy
6195	*H. abietinum*	2018	Chiusa di Pesio, CN, Italy
6196	*H. abietinum*	2018	Chiusa di Pesio, CN, Italy
6197	*H. abietinum*	2018	Chiusa di Pesio, CN, Italy
6198	*H. abietinum*	2016	Chabodey, AO, Italy
3543 *	*H. annosum*	2006	Mesola, FE, Italy
1204	*H. annosum*	2005	Sabaudia, LT, Italy
3538	*H. annosum*	2006	Ansedonia, GR, Italy
3656	*H. annosum*	2006	Sabaudia, LT, Italy
6191	*H. annosum*	2015	Saint-Denis, AO, Italy
1193 *	*H. irregulare*	2005	Castelfusano, RM, Italy
1151	*H. irregulare*	2005	Sabaudia, LT, Italy
1197	*H. irregulare*	2005	Sabaudia, LT, Italy
3627	*H. irregulare*	2005	Sabaudia, LT, Italy
5666	*H. irregulare*	2006	Nettuno, RM, Italy
5612 *	*H. parviporum*	2006	Trasquera, VB, Italy
5605	*H. parviporum*	2006	Druogno, VB, Italy
5615	*H. parviporum*	1999	Charvensod, AO, Italy
6192	*H. parviporum*	2016	Chabodey, AO, Italy
6193	*H. parviporum*	2016	Chabodey, AO, Italy

[1] MUT: Mycotheca Universitatis Taurinensis.

4.2. Production of Monocultures of P. protegens (Strain DSMZ 13134) for the Quantification of 2,4-DAPG, PLT and PRN

A set of monocultures of *P. protegens* (strain DSMZ 13134) was subjected to different culture conditions to determine whether cultural parameters may affect secondary metabolite production. Monocultures were grown in the dark with constant shaking (100 rpm) in conical flasks (250 mL) containing 100 mL of LB, KB, or KBM broth. Previous studies had shown that LB broth was conducive to the production of an array of secondary metabolites able to inhibit *Heterobasidion* mycelial growth and conidial germination [12], leading to a reduction in both the colonized area and incidence of *Heterobasidion* on stumps [13]. King B broth-based media are routinely used in laboratory to enhance production of secondary metabolites in fluorescent *Pseudomonas* strains [6,29,41,42].

An aliquot (1 mL) of the fresh culture of *P. protegens* (strain DSMZ 13134) was inoculated in each conical flask. Monocultures were incubated at 4, 25, and 30 °C and harvested after 24 h and 7 days of incubation. Incubation temperatures of 4 and 30 °C were chosen to test the influence of low and high temperature stresses on secondary metabolites production by *P. protegens* (strain DSMZ 13134). An incubation temperature of 25 °C and harvesting at 24 h of growth were the conditions used in the previous studies, where the CFF was obtained by culturing *P. protegens* (strain DSMZ 13134) in LB broth with constant shaking at 25 °C for 24 h (OD_{600} of 1.1) [12,13]. The 7-day harvesting period was chosen because secondary metabolism usually occurs at the late growth phase of the producing microorganisms [43]. For each culture medium (LB, KB, and KBM broth) and temperature (4, 25, and 30 °C), triplicate samples were established.

Monocultures were filtered to obtain a sample free from bacterial cells for the analysis by HPLC-MS. Cells were pelleted by centrifugation at 4000 rpm for 10 min, and the supernatant was filtered aseptically through a 0.22 µm filter membrane.

4.3. Production of Co-Cultures of P. protegens (Strain DSMZ 13134) and Heterobasidion spp. for the Quantification of 2,4-DAPG, PLT and PRN

A set of co-cultures of *P. protegens* (strain DSMZ 13134) and *Heterobasidion* spp. was subjected to different temperatures to explore how the production of 2,4-DAPG, PLT and PRN by *P. protegens* (strain DSMZ 13134) was modulated during the interaction between the bacterium and the fungal pathogens. To prepare co-cultures of *P. protegens* (strain DSMZ 13134) and *Heterobasidion* spp., 250 mL conical flasks were used, containing 100 mL of KB broth.

For each species of *H. annosum s.l.*, the genotype displaying the highest growth rates in antagonism assays conducted previously [12] was selected for this experiment (Table 4). Three mycelial plugs (7 mm in diameter) from an actively growing colony of each fungal genotype were inoculated in each flask and incubated in the dark with constant shaking (100 rpm) at 25 °C for 7 days. An aliquot (1 mL) of *P. protegens* (strain DSMZ 13134) fresh culture was subsequently transferred into the culture broths of *Heterobasidion* spp. Co-cultures were then incubated in the dark at 4, 25, and 30 °C and harvested after 7 days. Incubation temperatures were chosen to simulate the range of temperatures at which stump treatments may be performed and hence the interaction between the bacterium and the fungal pathogens may occur. For each combination of culture medium (KB) and temperature (4, 25, and 30 °C), three conical flasks were established. Co-cultures were filtered to obtain a sample free from bacterial cells for analysis by HPLC-MS.

4.4. Quantification of 2,4-DAPG, PLT, PRN Produced by P. protegens (Strain DSMZ 13134)

All reagents used for the quantification of secondary metabolites were analytical or LC-MS grade and were provided by Sigma-Aldrich (Milan, Italy). Pure 2,4-DAPG and PLT were provided by D.B.A. ITALIA (Milan, Italy), while pure PRN by Merck Life Science (Milan, Italy). The CFF resulting from cultures were purified using solid phase extraction (SPE) cartridges (Strata C18-E, 500 mg, 6 mL Phenomenex, Torrance, CA, USA). The SPE were previously activated with 3 mL of acetonitrile, washed with 2 mL of acidified water at pH 2, and the samples were then eluted to a final volume of 3 mL with a water-acetonitrile mix (1/1 volume).

The HPLC-MS system was a Varian MS-310 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and 212 LC pump (Agilent, Milan, Italy). Separation was performed on a Kinetex C18 column (5 µm, 50 × 2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase solvents were water (A) and acetonitrile (B), both containing 0.1% (*v*/*v*) formic acid. The mobile phase gradient was from 90% to 10% A in 10 min (0.2 mL/min flow rate), then from 10% to 90% A in 2 min and the conditions were maintained for 3 min.

4.5. Inhibition of Mycelial Growth of Heterobasidion spp. by PLT

The poisoned food technique was used to assess the in vitro inhibitory activity against *Heterobasidion* spp. of secondary metabolites present in the CFF of monocultures of *P. protegens* (strain DSMZ 13134) cultured in LB broth for 24 h at 25 °C. This CFF corresponds to the CFF of *P. protegens* (strain DSMZ 13134) used in previous studies [12,13]. The only secondary metabolite detected was PLT at the concentration of 3.77 mg/L (see results); therefore, the antifungal activity of PLT was assessed on MEA amended with PLT at 3.77 mg/L concentration poured into 6 cm Petri plates. Pyoluteorin (D.B.A. ITALIA, Milan, Italy) was suspended in acetonitrile (100 mg/L) and added to the medium after autoclaving for 20 min at 121 °C, when the medium had cooled to approximately 50 °C, to yield the final concentration of 3.77 mg/L. Five genotypes for each species of *H. annosum s.l.* (Table 4) were used as target pathogens for the examination of antifungal activity of PLT. MEA plates not amended with PLT inoculated with fungal genotypes acted as controls. A MEA plug (5 mm in diameter) from an actively growing colony of each genotype was inoculated in the center of each Petri plate. Three Petri plates were prepared for each *Heterobasidion* genotype. Petri plates were incubated in the dark at 25 °C allowing a com-

parison with the results of the inhibition assays carried out with the raw CFF of *P. protegens* (strain DSMZ 13134) conducted under the same incubation temperature and with the same *Heterobasidion* genotypes [12].

Colony radii of *Heterobasidion* genotypes were measured (in mm) in treated (r_T) plates (i.e., amended with PLT) and control (r_C) plates (not amended with PLT) along two perpendicular axes after 4 and 7 days of incubation, and the two measurements for each day were averaged. As described previously [12], the MGI of *Heterobasidion* spp. was determined by calculating (in %) the radial reduction observed in treated plates in relation to the corresponding control plates with the following equation:

$$\text{MGI} = 100\% \cdot \frac{r_C - r_T}{r_C} \quad (2)$$

4.6. Statistical Analyses

The average values of MGI were calculated and compared among *Heterobasidion* species for each time point (i.e., 4 and 7 days), and between time points for each *Heterobasidion* species. The above comparisons were carried out with unbiased recursive partitioning tree models [44,45] set as described in Lione et al. [46].

The same model was used to test whether the concentration of PLT (i.e., C, response variable) produced by monocultures of *P. protegens* (strain DSMZ 13134) are significantly associated with any of the following factors (i.e., covariates): culture medium, incubation temperature, and incubation period (i.e., 24 h and 7 days). The *c* statistics and its related *p*-value were calculated for each covariate [44]. Since none of the covariates was significantly associated with the response variable (see results), the concentration of PLT produced by monocultures of *P. protegens* (strain DSMZ 13134) was analyzed as such by estimating its upper tail probability distribution function. For any concentration of PLT, this function provides an estimate of the probability $\Pr(x > C)$ that monocultures of *P. protegens* (strain DSMZ 13134) can release PLT with a concentration x higher than a given threshold C. The equation of the function was obtained through the fit of the distribution types 0-VII included in the Pearson system of generalized frequency curves [47–49]. The fit was performed through maximum likelihood, and for each distribution type, the Akaike Information Criterion (AIC) was calculated [50,51]. The distribution type minimizing AIC was selected as the optimal Pearson curve [52]. The probabilities of retrieving PLT from monocultures of *P. protegens* (strain DSMZ 13134) with a concentration over 0 and up to 3.77 mg/L (i.e., mean concentration measured in the CFF, see results) were calculated from the optimal curve equation.

The concentration of PLT produced by *P. protegens* (strain DSMZ 13134) growing in co-culture with the genotype of either species of *Heterobasidion* on KB broth was analyzed through a further unbiased recursive partitioning tree model set as described above. The model was fitted to test if the production of PLT released by the bacterium in co-cultures was significantly influenced by the co-culture type (i.e., *P. protegens* (strain DSMZ 13134) and the genotype of either *H. abietinum*, *H. annosum*, *H. irregulare*, or *H. parviporum*) and the incubation temperature.

The 95% confidence intervals (95% CI) of the average values of MGI and PLT concentration were calculated with the bootstrap bias-corrected and accelerated (BCa) method [53,54] based on the setting parameters reported in Lione et al. [46]. Statistical analyses were conducted with R version 3.6.0 [55] and with the associated packages bootstrap [56], strucchange [57], partykit [45], and PearsonDS [58]. The significance threshold was set to 0.05 for all tests.

Statistical analyses were not performed on data of concentrations of 2,4-DAPG and PRN, because such compounds were either under the LOQ or under the LOD (see results).

5. Conclusions

The current study revealed the presence of the antifungal compound PLT at a concentration of 3.77 mg/L in the CFF of *P. protegens* (strain DSMZ 13134). This concentration of

PLT has a remarkable antifungal activity in vitro against *Heterobasidion* spp., although all lines of evidence suggest that antibiosis does not rely on a single constitutively produced metabolite, but rather on a plethora of secondary metabolites. The instability and the loss of efficacy of PLT over time may be a constraint for using this molecule as a pesticide against *Heterobasidion* spp. We did not determine the optimal fermentation conditions for the production of 2,4-DAPG, PLT, or PRN by *P. protegens* (strain DSMZ 13134), but the ability of the bacterium to produce PLT and 2,4-DAPG was demonstrated. Finally, an increased production of PLT was observed when the bacterium was grown in co-culture with *Heterobasidion* spp. at 4 °C. This finding may suggest that Proradix® could perform better when stump treatments are performed at low temperatures or if the product is kept at low temperatures before use. In more general terms, the availability of further effective products for stump treatments against *Heterobasidion* species is relevant considering that the approved ones, including the chemical product urea and biological products based on *Phlebiopsis gigantea* (Fr.) Jülich, are either close to the expiration date [59] or not registered for use in several southern EU member states.

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Chapter IV

Short-term impact of biological and chemical treatments against *Heterobasidion irregulare* on microbial communities of *Pinus pinea* stumps

Pellicciaro, M., Lione, G., Gonthier, P. Short-term impact of biological and chemical treatments against *Heterobasidion irregulare* on microbial communities of *Pinus pinea* stumps. (Research paper to be submitted)

Short-term impact of biological and chemical treatments against *Heterobasidion irregulare* on microbial communities of *Pinus pinea* stumps

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Abstract

Stump treatments with either chemical or biological control agents have been recognized as effective measures in preventing airborne infections of coniferous stumps by *Heterobasidion* spp. Here, we report the short-term response of bacterial and fungal communities inhabiting stumps of *P. pinea* to biological (Proradix®, the cell-free filtrate of *P. protegens* [strain DSMZ 13134], the conidial suspension of *P. gigantea* [strain MUT 6212], Rotstop®) and chemical (urea) treatments targeting the alien invasive *H. irregulare*. Amplicon metagenomic sequencing of 16S and ITS gene was used to examine, four months after treatments, microbial communities of 15 stumps per each treatment and for controls. Treatments with Rotstop® and urea had a significant impact on fungi, and they were responsible for a significant decrease of both diversity and richness of fungal communities compared to control stumps and to other treatments. The effects of the other treatments on fungal communities inhabiting stumps were less pronounced. Bacteria were less affected by treatments as diversity and richness metrics of their communities were never significantly different from those of control stumps. However, correspondence analysis indicated that microbial community's composition was always shaped by treatments. The results provide additional insights on the risk associated with treatments against *Heterobasidion* spp. on natural ecosystems and improve our knowledge on microbial communities inhabiting stumps of *P. pinea*.

Introduction

The fungal species belonging to the *H. annosum* s.l. complex are among the most destructive pathogens of coniferous forests worldwide (Garbelotto and Gonthier, 2013). In Europe, *H. annosum* s.l. comprises four species, i.e. three native and one alien, each characterized by distinct host preference (Garbelotto and Gonthier, 2013). Italian stone pine (*P. pinea*) stands of central Italy are currently threatened by the alien invasive *H. irregulare* introduced from North America during WWII (Gonthier *et al*., 2004). Following a pest risk analysis performed by the European and Mediterranean Plant Protection Organisation (EPPO) in 2015 (EPPO, 2015), *H. irregulare* was included in the EPPO A2 list of pests recommended for regulation as quarantine pests.

Heterobasidion species produce airborne basidiospores serving as the main dispersal units (Garbelotto and Gonthier, 2013). Basidiospores infect stump surfaces after tree felling. Once these fungi have colonised the stump, they are able to spread to neighbouring healthy trees through root contacts or grafts (Garbelotto and Gonthier, 2013).

A common practice to control *Heterobasidion* spp. is to treat the stump surface immediately after cutting with either chemical or biological products to prevent the establishment and germination of basidiospores (Garbelotto and Gonthier, 2013). Because of its ability to competitively colonize conifer wood, an efficient biological control agent is the wood decay basidiomycete *P. gigantea* (Pratt *et al*., 1999; Asiegbu *et al*., 2005; Rönnberg *et al*., 2006). Several formulations containing suspensions of spores of *P. gigantea* are available, among which the most widespread and commonly used is the one registered as Rotstop® (Pratt *et al*., 1999; Gonthier and Thor, 2013). Conversely, urea stands among the most widely used chemical treatments (Gonthier and Thor, 2013; Gonthier, 2019). A solution of urea is able to increase the pH of the stump surface to a level that prevents the germination of *Heterobasidion* basidiospores (Johansson *et al.,* 2002). *Pseudomonas protegens* (strain DSMZ 13134), marketed as Proradix®, recently received attention for its efficacy against *Heterobasidion* spp. under various laboratory and field conditions (Gžibovska, 2016; Rönnberg *et al.,* 2018; Pellicciaro *et al.,* 2021a,b; Pellicciaro *et al.,* 2022). The greater performance of the cell-free filtrate (CFF) of *P. protegens* (strain DSMZ 13134) compared

to *P. protegens* (strain DSMZ 13134) made this biological control product potentially applicable as treatment against *H. annosum* s.l. (Pellicciaro *et al.*, 2021b).

Although the deliberate introduction of organisms as biological control agents offers an excellent and sustainable solution to manage plant pathogens and it is commonly considered to be safe and without side effects, every adopted practice can potentially induce a profound impact and may pose environmental and ecological hazards (Trumbore *et al*., 2015; Balla *et al.*, 2021; Elnahal *et al.*, 2022; Teixidó *et al.*, 2022). Thus, follow-up studies or long-term field observation need to be considered before commercialisation to properly assess the impact of biological control measures in field conditions (He *et al.*, 2021). The effects of biological or chemical stump treatments against *Heterobasidion* spp. on resident microbial communities have only been documented in few studies, mainly focused on Norway spruce (*P. abies*) and its fungal community structure (Varese *et al*., 1999 and 2003a,b; Vasiliauskas *et al*., 2004 and 2005). The diversity of fungi that inhabit stump surface has been reported to greatly decline following either chemical or biological treatments. In some cases, the decreased richness was greatest with treatments based on fungi, including those based on *P. gigantea*, instead of the chemical ones (Varese *et al*., 1999 and 2003a,b). However, evidences based on pyrosequencing instead of standard culture-based methods suggested a less severe scenario (Sun *et al.*, 2013; Terhonen *et al*., 2013). Sun and colleagues (2013) explored the effects of Rotstop® on bacterial communities of *P. abies* stumps during a time period covering 13 years. The main finding of this study was that the bacterial community gradually recovered the decreased initial richness. No adverse effects on fungal communities were reported in the same experiments (Terhonen *et al.*, 2013). To date, no study has addressed the effects of different treatments against *Heterobasidion* spp. on stumps of tree species other than Norway spruce.

The present work is part of a project aiming to assess both the efficacy and the side effects of state-of-the-art and new biological stump treatments against *H. annosum* s.l. An investigation on the efficacy of different stump treatments has already been conducted testing both biological (Proradix®, the CFF of *P. protegens* [strain DSMZ 13134], the conidial suspension of *P. gigantea* [strain MUT 6212], Rotstop®) and chemical (urea) treatments in forests of *P. pinea* infested by the invasive *H. irregulare* in central Italy

(Pellicciaro *et al.*, 2021b). In this work, a metagenomic approach was used to investigate the short-term impact of such treatments on both bacterial and fungal communities inhabiting the stump surfaces. Our hypothesis was that these treatments could led to a decrease in microbial diversity and richness.

Materials and methods

Study sites and sample collection

The study was conducted at the Gallinara Park (Rome, Italy), a Site of Community Importance (SCI) located in central Italy (41.53156, 12.56187). The study site is an even-aged *P. pinea* stand, where a field experiment was established in January 2020 during thinning operations. A detailed description of the field experiment was reported in Pellicciaro *et al.* (2021b). Briefly, freshly cut stump surfaces of *P. pinea* created during thinnings were treated with biological and chemical treatments against *Heterobasidion* spp. The tested treatments were the biological control product Proradix® (SP Sourcon Padena GmbH, Tübeningen, Germany), the cell-free filtrate (CFF) of *P. protegens* (strain DSMZ 13134), a conidial suspension of *P. gigantea* (strain MUT 6212) originally isolated from the same stand, the biological control product Rotstop® (Verdera Oy, Espoo, Finland), and aqueous urea (Fluka, Cologno Monzese, Italy) solution. Control stumps received sterile water instead of treatments; we will refer to water as one of the six treatments.

The effect of biological and chemical stump treatments on the fungal and bacterial communities inhabiting the stump surface was investigated 4 months after the application of treatments, i.e. June 2020. Samples were collected from 15 treated stumps for each treatment, resulting in a total of 90 individual samples (15 samples x 6 treatments). Using a gouge chisel, 6 wood chips (1 x 1 x 2 cm) were randomly collected from both heartwood and sapwood of each stump surface. The sampling was done from one half of the stump surface due to other concomitant samplings as described by Pellicciaro *et al.* (2021b). To avoid contaminations, the gouge chisel was cleaned with a 2% (v/v) NaClO solution between samplings on different stumps. Wood samples were placed in a paper envelope, frozen in liquid nitrogen and stored at -80 °C until DNA extraction.

DNA extraction

The wood chips were pulverised using a laboratory mixer mill (Terzano & C. spa, Italy): one sterile tungsten bead (1 cm in diameter) was added to the grinding jars to achieve an accurate disruption of the woody tissue. Extraction of genomic DNA from each of the 90 wood samples was performed with the DNeasy® PowerSoil® Pro Kit (Qiagen GmbH, Germany) following the manufacturer's instructions with some minor modifications: DNA was isolated from 100 mg of wood powder, and after adding Solution CD1 (causing cell lysis), the vortexing time was increased to 30 min to achieve an accurate disruption of the woody tissue, and elution in 100 µl of Solution C6 (releasing DNA from filter membrane) was done twice collecting the DNA after the first centrifugation and placed it back in the membrane. The DNA extracted was stored at -20 °C. Quality and quantity of DNA were checked with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Quality of genomic DNA was also visualised on 1.5% (w/v) agarose gel. After DNA extraction and quantification, genomic DNA was pooled in equal volumes as 3 individuals/pool in 30 pools resulting in 5 replicates for each stump treatment (6 treatments x 5 pooled DNA samples). A representative scheme of the pooling approach is reported in Figure 1.

Illumina NovaSeq sequencing

Genomic DNA samples were submitted to Novogene (UK) Company Limited for 250 bp paired-end (PE) sequencing of the bacterial 16S V3–V4 region and the fungal ITS2 region on the Illumina NovaSeq 6000 system. Assessment of DNA quality control for library preparation was done with agarose gel electrophoresis evaluating DNA integrity, purity, fragment size and concentration. All 30 samples were then subjected to library construction. The DNA was subjected to polymerase chain reaction (PCR) amplification of V3–V4 and ITS2 regions using specific primers connecting with barcodes. For bacterial communities, the V3-V4 region of 16S DNA was amplified with the forward 341F (5'-CCTAYGGGRBGCASCAG-3') and reverse 806R (5'-GGACTACNNGGGTATCTAAT-3') primers (Yu *et al.*, 2005). Amplification of fungal ITS2 region was performed using the forward ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and reverse ITS4 (5'-CCTCCGCTTATTGATATGC-3') primers (White *et al.,* 1990). The PCR products

with proper size were selected by running a 2% agarose gel electrophoresis. A same amount of PCR products from each sample was pooled, end-repaired, A-tailed and further ligated with Illumina adapters. The library was checked with Qubit and real-time PCR for quantification and with bioanalyzer for size distribution detection. Library was sequenced on Illumina NovaSeq 6000 platform to generate 250 bp paired-end raw reads.

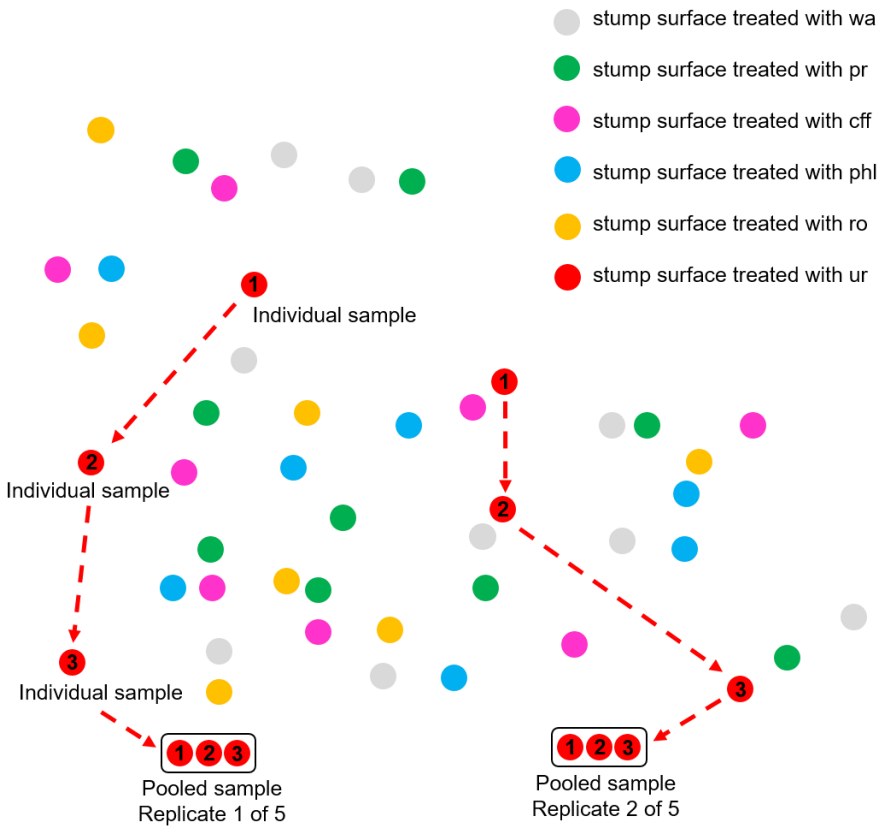


Figure 1. *Sample pooling approach: samples of genomic DNA were pooled as 3 individuals/pool in 30 pools resulting in 5 replicates for each of the six stump treatments. The coloured circles represent stumps treated with: wa-water (controls)-grey circles; pr-Proradix®-green circles; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134)-pink circles; phl-conidial suspension of* P. gigantea *(strain MUT 6212)-light blue circles; ro-Rotstop®-yellow circles; and ur-urea-red circles.*

Sequencing data processing

Paired-end reads were assigned to samples based on their unique barcodes. They were truncated by cutting off the barcodes and primer sequences and were merged using FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). The splicing sequences were called raw tags. Quality filtering on the raw tags was performed using the fastp (Version 0.20.0) (Chen *et al.,* 2018) software to obtain high-quality clean tags. Vsearch software (Version 2.15.0) (Rognes *et al*., 2016) was used to blast clean tags to the reference database to detect chimera sequences. In this study, we used Silva database (https://www.arb-silva.de/) as the reference database for bacterial 16S region (Quast *et al.,* 2013; Yilmaz *et al*., 2014; Glöckner *et al.,* 2017; Pruesse *et al.,* 2007) and UNITE database (https://unite.ut.ee/) as the reference for fungal ITS region (Kõljalg *et al.,* 2020; Nilsson *et al.*, 2018). According to Haas *et al.* (2011), chimera sequences were then removed to obtain the effective tags.

Data denoise of effective tags was performed with DADA2 (Callahan *et al.*, 2016) or deblur in the QIIME2 software (Version QIIME2-202006) (Bolyen *et al.,* 2019) to obtain initial ASVs (default: DADA2), and the sequences with abundance less than 5 were filtered out to obtain the final ASVs (Li *et al.,* 2020). Species annotation was performed using Silva Database for 16S and UNITE Database for ITS in the QIIME2 software. Multiple sequence alignment was performed using QIIME2 software to study the phylogenetic relationship of each ASV and the differences of the dominant species among different samples. The absolute abundance of ASVs was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity and beta diversity were all performed based on this output-normalized data. Alpha-diversity metrics (Simpson, Shannon and Chao1) were calculated in QIIME2 to analyse the evenness, richness and diversity of both bacterial and fungal communities. To more comprehensively evaluate the sequencing effort reached in our study, we created rarefaction curves using the Good's coverage index in QIIME2 and MS Excel.

Statistical analyses

The average values of the Simpson, Shannon and Chao 1 diversity indices were calculated for each treatment along with the corresponding 95% Bias Corrected and accelerated (BCa) bootstrap confidence interval ($CI_{95\%}$) based on 10^5 iterations (DiCiccio and Efron, 1996). Comparisons of the above indices were conducted among treatments by fitting unbiased recursive partitioning tree models based on conditional inference (Hothorn *et al.*, 2006) by using the algorithms of Hothorn *et al.* (2015). The algorithms were set as described in Lione *et al.* (2020) with the diversity indices included as output variables and the treatments as factor covariates (Crawley, 2013). The effects of treatments on the three diversity indices were assessed by computing the Bonferroni-corrected *P*-value associated with the *c* statistics (Zeileis *et al.*, 2002; Hothorn *et al.*, 2006) resulting from the presence/absence of binary splits in the corresponding tree model (Lione *et al.*, 2022).

A χ^2 test analysis for categorical association (Agresti, 2002) followed by a correspondence analysis (CA) (Kassambara, 2017) were conducted to assess: I) whether different treatments were associated with different bacterial and fungal communities (i.e. beta diversity); II) which treatment levels (i.e. water, Proradix®, CFF of *P. protegens* [strain DSMZ 13134], conidial suspension of *P. gigantea* (MUT 6212), Rotstop®, urea) and taxonomic group levels (phyla for bacteria and orders for fungi) were associated; and III) which treatments were associated with similar bacterial and fungal communities. Separate χ^2 test analysis and CA were run for bacterial and fungal communities. The association between treatments and microbial communities was deemed significant if the condition $P(\chi^2)<0.05$ was met (Agresti, 2002). In the above inequality, $P(\chi^2)$ represents the right tailed integral of the chi-squared curve associated with the contingency table of the absolute abundance of ASVs at the specified taxonomic level (phyla for bacteria and orders for fungi) after cross-tabulation between treatments and taxonomic groups. A heatmap rendering of the above-mentioned contingency table was built. The number of CA dimensions to retain was determined by considering the percent of cumulative variance explained, as described in Kassambara (2017), with a threshold of 80%. A vector-based and a dot-based representation was used for treatments and taxonomic groups, respectively. Such representation was embedded within asymmetric contribution biplots (one biplot for each pair of retained CA dimensions)

(Greenacre, 2013). Vectors orientations and their orthogonal projections on the retained CA dimensions (Kassambara, 2017) were used to interpret treatment similarity/dissimilarity, while dot positions in relation to treatment vectors were used as a proxy to interpret the association between treatments and taxonomic levels.

The partitioning of taxonomic levels among treatments was represented as a Venn diagram (Gao, 2021) to refine the interpretation of the CA results. The Venn diagram was built by fitting irregular border shapes to account for all possible set intersections (Gao, 2021).

Results

Bacterial community

Illumina sequencing data

After filtering chimeric sequences, the total number of bacterial PE reads obtained from 30 samples was 1,113,411 (54% of the total 2,050,511 raw PE reads) with an average of 37,114 ± 4,092 PE reads per sample (Table 1, Table A1). By pooling the PE reads from all the five replicates in each treatment, about 185,569 PE reads per treatment (range 163,402-202,752) were obtained (Table 1, Table A1).

Table 1. *Total number of PE reads for the bacterial 16S V3–V4 region and the fungal ITS2 region before (raw PE reads) and after (effective PE reads) data cleaning in control and treated stumps of* P. pinea*. Samples groups (i.e. stump treatments) abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Sample groups	Total number of raw PE reads		Total number of effective PE reads	
	16S V3–V4 region	ITS2 region	16S V3–V4 region	ITS2 region
wa	353,116	354,703	202,752	300,765
pr	331,508	360,619	163,402	303,828
cff	323,340	335,201	176,342	288,221
phl	360,871	367,719	195,513	312,393
ro	340,865	341,191	191,763	290,967
ur	340,811	375,150	183,639	325,927
total	2,050,511	2,134,583	1,113,411	1,822,101

The rarefaction curves of bacteria demonstrated a similar pattern and reached a saturation stage, indicating that most of the species in each sample group (i.e. stump treatment) was observed (Figure 2).

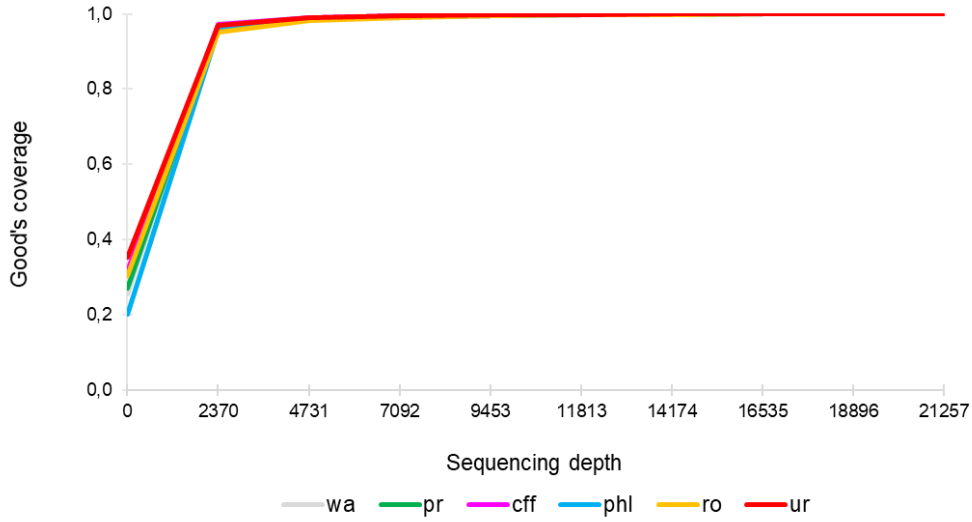


Figure 2. *Rarefaction curves for each sample group (i.e. stump treatment) depicting the effect of bacterial 16S V3-V4 region sequence number on the Good's coverage. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Bacterial community composition and structure

More than 99.9% of total bacterial sequences were classified to the bacterial domain. The 3,674 bacterial ASVs found in treated and control stumps of *P. pinea* were classified into 20 phyla, 50 classes, 128 orders, 222 families, and 451 genera. The relative abundance of phyla detected is shown in Figure 3. The most abundant phylum was *Proteobacteria* (from 60.5% to 70.1%) in all sample groups, followed by *Actinobacteriota* (from 6.0% to 14.6%), *Cyanobacteria* (from 3.9% to 12.6%), *Firmicutes* (from 1.4% to 6.9%), and *Acidobacteriota* (from 1.0% to 2.3%) except in stumps treated with urea, where the abundance of *Bacteroidota* was higher than in other groups, accounting for 1.6% (Figure 3). Besides, the quota of unidentified ASVs ranged from 4.4% and 16.7% (Figure 3). The relative abundance of the 30 most abundant (>1% of the total bacterial community on average) genera is shown in Table A2. *Sphingomonas* (from 7% to 19.2%), *Robbsia* (from 1.4% to 12.4%), *Pseudomonas* (from 5.8% to 25.2%), and *Endobacter* (from 1.5% to 7.7%) were the common predominant genera in all sample groups (Table A2). Sequences of *P. protegens* were not observed in this study.

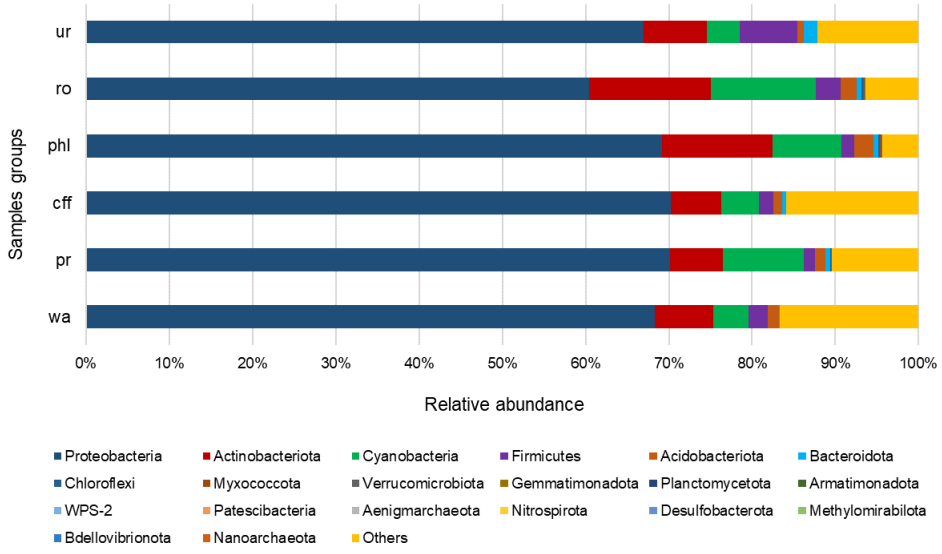


Figure 3. *Bacterial taxa distribution at phylum level associated with the six samples groups (i.e. stump treatments). Others are ASVs that remained unclassified at the phylum level. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

The Venn diagram shows that the distribution of ASVs in the bacterial community varied among the different treatments (Figure 4). A total of 150 ASVs were shared among the six sample groups, accounting for 4.1% of the total 3,674 ASVs (Figure 4). In addition, 161, 517, 245, 432, 761 and 218 ASVs were unique in the control, Proradix®, CFF of *P. protegens* (strain DSMZ 13134), conidial suspension of *P. gigantea* (strain MUT 6212), Rotstop® and urea treatments, respectively (Figure 4).

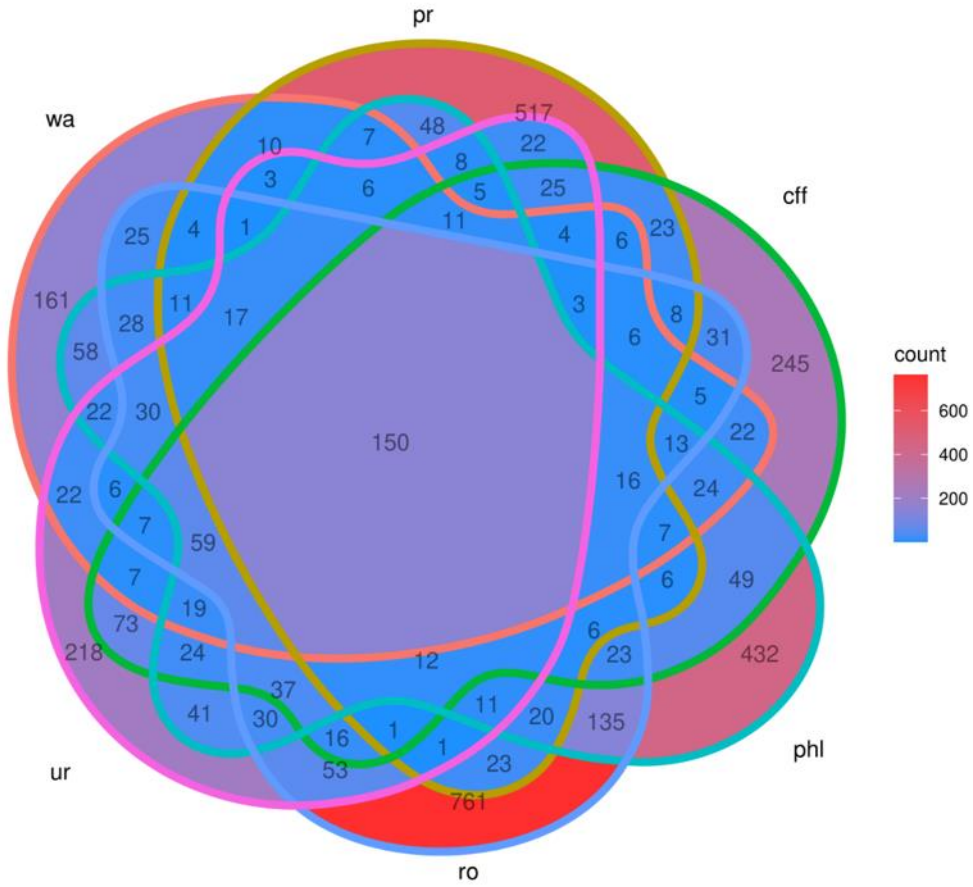


Figure 4. *Venn diagram of bacterial ASVs associated with the six sample groups (i.e. stump treatments). The numbers in one circle indicate unique ASVs, and numbers in two or more intersecting circles indicate shared ASVs. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Alpha-diversity analysis showed no significant differences in the bacterial community diversity and richness among the six treatments for any of the diversity indices ($P > 0.05$) (Figure 5, Table A1). Control stumps showed the lowest value of diversity and richness based on Shannon (mean value of 5.8) and Chao1 (mean value of 276.5) metrics (Figure 5, Table A1). While, the lowest community diversity with the Simpson index was found in stumps treated with urea (mean value of 0.9) (Figure 5, Table A1).

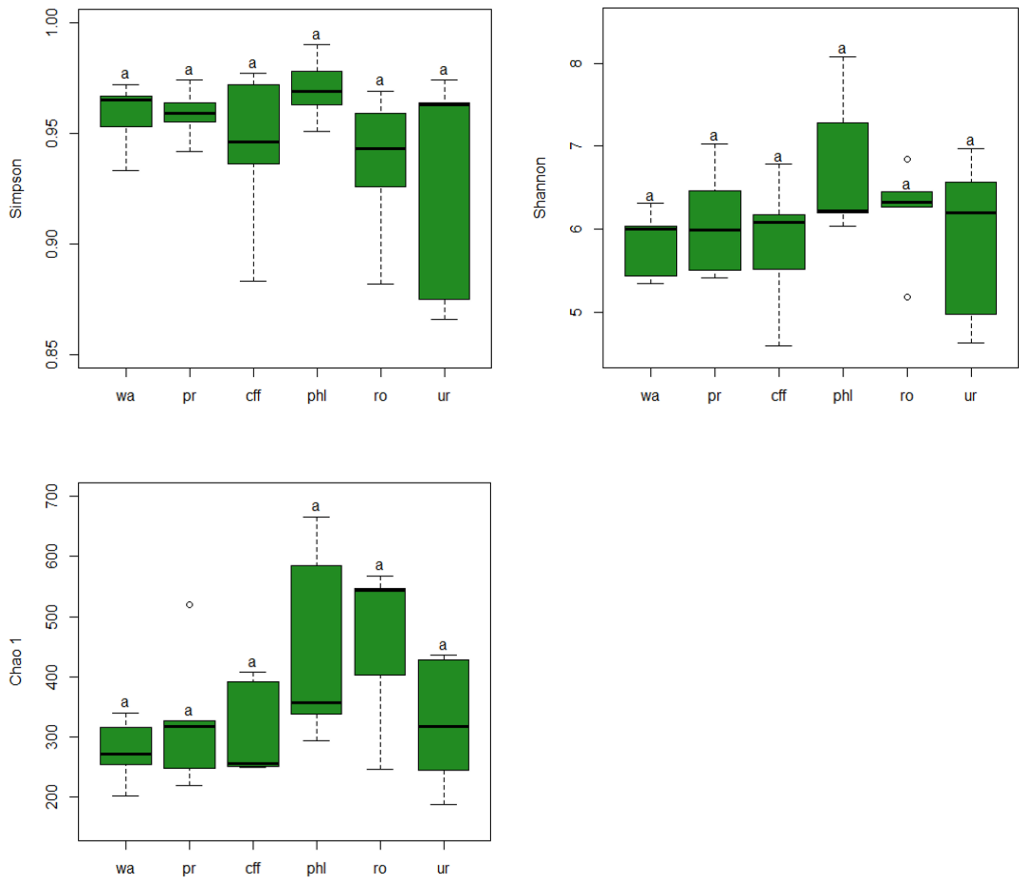


Figure 5. *Comparison among the values of alpha diversity indices (Simpson, Shannon and Chao1) of bacterial community for each sample group (i.e. stump treatment). The boxplot associated with the alpha index of all sample groups is reported. The boxes display the values ranging between the 25th and 75th percentiles, the horizontal thick line in between marks the median value, the t-shaped whiskers outside the boxes identify the minimum and maximum values, while dots are outliers. Same letters indicate non-significant differences ($P > 0.05$). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Overall, χ^2 test analysis for categorical association revealed an association between stump treatments and bacterial community composition since the condition $P(\chi^2)<0.05$ was met (Figure 6A and Figure A1). The key of bacterial phyla acronyms is in Table A3 while heatmaps of absolute and relative frequencies of phyla are plotted in Figure 6B and 6C. The first two axes of the correspondence analysis (CA) explained a cumulated variance of 86.7%, hence only the first two CA dimensions were reteined (Figure 6A). The less disturbed community with respect to control was the one resulting from CFF treatment (Figure 6A and Figure A1). Any phylum showed a clear tendency to colonize control or CFF-treated stumps (Figure 6A). Communities from Rotstop® and *P. gigantea* (strain MUT 6212) treatment were found to be similarly associated with dimension 1, which means that these treatments consisted of similar bacterial communities (Figure 6A and Figure A1). Both these treatments were associated with *Actinobacteriota*, *Cyanobacteria* and *Acidobacteriota* (phyla no. 2, 3 and 5, respectively) (Figure 6 and Figure A1, Table A3). Community composition associated with Proradix® and urea treatment reflected more divergent communities. The dimension 2 revealed an association with the phyla *Firmicutes* and *Bacteroidota* (phyla no. 4 and 6, respectively) mainly on stumps treated with urea (Figure 6 and A1, Table A3).

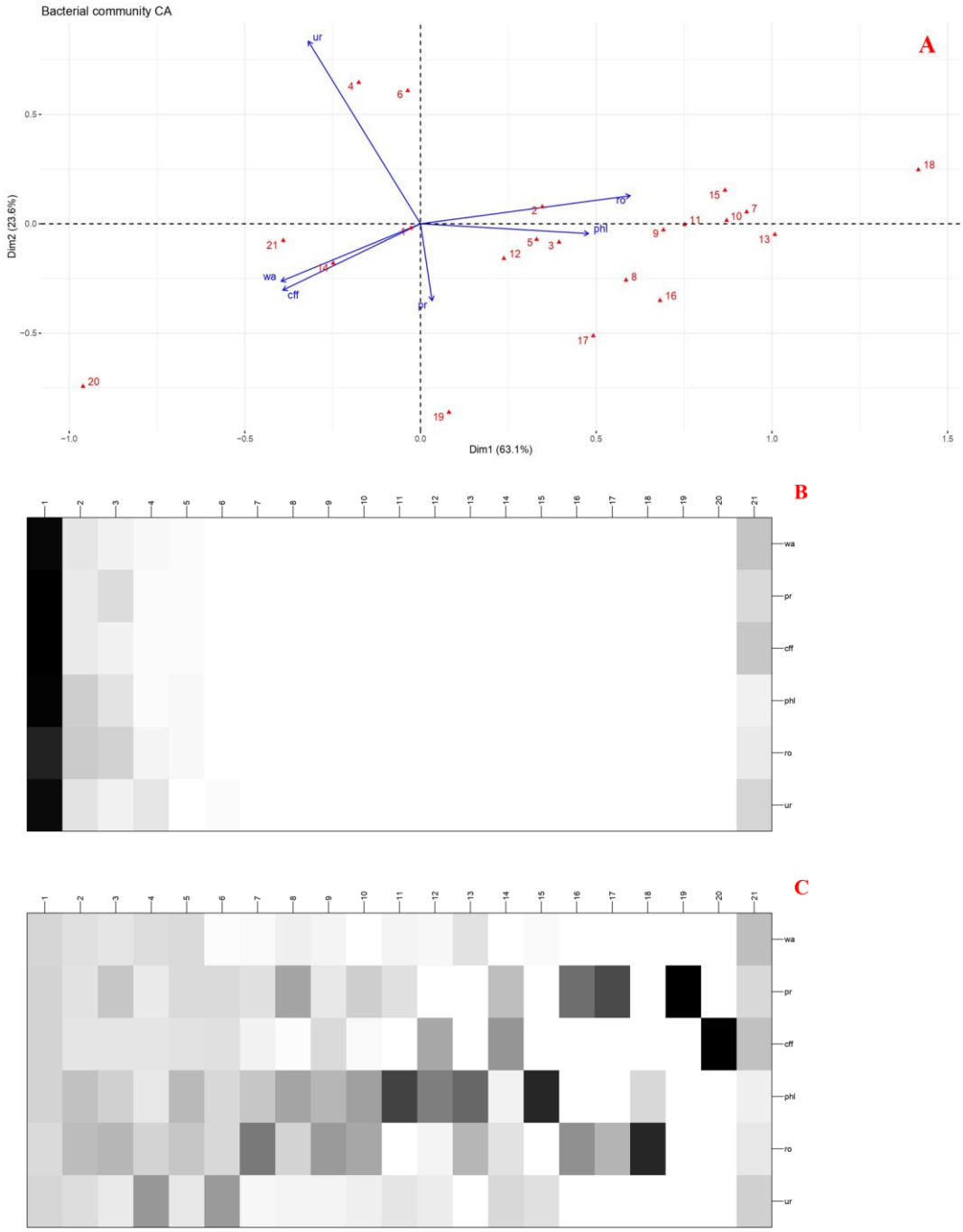


Figure 6. *Correspondence analysis of bacterial beta diversity based on phyla composition across samples groups (i.e. stump treatments). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea. (A) Biplot of the first two dimensions: stump treatments are fitted as blue vectors; phyla correspond to numbered red triangles. Greyscale heatmap of absolute (B) and relative (C) frequencies of phyla across samples groups. Darkest colours indicate the higher values and vice-versa. The numbered phyla are listed in Table A3.*

Fungal community

Illumina sequencing data

A total of 2,134,583 fungal raw PE reads were generated by Illumina sequencing from 30 pooled samples (Table 1, Table A4). After data cleaning, 1,822,101 effective PE reads were obtained representing 85% of the original raw PE reads. We detected an average of 60,737 ± 4,688 PE reads per sample (range 50,905-67,145) (Table 1, Table A4).

The rarefaction curves of fungi showed saturation for all sample groups indicating that the current sequencing depth was accurate and reliable (Figure 7).

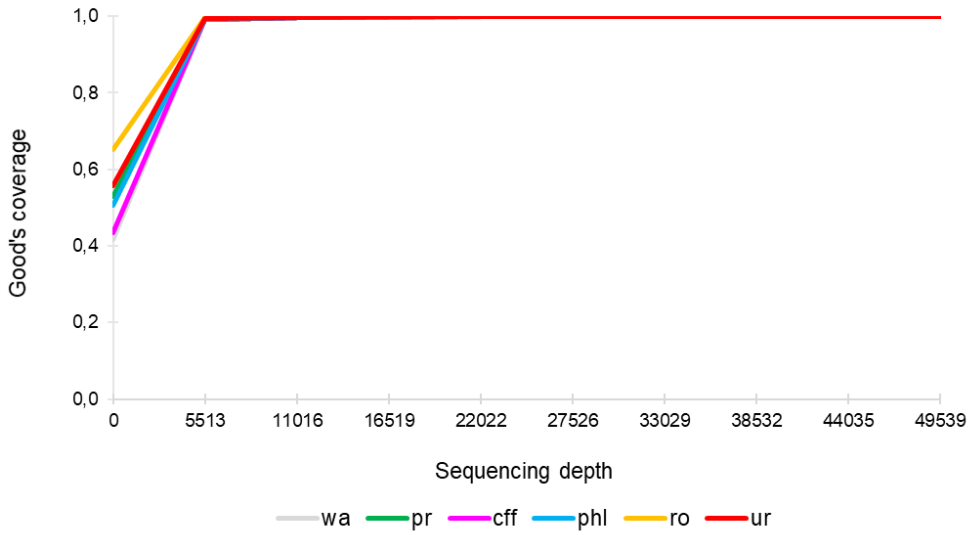


Figure 7. *Rarefaction curves for each sample group (i.e. stump treatment) depicting the effect of fungal ITS2 region sequence number on the Good's coverage. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Fungal community composition and structure

A total of 1,292 different fungal ASVs were obtained from treated and control stumps of *P. pinea*. According to the taxonomic identification, fungal ASVs could be classified into 3 phyla, 17 classes, 53 orders, 123 families and 206 genera. The relative abundance of phyla detected is shown in Figure 8. Fungi were dominated by *Ascomycota* (from 65.5% to 87.5%) and *Basidiomycota* (from 12.2% to 33.9%). The remaining ASVs (from 0.6% to 1.1%) was assigned to *Mucoromycota* or remained unclassified (Figure 8).

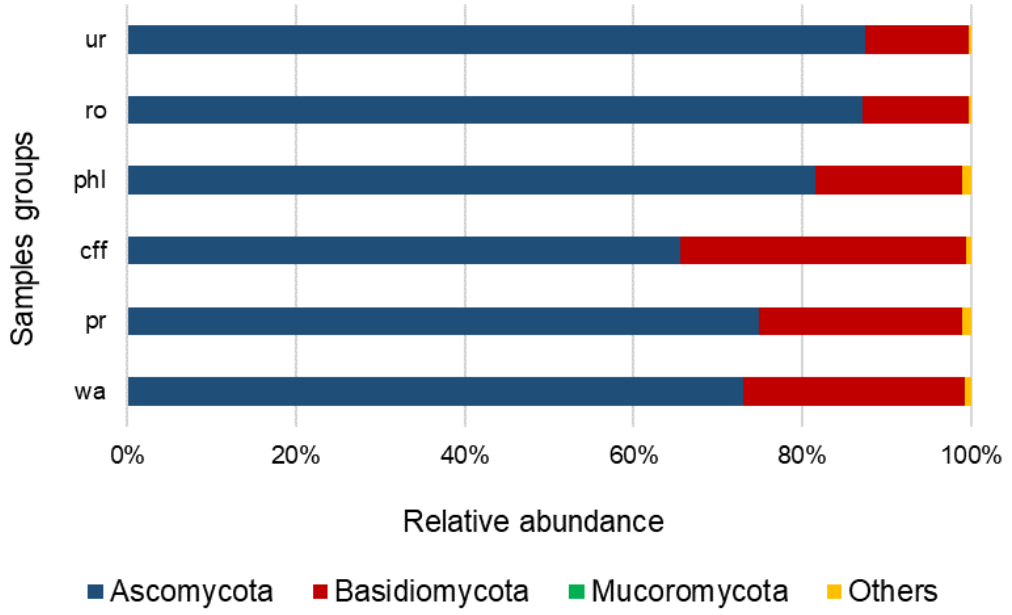


Figure 8. *Fungal taxa distribution at phylum level associated with the six samples groups (i.e. stump treatments). Others are ASVs that remained unclassified at the phylum level. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

The top 30 fungal genera on treated and control stumps are listed in Table A5. The relative abundance of the genus *Phlebiopsis* ranged from 0.04% (of the total fungal community on average) in stumps treated with Proradix® to 2.72% in stumps treated with Rotstop®. The genus *Heterobasidion* accounted for $<10^{-4}$ to 0.37% of total fungal community of stumps depending on treatments. The average relative abundances of the biological control agent *P. gigantea* were 0.67% (control), 0.05% (Proradix®), 0.88% (CFF of *P. protegens* [strain DSMZ 13134]), 0.77% (conidial suspension of *P. gigantea* [strain MUT 6212]), 2.72% (Rotstop®) and 0.04% (urea). *Heterobasidion irregulare* was detected in stumps treated with water (i.e. control, 0.04%), the CFF of *P. protegens* (strain DSMZ 13134) (0.08%), the conidial suspension of *P. gigantea* (strain MUT 6212) (0.04%) and with Rotstop® (0.37%). Other species of the genus *Heterobasidion* were not detected in this study.

A total of 133 ASVs were shared among the six sample groups, accounting for 10.3% of the total ASVs (Figure 9). There were 114, 133, 147, 142, 64 and 89 unique ASVs for control, Proradix®, CFF of *P. protegens* (strain DSMZ 13134), conidial suspension of *P. gigantea* (strain MUT 6212), Rotstop® and urea treatments, respectively (Figure 9).

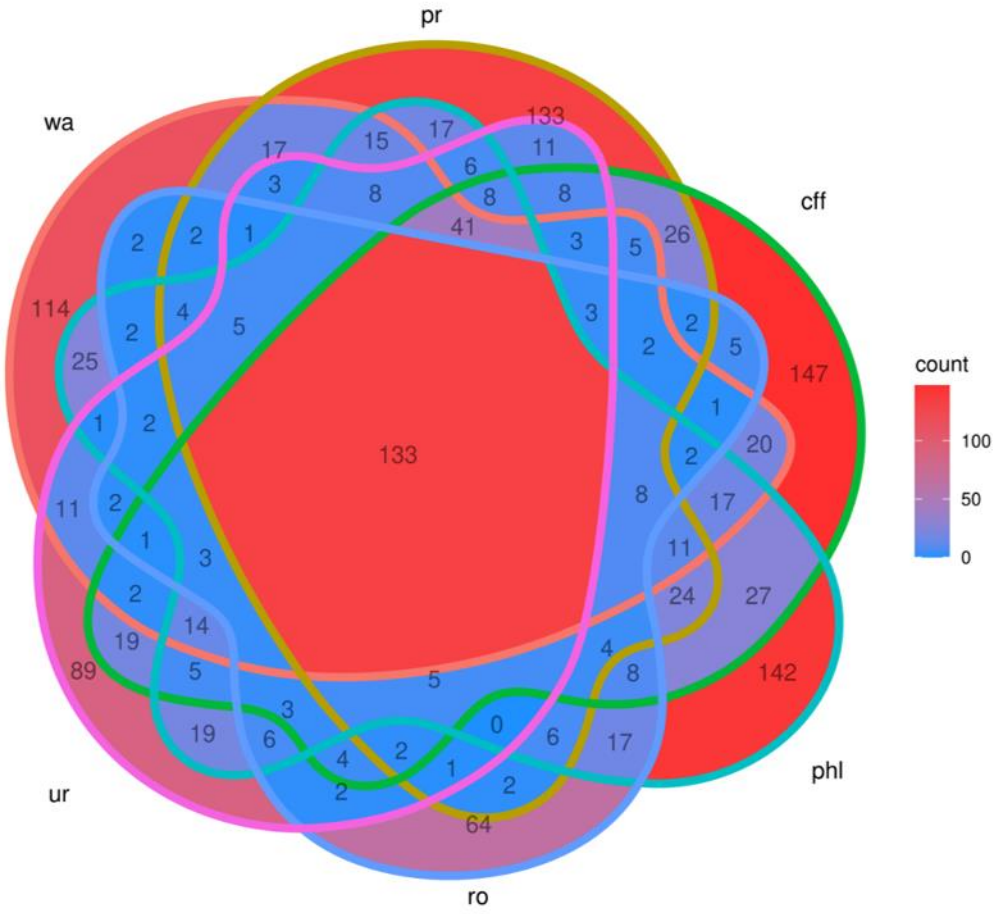


Figure 9. *Venn diagram of fungal ASVs associated with the six sample groups (i.e. stump treatments). The numbers in one circle indicate unique ASVs, and numbers in two or more intersecting circles indicate shared ASVs. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

All alpha diversity metrics showed that the fungal diversity and richness in stumps treated with Rotstop® and urea were significantly lower than in control stumps and in stumps treated with the other stump treatments ($P < 0.05$) (Figure 10 and Table A4). The fungal community of stumps treated with Rotstop® displayed the lowest values of Simpson (mean value of 0.8), Shannon (mean value of 3.3), and Chao1 (mean value of 119.2) indices (Figure 10 and Table A4).

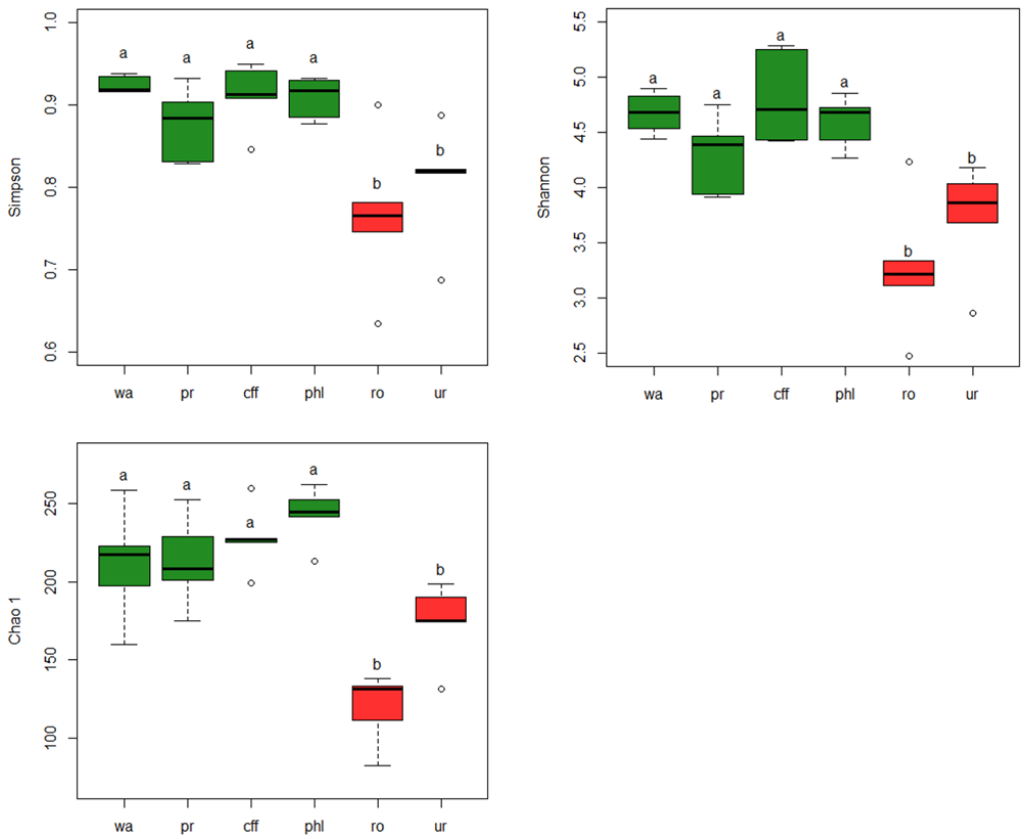
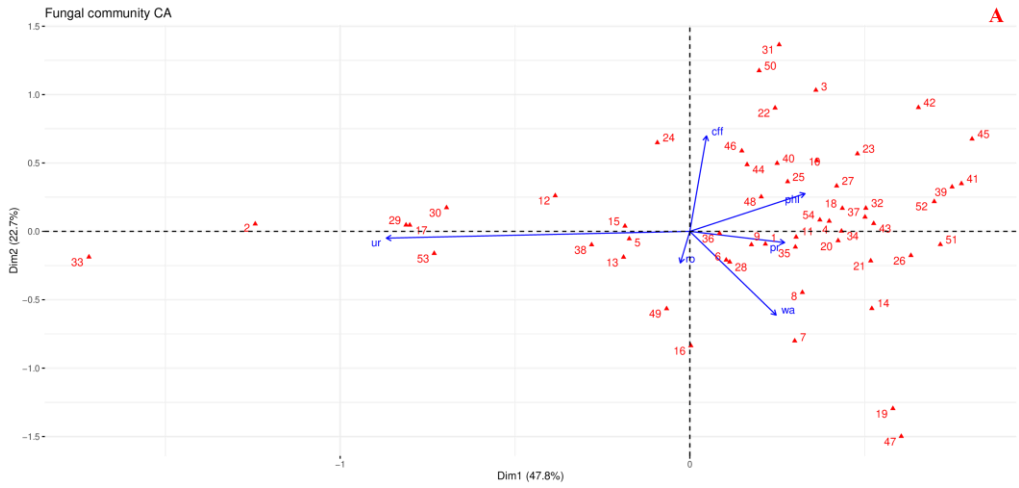
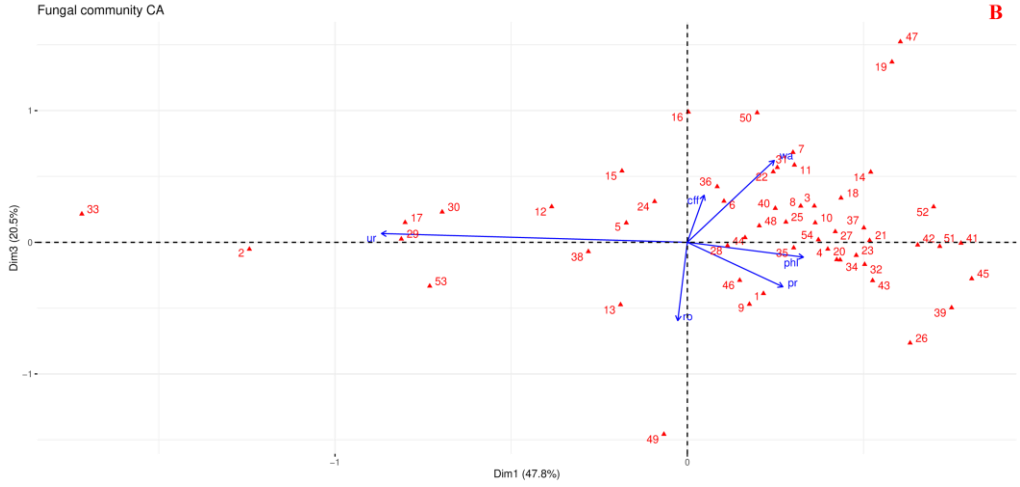
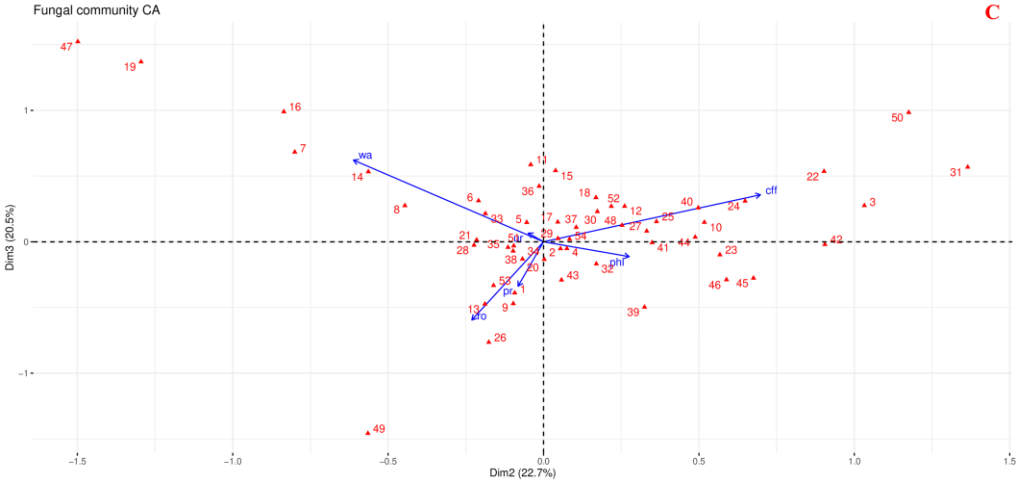


Figure 10. *Comparison among the values of alpha diversity indices (Simpson, Shannon and Chao1) of fungal community for each sample group (i.e. stump treatment). The boxplot associated with the alpha index of all sample groups is reported. The boxes display the values ranging between the 25th and 75th percentiles, the horizontal thick line in between marks the median value, the t-shaped whiskers outside the boxes identify the minimum and maximum values, while dots are outliers. Different letters indicate significant differences of the associated average values ($P < 0.05$). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Overall, the χ^2 test analysis for categorical association revealed an association between stump treatments and fungal community composition since the condition $P(\chi^2)<0.05$ was met (Figure 11A,B,C and Figure A2). The key of fungal orders acronyms is in Table A6. Heatmaps of absolute and relative frequencies of orders are depicted in Figure 11D and 11E, respectively. Three dimensions of the CA were retained, accounting for 90.9% of the total variance within the fungal orders composition (Figure 11A,B,C). No convergence of community composition was found between control stumps and any of the other stump treatments (Figure 11A,B,C). For communities associated with the urea treatment, more than 98% of the variance was explained by the first dimension (Figure 11A,B and Figure A2). Correspondence analysis revealed significant differences in the fungal community composition between stumps treated with urea and those treated with the other stump treatments (Figure 11A,B). *Hypocreales, Capnodiales, Cystobasidiales, Agaricostilbales, Agaricales*, *Ophiostomatales* (orders no. 2, 5, 17, 29, 30, and 33, respectively) showed a clear tendency to colonize stumps treated with urea (Figure 11A,B,D,E and Figure A2, Table A6). Stump surfaces treated with Proradix® and *P. gigantea* (strain MUT 6212) resulted in similar fungal communities (Figure 11A). Similarly to urea, the CFF resulted in a fungal community shift (Figure 11A and A2). The dimension 2 revealed an association of fungi of the order *Russulales* (order no. 3; *Heterobasidion* spp.) mainly with the CFF treatment (Figure 11D,E and Figure A2, Table A6). Significant changes in the community were also detected following Rotstop® treatment (Figure 11B and Figure A2). Despite the higher abundance of *Polyporales* (order no. 9; *Phlebiopsis* spp.) in samples from stumps treated with Rotstop®, no association was observed between this sample group and such fungal order (Figure 11C,E and A2, Table A6).


Fungal community CA
A
Dim2 (22.7%)
Dim1 (47.8%)
cff
phl
pr
ro
wa
ur


Fungal community CA
B
Dim3 (20.5%)
Dim1 (47.8%)
cff
phl
pr
ro
wa
ur


Fungal community CA
C
Dim3 (20.5%)
Dim2 (22.7%)
cff
phl
pr
ro
wa
ur

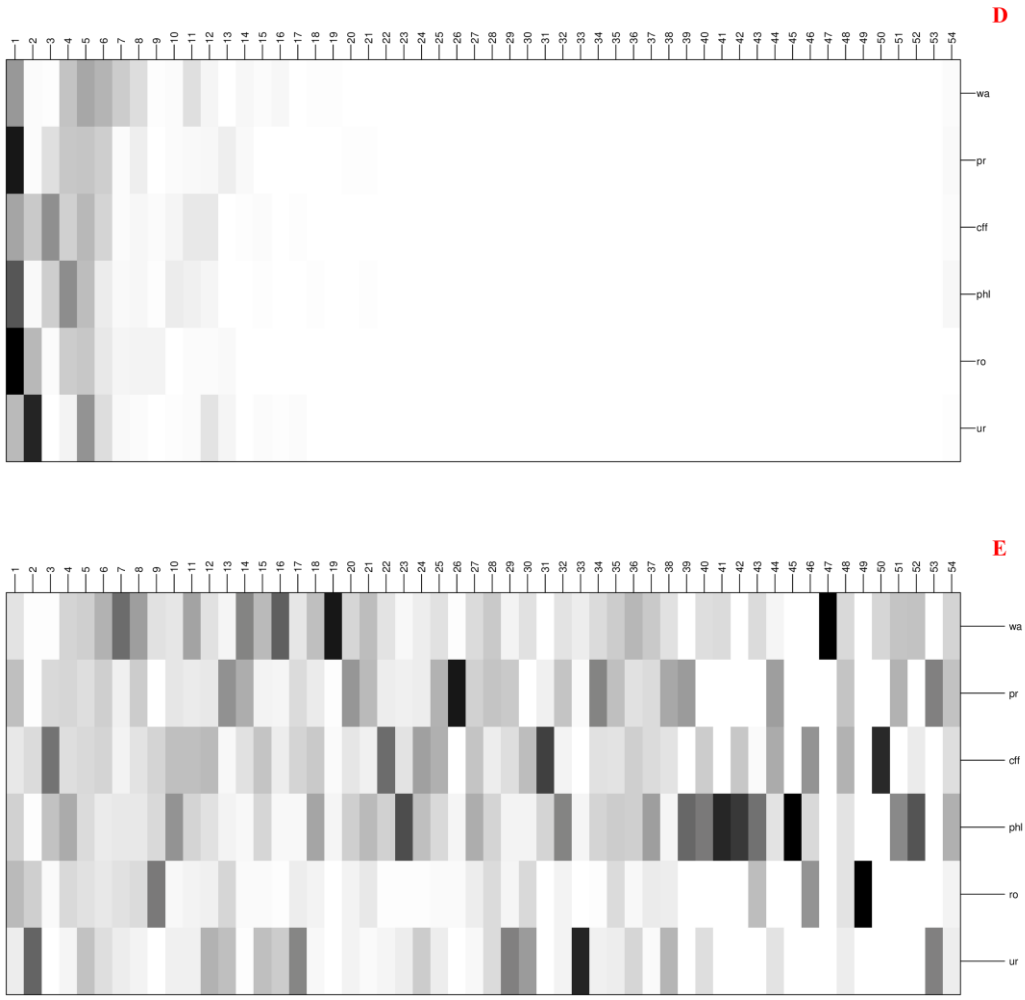


Figure 11. *Correspondence analysis of fungal beta diversity based on orders composition across samples groups (i.e. stump treatments). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea. Biplot of dimensions 1 and 2(A), 1 and 3 (B), 2 and 3 (C): stump treatments are fitted as blue vectors; orders correspond to numbered red triangles. Greyscale heatmap of absolute (D) and relative (E) frequencies of orders across samples groups. Darkest colours indicate the higher values and vice-versa. The numbered orders are listed in Table A6.*

Discussion

A large body of literature recognizes stump treatments with either chemical or biological control products as an effective measure in preventing primary infections by *Heterobasidion* spp. (Holdenrieder and Greig, 1998; Nicolotti *et al*., 1999; Pratt *et al*., 1999; Garbelotto and Gonthier, 2013; Gonthier and Thor, 2013; Annesi *et al*., 2005; Nicolotti and Gonthier, 2005; Gonthier, 2019; Blomquist *et al.,* 2020; Poloni *et al.,* 2021; Zaluma *et al.,* 2021). In a previous study, we demonstrated that the CFF of *P. protegens* (strain DSMZ 13134), the Rotstop® and the urea solution were equally effective in reducing airborne infections of *H. irregulare* on *P. pinea* stumps, which is currently a key host of this invasive *Heterobasidion* species (Pellicciaro *et al.,* 2021b). Whereas, a relatively low efficacy of *P. protegens* (strain DSMZ 13134) and *P. gigantea* (strain MUT 6212) was observed in the same experiment. In this study, by working on the same stumps, we investigated the impact of treatments focusing on bacterial and fungal communities inhabiting the stump surfaces 4 months after treatment application.

We observed a decrease in fungal diversity and richness in Rotstop® and urea-treated stumps compared to control stumps and stumps treated with other products, whereas bacterial diversity and richness were not significantly different among treatments, including controls. Studies on *P. abies* stumps have shown significant shifts in the fungal community composition following both Rotstop® and urea treatments soon after application (from 7 weeks to 1 year after treatment) (Varese *et al.,* 2003a; Vasiliauskas *et al*., 2004). However, these results partially contradict the study conducted in Finland on *P. abies* stumps by Terhonen *et al*. (2013), where fungal diversity did not decrease significantly following Rotstop® treatment soon after application (1 year after treatment). Using a comparable approach based on pyrosequencing, they observed a lower Shannon diversity index compared to our study, both in control (3.30 vs. 4.68, respectively) and Rotstop®-treated stumps (2.75 vs. 3.27, respectively). The complementary study in which the adverse effects towards bacterial community on the same stumps have been reported is not in agreement with our results (Sun *et al*., 2013). Differently from our outcomes, the application of Rotstop® caused a lowered bacterial richness soon after application (1 year after treatment). However, it should be noted that non-negligible aspects differentiate our study from these

similar ones (Sun *et al*., 2013; Terhonen *et al*., 2013) that were conducted in different ecoregions of Europe (i.e., northern Europe) as well as with reference to different pathosystems (i.e. *P. abies* and *H. parviporum*). For example, host tree species is reported as an important factor affecting wood-inhabiting fungi (Yuan *et al.*, 2017).

The aggressive nature of *P. gigantea* as a saprobic white-rot fungus has been widely reported (Behrendt and Blanchette, 1997; Boddy *et al.,* 2000; Żółciak *et al.,* 2020). Therefore, it is expected that wood colonization by *P. gigantea* could affect wood-inhabiting fungi, as we documented in this study. In contrast, our findings suggest that the early stage of stump decomposition by *P. gigantea* in Rotstop®-treated stumps may favour bacteria, possibly thanks to the use of derivatives of lignin decomposition and sugars released by fungal extracellular-enzymes (de Boer and van der Wal, 2008; Hervé *et al.,* 2014). Interestingly, the number of total and unique bacterial ASVs in Rotstop® samples were 1549 and 761, both values higher than those found in the other sample groups, included the controls (770 total ASVs, 161 unique ASVs). However, this did not result in significant higher values of richness and diversity, based on alpha-metrics calculated.

It should be noted that the inoculation of the stump surface with a different strain of *P. gigantea* (i.e. *P. gigantea* strain MUT 6212) did not led to the same effects on microbial communities of the Rotstop® application. Assuming as previously suggested that the success of *P. gigantea* as a biocontrol agent is dependent on the ability of the fungal strain to colonize wood (Żółciak *et al.*, 2020), we can only speculate that the impact of *P. gigantea* (strain MUT 6212) on fungal communities was less pronounced because of its low colonization rate of *P. pinea* stumps. However, such assumption is supported by the weak efficacy of *P. gigantea* (strain MUT 6212) against *H. irregulare* on stumps (Pellicciaro *et al.*, 2021b).

Not surprisingly, bacterial communities seems to be more resilient than fungal communities to the drastic increase of pH expected after the application of urea (Kielak *et al.*, 2016; Moll *et al.*, 2018). Adverse effects on non-target microorganisms seem to be negligible in the case of *P. protegens* (strain DSMZ 13134). However, that treatment did not performed well against *H. irregulare* in a previous study (Pellicciaro *et al.*, 2021b). Overall, stump treatment with the CFF of *P. protegens* (strain DSMZ 13134) combined good efficacy

against *H. irregulare* (Pellicciaro *et al*., 2021b) and little impact on microbial communities in this study, which makes it the best candidate treatment based on these short-term results.

Interestingly, the Venn diagrams indicated a number of both bacterial and fungal ASVs always higher in treated stumps than in the controls, except for fungal communities in stumps treated with Rotstop® and urea, as confirmed by alpha-diversity metrics. Therefore, application of all tested treatments at least in some cases, seems to promote microbial diversity.

Correspondence analysis indicated that microbial communities are shaped by different treatments, which is consistent with previous findings (Varese *et al.,* 1999, 2003a,b; Sun *et al*., 2013; Terhonen *et al*., 2013). *Proteobacteria* and *Ascomycota* were the predominant phyla among bacteria and fungi, respectively. *Proteobacteria* is known as the most ubiquitous phylum worldwide and has been found as the predominant also in wood (Sun *et al*., 2013; Hervé *et al.,* 2014; Kielak *et al.,* 2016; Moll *et al.,* 2018; Ren *et al.,* 2019). *Ascomycota* is ubiquitous as well, and it is widely documented as the largest and the most diverse phylum of Fungi (Schoch *et al.,* 2009). The predominance of *Ascomycota* and *Basidiomycota*, among fungal phyla, in wood supports the outcomes of previous studies (Yuan *et al.,* 2017; Leonhardt *et al.,* 2019; Behnke-Borowczyk *et al.,* 2021; Zöhrer *et al.,* 2021).

Fungal community composition selected by biological treatments was clearly different from that promoted by the chemical treatment with urea, as already suggested by alpha-diversity metrics. This can be explained by the raise of pH caused by the application of urea (Johansson *et al.,* 2002). Indeed, the pH has been reported as the most important driver of microbial community structure (Moll *et al.,* 2018).

Rotstop® and *P. gigantea* (strain MUT 6212) selected similar bacterial communities, characterized by the presence of *Actinobacteriota* and *Acidobacteriota* among other phyla. *Actinobacteriota* are reported to degrade lignin and cellulose and to play an important role in the maintenance of forest ecosystem functioning (Hervé *et al.,* 2014; Moll *et al.,* 2018; Mu *et al*., 2021). *Acidobacteria* is known to be associated with the initial stages of wood degradation (Mu *et al*., 2021; Sun *et al.,* 2013). Therefore, these treatments may improve wood degradation process also by favoring, in a similar manner, suitable wood degrading

bacteria. On the contrary, their effects on fungal communities were very different, with those selected by *P. gigantea* (strain MUT 6212) similar to the ones resulting from treatments with *P. protegens* (strain DSMZ 13134) rather than with Rotstop®. It should be noted that both *P. gigantea* (strain MUT 6212) and *P. protegens* (strain DSMZ 13134) showed little efficacy against *Heterobasidion* spp. in previous studies as opposed to Rotstop® (Pellicciaro *et al.,* 2021b), whose strain is known as a rapid colonizer of stumps outcompeting *Heterobasidion* spp. as well as other fungi (Pratt *et al*., 1999; Asiegbu *et al*., 2005; Rönnberg *et al*., 2006).

In this study we considered the taxonomic levels of phyla for bacteria and orders for fungi to decipher the effects of stump treatments on microbial communities inhabiting stumps. The effects of treatments on microbes, both positive and adverse, could be evaluated more comprehensively and in a greater detail. Moreover, reports on the long-term effects of such treatments would be desirable as well, because the impacts of stump treatments on microbial communities is known to vary over time (Varese *et al*., 2003a,b; Sun *et al*., 2013; Terhonen *et al*., 2013). Meanwhile, our study was limited to the short-term impact of such treatments. Based on our initial hypothesis, strong microbial selection was expected to occur on both fungi and bacteria. Instead, our results reveal that only stump treatments with Rotstop® and urea had a significant negative influence on fungal communities inhabiting stumps, whereas the effects of the other treatments were less pronounced. Bacteria were less affected by treatments and revealed unexpected resilience. The results obtained in this work provide additional insights on the risk associated with treatments against *Heterobasidion* spp. on natural ecosystems, and they may come in support of the process of registration of phytosanitary products. In addition, they improve our knowledge on microbial communities inhabiting Italian stone pine stumps.

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General discussion and conclusions

General discussion and conclusions

In this thesis, investigations on the biological control of *P. protegens* (strain DSMZ 13134) were conducted under both laboratory and field conditions to determine the antagonistic potential of the bacterium against the four species of *H. annosum* s.l. currently known to occur in Europe. Based on the results provided in Chapter I and II, the bacterium proved to be effective as a biological control agent. Similarly, the cell-free filtrate (CFF) of the bacterium containing its extracellular secondary metabolites was also found to be effective against *Heterobasidion* species. However, the efficacy of both the bacterium and its CFF was not uniform towards *Heterobasidion* species. The reasons for that are unknown. Nevertheless, variability in performances has been reported for several biological control agents and it is one of the main reasons why these agents are not used routinely and systematically in practice against pathogens of agricultural crops and forests (Köhl *et al*., 2019; Teixidó *et al.*, 2022). However, it should be noted that the results obtained in this thesis are not meant to be definitive with respect to the effectiveness of the tested biological control agents. Timing of assessment of field trials can be critical because sampling after only a few months, as in our study, may give misleading results compared with outcomes over a long time period (Holdenrieder and Greig, 1998). Moreover, natural infection by *Heterobasidion* spp. on which our field experiments relied is obviously subjected to variations in time and space that are beyond the control of our experimental setup and can generate variability in the results (Holdenrieder and Greig, 1998). In addition, *P. protegens* (strain DSMZ 13134), its CFF, and *P. gigantea* (strain MUT 6212) were tested at a single concentration while, various concentrations of the potential biological control agent should be tested to find the most appropriate one (Holdenrieder and Greig, 1998; Teixidó *et al.*, 2022). Further research is needed to draw conclusions on the level of efficacy of the tested biological control agents.

Based on the outcomes of the experiments *in vitro* presented in Chapter I, the thesis was shaped to deepen the knowledge on the level of efficacy of the CFF of *P. protegens* (strain DSMZ 13134), which resulted particularly promising. Different alternatives could be taken into account while attempting to optimise strategies based on biological control against *Heterobasidion* species. The commercial biological control agent may contain the

bacterium alone, thus the optimization can hinge on the development of the most suitable formulation and dosage (Teixidó *et al.*, 2022). Alternatively, as discussed in Chapter III, formulations containing both the living bacterium and its secondary metabolites can be developed that combine the direct effects of the secondary metabolites with the antagonistic activity of the bacterium, which may rely on the production of additional metabolites *in situ* as a result of the interaction of the bacterium with resident pathogens (Köhl *et al.*, 2019). Metabolites-based formulations are particularly useful in the case of bacteria known to produce a variety of antibiotic secondary metabolites. Such type of products, lacking dead or living cells of the bacterium, may need an extensive characterisation for risk assessment as each metabolite is considered as an 'active substance' of the biocontrol product (Mishra and Arora, 2018; Köhl *et al.*, 2019). A further option, yet not explored in this thesis, hinges on the concept of synergism (Gabriel *et al.*, 2018). Within this perspective, the synergism occurs when the antagonistic effects observed from a formulation composed of several microorganisms are greater than those expressed by the same microorganisms taken individually (Gabriel *et al.*, 2018; Köhl *et al.*, 2019). In the recent years, such concept was translated into the use of assembled consortia or helper strains (Massart *et al.*, 2015; Bhatia *et al*., 2018; Köhl *et al*., 2019; Niu *et al.*, 2020; Balla *et al*., 2021). However, difficulties in the registration of such types of biological control agents have to be considered as well (Köhl *et al.*, 2019).

Volatile compounds produced by bacteria such as *Pseudomonas* spp. or *Bacillus* spp. are known for their broad spectrum antifungal activity and therefore for their potential in the management of fungal plant pathogens (Fernando *et al*., 2005; Yuan *et al*., 2012; Cornelison *et al*., 2014; Gabriel *et al.*, 2018). In this thesis, the antagonistic activity of volatile compounds produced by *P. protegens* (strain DSMZ 13134) was only partially explored due to the most promising effects of the diffusible secondary metabolites released by the bacterium and present in its CFF.

Understanding the modes of action of a biological control agent is crucial to achieve optimum control of the target pathogen (Köhl *et al.*, 2019). In Chapter I, II and III, investigations were carried out to explore the possible modes of action of *P. protegens* (strain DSMZ 13134) against *Heterobasidon* species. Assays *in vitro*, i.e. dual cultures, are

commonly used to assess the antagonistic activity of promising microorganisms against plant pathogens, yet they may provide biased results (Köhl *et al.*, 2019). Such assays allow an easy visualisation of the resulting inhibition zone between the biological control agent and the target pathogen, mainly due to secondary metabolites released by the former, but they systematically exclude other modes of action (Köhl *et al.*, 2019; Raymaekers *et al.*, 2020). Hence, *in vitro* assays do not allow the evaluation of the full spectrum of modes of action of the promising microorganism (Köhl *et al.*, 2019; Raymaekers *et al.*, 2020). However, a robust line of evidence pointing to antibiosis as the main mode of action of *P. protegens* (strain DSMZ 13134) against *Heterobasidion* spp. was the observation that its CFF provided similar or even better levels of stump protection than the bacterium itself in field experiments (Chapter II). Indeed, results provided in Chapter III proved the ability of *P. protegens* (strain DSMZ 13134) to produce and release two secondary metabolites previously known to be expressed in the genus *Pseudomonas*, namely pyoluteorin and 2,4-diacetylphloroglucinol. Moreover, a remarkable antifungal activity of pyoluteorin was demonstrated by conducting assays *in vitro* against all the four *Heterobasidion* species tested, yet the precise modes of action of such metabolites against the pathogens still need to be elucidated. The best way to further confirm the crucial role of antibiosis will be to prove the role of antifungal secondary metabolites *in situ* during the interaction between *P. protegens* (strain DSMZ 13134) and *Heterobasidion* species on the stump surface (Raaijmakers *et al.*, 2002). To date, analytical techniques, like thin layer chromatography, high-performance liquid chromatography coupled with mass spectrometry have been successfully used to detect and quantify *in situ* a variety of secondary metabolites including pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol (Raaijmakers *et al.*, 2002; Raaijmakers and Mazzola 2012). However, recovery and detection of metabolites may be hampered by chemical instability of the compounds, as suggested by the instability of pyoluteorin in the experiments presented in Chapter III, by the wood decomposition mediated by microorganisms, and by several inherent difficulties in detecting secondary metabolites in wood (Raaijmakers *et al.*, 2002; Raaijmakers and Mazzola 2012).

Chemical and biological stump treatments may affect microbial communities of stumps. Understanding the impact that such treatments may have on non-target microorganisms would be important for gaining knowledge on the environmental hazards linked to the

application of these treatments in the forest. In Chapter IV, we provide evidence that treatments with *P. protegens* (strain DSMZ 13134) or with its CFF did not alter significantly the microbial communities inhabiting stump surfaces of *P. pinea*. Reservations should only be maintained concerning the application of Rotstop® and urea because fungal communities significantly decreased after these treatments in diversity and richness compared to control stumps. However, in this thesis we investigated only the short-term effects of treatments on stumps of a single tree species. One may argue that the effects in the medium or long term or on stumps of other tree species may be different. It should be noted that the knowledge about the long-term effects of stump treatments on non-target microorganisms is still largely insufficient for all the host tree species considered in this thesis (Sun *et al.,* 2013; Terhonen *et al.,* 2013).

The development of alternative control options to fight against *Heterobasidion* spp. is pivotal. The current restrictions on the application of Rotstop® and urea against these pathogens in Southern Europe have been discussed earlier in this thesis. Moreover, the availability of additional treatments is desirable and relevant as a result of the ongoing rising price of energy and fertilisers (Berkhout *et al.,* 2022). In Italy, as an example, the price of urea raised from about 400€/ton of May 2021 to 1040€/ton of May 2022 (Chamber of Commerce of Turin). Whereas, from an ecological perspective, large scale and long term application of a single stump treatment, regardless of being either biological or chemical, might reduce diversity, richness and the balancing power of resident microbial communities, with unknown environmental consequences (Nicolotti and Gonthier, 2005; Sun *et al.,* 2013; Terhonen *et al.,* 2013).

In conclusion, the results presented in this thesis support the use of both *P. protegens* (strain DSMZ 13134) and its CFF against the four species of *H. annosum* s.l. currently occurring in Europe. Early investigations *in vitro* and in controlled conditions supported the hypothesis that antibiosis is the main mode of action displayed by this bacterium against these forest pathogens. A plethora of secondary metabolites is likely implicated and may explain the effectiveness of the CFF, in which the antifungal compound pyoluteorin was quantified at remarkable concentration. Results showed that the bacterium is also able to produce the antifungal compound 2,4-diacetylphloroglucinol. Preliminary investigations on

the short-term impact of treatments based on *P. protegens* (strain DSMZ 13134) and its CFF on *P. pinea* stumps support their negligible effects on microbial communities inhabiting stumps. Overall, stump treatment with the CFF of *P. protegens* (strain DSMZ 13134) combining good effectiveness against *H. irregulare* and no adverse effects on microbial communities, can be considered as the best candidate treatment on *P. pinea*. However, comprehensive studies and long-term monitoring of the same stumps are desirable to exclude that adverse effect will occur later. Moreover, follow-up studies are needed to exclude any environmental hazard associated with the use of treatments on stumps of the other tree species included in the field trials of this thesis. This would be crucial to support a safe use of these stump treatments in practical forestry.

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Appendix

Appendix – Chapter IV

Table A1. *Number of PE reads before (raw) and after (effective) data cleaning, effectiveness, alpha-metrics (Simpson, Shannon, Chao1 and Good's coverage) for the bacterial 16S V3–V4 region in control and treated stumps of* P. pinea*. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Samples groups	Replicate	Number of raw PE reads	Number of effective PE reads	Effective (%)	Simpson	Shannon	Chao1	Good's coverage
wa	1	76,559	40,570	53	0.933	5.343	254	0.999
	2	72,693	46,384	64	0.972	6.034	271	1.000
	3	66,177	38,435	58	0.967	6.312	340	1.000
	4	62,660	38,233	61	0.965	6.001	316	1.000
	5	75,027	39,130	52	0.953	5.438	202	1.000
pr	1	63,354	31,921	50	0.959	7.029	520	1.000
	2	66,670	32,547	49	0.942	5.507	247	1.000
	3	64,838	26,309	41	0.974	6.463	326	1.000
	4	73,035	38,440	53	0.964	5.991	317	1.000
	5	63,611	34,185	54	0.955	5.419	219	1.000
cff	1	67,276	36,266	54	0.946	6.089	408	1.000
	2	68,235	40,868	60	0.883	4.600	250	1.000
	3	60,089	29,985	50	0.936	5.520	250	1.000
	4	62,574	32,935	53	0.977	6.782	391	1.000
	5	65,166	36,288	56	0.972	6.179	255	1.000
phl	1	79,610	44,095	55	0.990	8.078	666	1.000
	2	71,197	39,731	56	0.978	7.284	585	1.000
	3	65,353	37,074	57	0.951	6.042	338	1.000
	4	71,941	38,808	54	0.963	6.219	357	0.999
	5	72,770	35,805	49	0.969	6.194	293	1.000
ro	1	70,735	39,294	56	0.926	6.267	568	1.000
	2	66,184	36,193	55	0.943	6.449	546	1.000
	3	71,242	42,421	60	0.969	6.848	544	1.000
	4	64,663	34,316	53	0.882	5.184	246	1.000
	5	68,041	39,539	58	0.959	6.325	403	1.000
ur	1	71,801	37,165	52	0.974	6.969	435	1.000
	2	61,358	35,364	58	0.875	4.640	188	1.000
	3	72,827	35,685	49	0.963	6.202	317	1.000
	4	72,598	39,771	55	0.866	4.978	244	1.000
	5	62,227	35,654	57	0.964	6.565	427	1.000

Table A2. *Relative abundance (%) of 30 most abundant bacterial genera found among the six sample groups (i.e. stump treatments). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Genus	Mean relative abundance ± SE (%)[a]					
	wa	pr	cff	phl	ro	ur
Sphingomonas	15.18 ± 0.07	13.85 ± 0.07	10.18 ± 0.09	19.16 ± 0.12	7.75 ± 0.06	6.98 ± 0.05
Robbsia	12.39 ± 0.18	3.89 ± 0.01	2.04 ± 0.02	4.36 ± 0.02	1.43 ± 0.01	1.41 ± 0.01
Pseudomonas	8.87 ± 0.04	5.84 ± 0.03	15.69 ± 0.09	8.50 ± 0.04	14.76 ± 0.16	25.24 ± 0.14
Endobacter	5.76 ± 0.03	7.65 ± 0.06	3.06 ± 0.09	3.58 ± 0.01	2.98 ± 0.02	1.48 ± 0.01
Chloroplast	4.34 ± 0.02	9.74 ± 0.06	4.60 ± 0.09	8.19 ± 0.08	12.55 ± 0.10	3.88 ± 0.05
Pantoea	4.13 ± 0.03	3.97 ± 0.05	2.35 ± 0.09	2.34 ± 0.02	3.64 ± 0.05	6.51 ± 0.06
Curtobacterium	4.08 ± 0.04	0.10 ± 0.00	1.12 ± 0.09	1.12 ± 0.01	1.32 ± 0.01	0.67 ± 0.00
1174-901-12	3.35 ± 0.03	6.71 ± 0.03	1.51 ± 0.09	3.68 ± 0.02	2.75 ± 0.02	1.33 ± 0.01
Mitochondria	2.78 ± 0.03	5.52 ± 0.04	0.88 ± 0.09	1.19 ± 0.01	6.10 ± 0.05	0.50 ± 0.01
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	1.97 ± 0.02	0.68± 0.00	0.95 ± 0.09	2.69 ± 0.02	0.52 ± 0.00	0.80 ± 0.00
Luteibacter	1.41 ± 0.00	1.12 ± 0.01	4.33 ± 0.09	2.43 ± 0.01	0.53 ± 0.00	1.24 ± 0.01
Methylobacterium-Methylorubrum	1.22 ± 0.01	2.69 ± 0.01	1.92 ± 0.09	1.73 ± 0.01	2.11 ± 0.01	1.63 ± 0.01
Massilia	0.89 ± 0.00	1.93 ± 0.02	0.98 ± 0.09	0.88 ± 0.01	0.89 ± 0.00	1.07 ± 0.01
Terriglobus	0.87 ± 0.02	0.38 ± 0.00	0.22 ± 0.09	0.67 ± 0.00	0.19 ± 0.00	0.17 ± 0.00
Escherichia-Shigella	0.79 ± 0.02	1.12 ± 0.00	0.35 ± 0.09	0.26 ± 0.00	0.16 ± 0.00	0.56 ± 0.01
Burkholderia-Caballeronia-Paraburkholderia	0.71 ± 0.01	0.52 ± 0.00	1.33 ± 0.09	1.49 ± 0.01	0.29 ± 0.00	0.36 ± 0.00

Enterococcus	0.69 ± 0.02	0.00 ± 0.00	0.00 ± 0.09	0.03 ± 0.00	0.04 ± 0.00	0.06 ± 0.00
Clostridium_sensu_stricto_1	0.41 ± 0.00	0.02 ± 0.00	0.20 ± 0.09	0.39 ± 0.00	0.04 ± 0.00	2.84 ± 0.06
Novosphingobium	0.32 ± 0.00	0.09 ± 0.00	0.71 ± 0.09	1.29 ± 0.02	0.38 ± 0.00	0.84 ± 0.00
Acinetobacter	0.26 ± 0.00	3.31 ± 0.06	0.94 ± 0.09	0.27 ± 0.00	0.73 ± 0.01	0.19 ± 0.00
Kosakonia	0.26 ± 0.00	0.16 ± 0.00	0.77 ± 0.09	0.53 ± 0.00	0.40 ± 0.00	1.07 ± 0.01
Jatrophihabitans	0.21 ± 0.00	0.58 ± 0.01	0.60 ± 0.09	1.75 ± 0.02	0.90 ± 0.01	0.43 ± 0.01
Pseudarthrobacter	0.20 ± 0.00	0.00 ± 0.00	0.00 ± 0.09	0.25 ± 0.00	1.98 ± 0.02	$<10^{-4}$ ± 0.00
Microbacterium	0.19 ± 0.00	0.06 ± 0.00	0.05 ± 0.09	0.76 ± 0.01	0.30 ± 0.00	0.46 ± 0.01
Bacillus	0.16 ± 0.00	0.09 ± 0.00	0.32 ± 0.09	0.32 ± 0.00	1.47 ± 0.01	0.31 ± 0.00
Nocardioides	0.11 ± 0.00	0.27 ± 0.01	0.18 ± 0.09	0.71 ± 0.01	1.13 ± 0.01	0.08 ± 0.00
Romboutsia	0.10 ± 0.00	0.11 ± 0.00	0.11 ± 0.09	0.31 ± 0.00	0.02 ± 0.00	1.72 ± 0.03
Serratia	0.09 ± 0.00	0.20 ± 0.00	6.02 ± 0.09	0.28 ± 0.00	0.45 ± 0.00	0.90 ± 0.01
Mycobacterium	0.08 ± 0.00	0.13 ± 0.00	0.26 ± 0.09	0.26 ± 0.00	0.16 ± 0.00	1.19 ± 0.02
Sanguibacter	0.06 ± 0.00	0.00 ± 0.00	0.00 ± 0.09	0.10 ± 0.00	0.00 ± 0.00	0.65 ± 0.01

[a] Data were calculated from the five replicates from each stump treatment with standard error.

Table A3. *List of bacterial phyla analysed in the correspondence analysis of bacterial community (Figure 6). Others are sequences that remained unidentified at the phylum level.*

Phylum	code
Proteobacteria	1
Actinobacteriota	2
Cyanobacteria	3
Firmicutes	4
Acidobacteriota	5
Bacteroidota	6
Chloroflexi	7
Myxococcota	8
Verrucomicrobiota	9
Gemmatimonadota	10
Planctomycetota	11
Armatimonadota	12
WPS-2	13
Patescibacteria	14
Aenigmarchaeota	15
Desulfobacterota	16
Nitrospirota	17
Bdellovibrionota	18
Methylomirabilota	19
Nanoarchaeota	20
Others	21

Figure A1. *Histograms of associations depicted in the correspondence analysis of bacterial communities (Figure 6). Association (%) of stump treatments to dimension 1 (A) and 2 (B). Association (%) of phyla to dimension 1 (C) and 2 (D). The numbered phyla are listed in Table A3***.** *Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

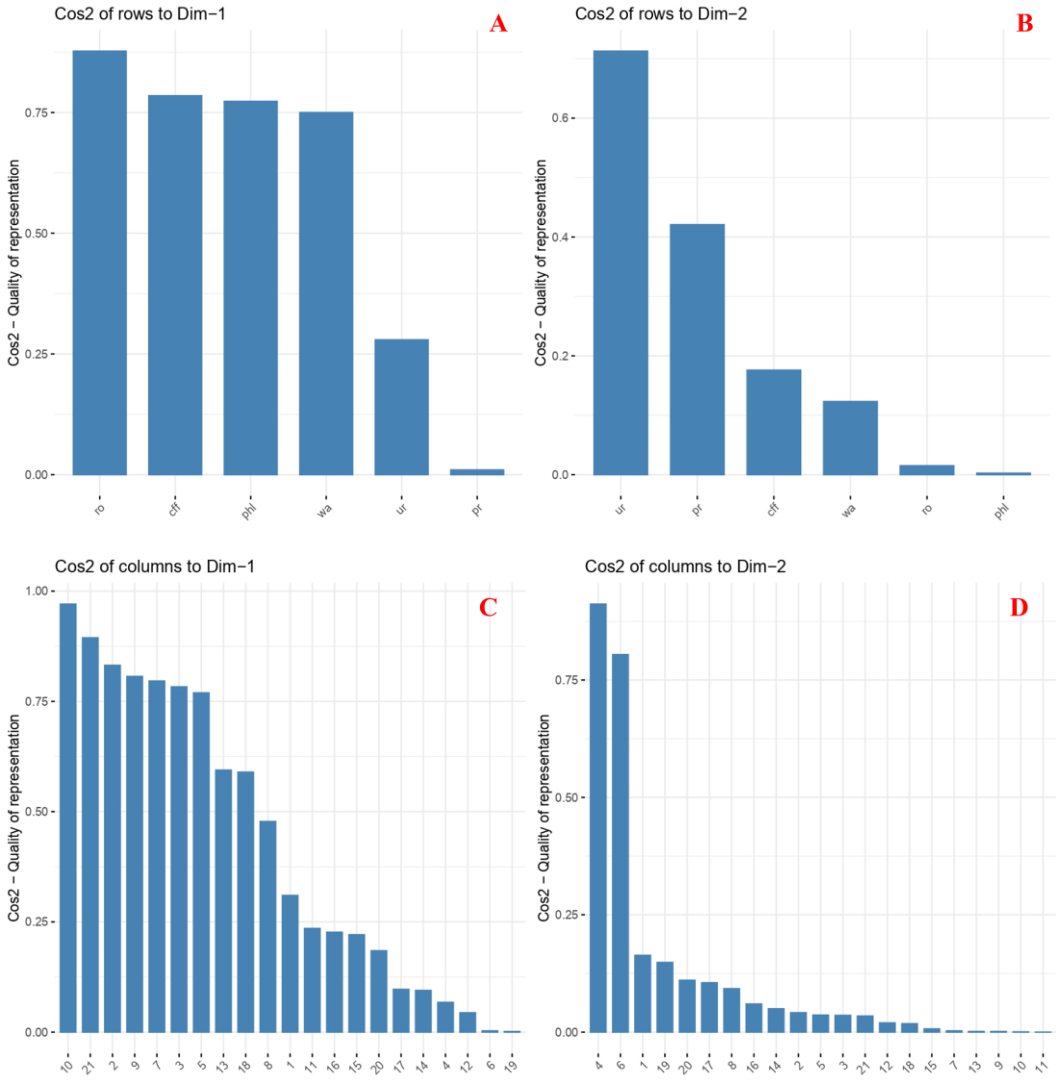


Table A4. *Number of PE reads before (raw) and after (effective) data cleaning, effectiveness, alpha-metrics (Simpson, Shannon, Chao1 and Good's coverage) for the fungal ITS2 region in control and treated stumps of* P. pinea*. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Samples groups	Replicate	Number of raw PE reads	Number of effective PE reads	Effective (%)	Simpson	Shannon	Chao1	Good's coverage
wa	1	72,155	63,313	88	0.934	4.828	217	1.000
	2	63,370	53,504	84	0.918	4.443	198	1.000
	3	68,805	58,357	85	0.938	4.894	223	1.000
	4	79,306	65,505	83	0.916	4.680	258	1.000
	5	71,067	60,086	85	0.916	4.536	160	1.000
pr	1	72,319	60,874	84	0.829	3.917	201	1.000
	2	70,691	58,000	82	0.884	4.462	252	1.000
	3	74,890	63,513	85	0.903	4.384	208	1.000
	4	69,069	58,130	84	0.932	4.751	175	1.000
	5	73,650	63,311	86	0.831	3.943	229	1.000
cff	1	69,757	60,517	87	0.942	5.282	259	1.000
	2	60,876	53,263	87	0.846	4.428	228	1.000
	3	73,162	62,544	85	0.908	4.426	199	1.000
	4	67,050	57,009	85	0.913	4.707	225	1.000
	5	64,356	54,888	85	0.950	5.249	227	1.000
phl	1	64,206	53,574	83	0.885	4.427	252	1.000
	2	77,174	64,285	83	0.877	4.267	213	1.000
	3	77,660	66,870	86	0.917	4.684	262	1.000
	4	74,490	64,805	87	0.930	4.722	244	1.000
	5	74,189	62,859	85	0.932	4.856	241	1.000
ro	1	72,404	61,878	85	0.765	3.212	131	1.000
	2	64,211	54,500	85	0.781	3.338	133	1.000
	3	60,235	50,905	85	0.900	4.229	138	1.000
	4	66,982	57,987	87	0.634	2.474	83	1.000
	5	77,359	65,697	85	0.746	3.113	111	1.000
ur	1	70,319	61,079	87	0.822	3.865	175	1.000
	2	75,260	65,671	87	0.819	4.036	190	1.000
	3	75,559	66,657	88	0.817	3.682	174	1.000
	4	78,079	67,145	86	0.687	2.862	131	1.000
	5	75,933	65,375	86	0.887	4.180	198	1.000

Table A5. *Relative abundance (%) of 30 most abundant fungal genera found among the six sample groups (i.e. stump treatments). Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*

Genus	Mean relative abundance ± SE (%)[a]					
	wa	pr	cff	phl	ro	ur
Aureobasidium	10.23 ± 0.10	18.12 ± 0.00	9.18 ± 0.10	16.6 ± 0.00	19.73 ± 0.10	10.32 ± 0.10
Aequabiliella	10.11 ± 0.00	8.92 ± 0.00	8.43 ± 0.10	18.74 ± 0.10	8.20 ± 0.10	2.25 ± 0.00
Diplodia	9.67 ± 0.10	1.13 ± 0.00	0.96 ± 0.00	1.55 ± 0.00	2.08 ± 0.00	1.32 ± 0.00
Hormonema	9.36 ± 0.10	25.05 ± 0.10	7.98 ± 0.10	14.74 ± 0.10	27.88 ± 0.20	2.65 ± 0.00
Cladosporium	9.29 ± 0.00	6.08 ± 0.00	8.63 ± 0.00	5.45 ± 0.00	3.17 ± 0.00	10.33 ± 0.10
Vishniacozyma	7.43 ± 0.10	0.93 ± 0.00	1.34 ± 0.00	0.52 ± 0.00	1.11 ± 0.00	1.41 ± 0.00
Curvibasidium	6.41 ± 0.00	3.45 ± 0.00	1.78 ± 0.00	1.60 ± 0.00	2.53 ± 0.00	0.99 ± 0.00
Heterotruncatella	5.79 ± 0.00	1.38 ± 0.00	4.01 ± 0.00	2.82 ± 0.00	0.86 ± 0.00	0.85 ± 0.00
Kwoniella	5.72 ± 0.00	7.60 ± 0.10	6.27 ± 0.00	2.80 ± 0.00	3.20 ± 0.00	4.53 ± 0.00
Mycosphaerella	5.03 ± 0.00	2.56 ± 0.00	3.14 ± 0.00	3.29 ± 0.00	4.30 ± 0.10	7.82 ± 0.00
Neocatenulostroma	2.45 ± 0.00	2.46 ± 0.00	1.43 ± 0.00	3.67 ± 0.00	2.97 ± 0.00	2.20 ± 0.00
Meyerozyma	1.82 ± 0.00	0.04 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.08 ± 0.00	0.04 ± 0.00
Leucosporidium	1.76 ± 0.00	0.12 ± 0.00	0.20 ± 0.00	0.10 ± 0.00	0.03 ± 0.00	0.58 ± 0.00
Penicillium	1.11 ± 0.00	0.21 ± 0.00	0.93 ± 0.00	0.66 ± 0.00	0.08 ± 0.00	1.03 ± 0.00
Fonsecazyma	0.95 ± 0.00	0.61 ± 0.00	0.36 ± 0.00	0.51 ± 0.00	0.24 ± 0.00	0.21 ± 0.00
Alternaria	0.91 ± 0.00	0.64 ± 0.00	1.70 ± 0.00	0.51 ± 0.00	0.49 ± 0.00	2.18 ± 0.00
Gibberella	0.70 ± 0.00	0.74 ± 0.00	8.94 ± 0.20	0.87 ± 0.00	12.03 ± 0.30	35.13 ± 0.20
Phlebiopsis	0.67 ± 0.00	0.05 ± 0.00	0.88 ± 0.00	0.77 ± 0.00	2.72 ± 0.10	0.04 ± 0.00
Peniophora	0.55 ± 0.00	5.89 ± 0.10	20.72 ± 0.20	8.33 ± 0.10	0.17 ± 0.00	0.28 ± 0.00

Filobasidium	0.38 ± 0.00	2.99 ± 0.00	0.30 ± 0.00	0.40 ± 0.00	1.34 ± 0.00	2.04 ± 0.00
Fusarium	0.33 ± 0.00	0.18 ± 0.00	0.52 ± 0.00	0.20 ± 0.00	1.41 ± 0.00	5.18 ± 0.00
Pragmopora	0.25 ± 0.00	0.22 ± 0.00	0.18 ± 0.00	1.10 ± 0.00	0.19 ± 0.00	0.11 ± 0.00
Neocucurbitaria	0.23 ± 0.00	0.13 ± 0.00	1.36 ± 0.00	0.13 ± 0.00	0.03 ± 0.00	0.15 ± 0.00
Paraconiothyrium	0.23 ± 0.00	0.13 ± 0.00	0.23 ± 0.00	0.77 ± 0.00	0.09 ± 0.00	0.37 ± 0.00
Cystobasidium	0.23 ± 0.00	0.32 ± 0.00	0.39 ± 0.00	0.08 ± 0.00	0.16 ± 0.00	1.05 ± 0.00
Lachnellula	0.19 ± 0.00	0.26 ± 0.00	0.58 ± 0.00	1.94 ± 0.00	0.07 ± 0.00	0.21 ± 0.00
Stereum	0.06 ± 0.00	0.17 ± 0.00	0.25 ± 0.00	1.04 ± 0.00	0.30 ± 0.00	0.03 ± 0.00
Heterobasidion	0.04 ± 0.00	$<10^{-4}$ ± 0.00	$<10^{-4}$ ± 0.00	$<10^{-4}$ ± 0.00	0.37 ± 0.00	$<10^{-4}$ ± 0.00
Saccharomyces	$<10^{-4}$ ± 0.00	1.10 ± 0.00	0.21 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Naganishia	$<10^{-4}$ ± 0.00	$<10^{-4}$ ± 0.00	$<10^{-4}$ ± 0.00	0.00 ± 0.00	0.40 ± 0.00	0.00 ± 0.00

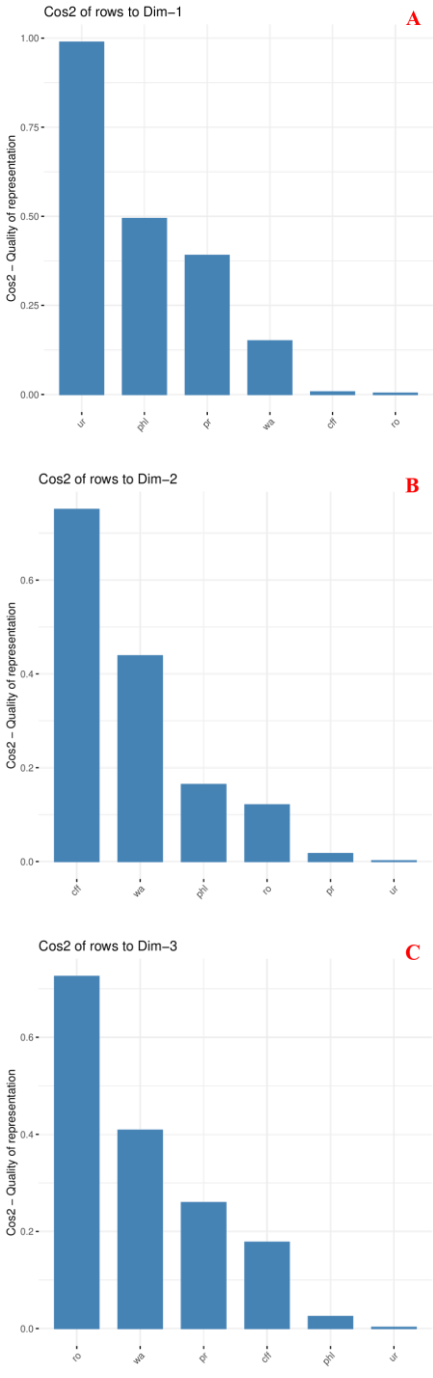
[a] Data were calculated from the five replicates from each stump treatment with standard error.

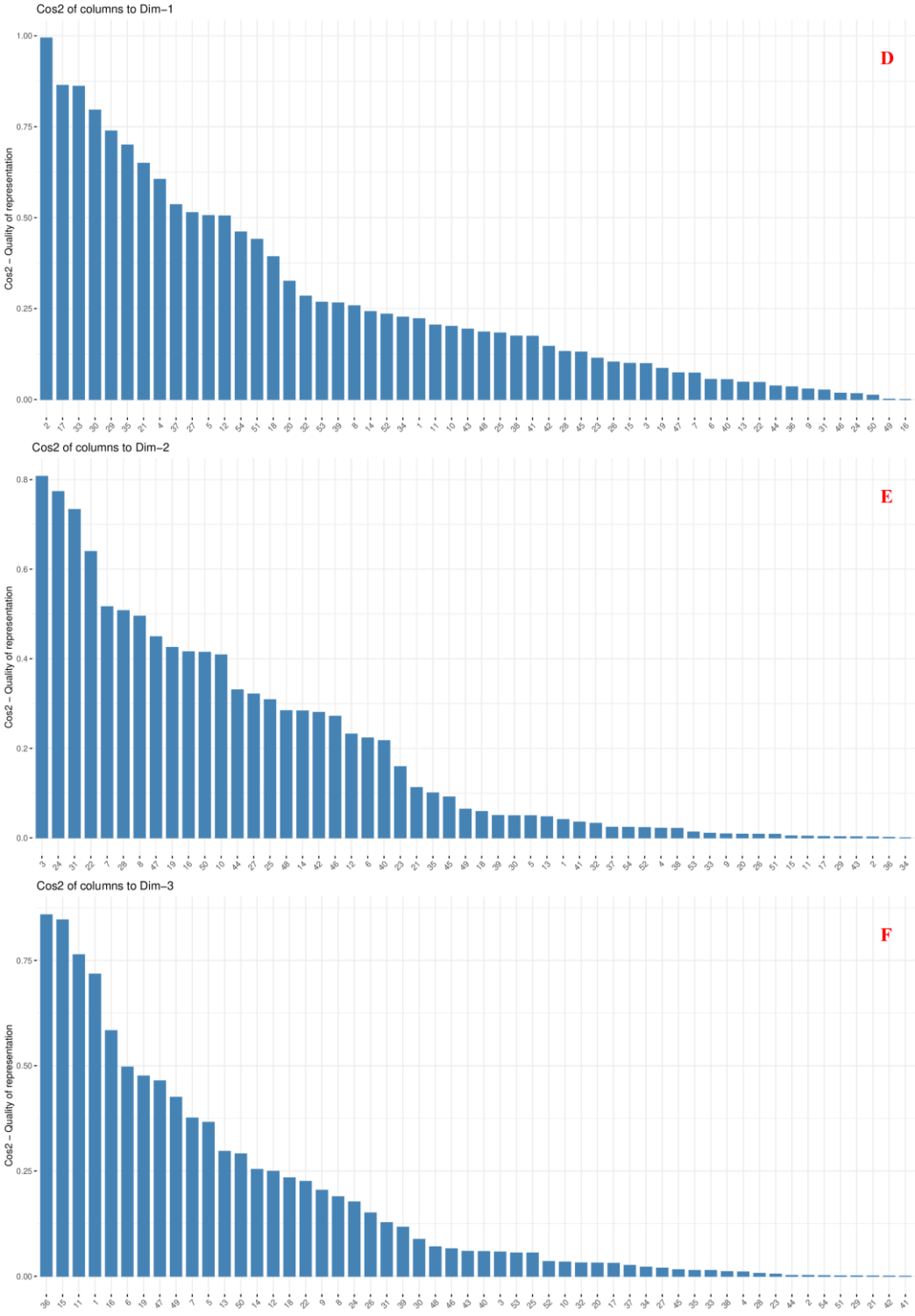
Table A6. *List of fungal orders analysed in the correspondence analysis of fungal community (Figure 11). Others are sequences that remained unidentified at the order level.*

Order	code
Dothideales	1
Hypocreales	2
Russulales	3
Phaeomoniellales	4
Capnodiales	5
Tremellales	6
Botryosphaeriales	7
Microbotryomycetes_ord_Incertae_sedis	8
Polyporales	9
Helotiales	10
Xylariales	11
Pleosporales	12
Filobasidiales	13
Saccharomycetales	14
Eurotiales	15
Leucosporidiales	16
Cystobasidiales	17
Lecanoromycetes_ord_Incertae_sedis	18
Cystofilobasidiales	19
Coryneliales	20
Microstromatales	21
Calosphaeriales	22
Ostropales	23
Togniniales	24
Sporidiobolales	25
Taphrinales	26
Chaetothyriales	27
Cystobasidiomycetes_ord_Incertae_sedis	28
Agaricostilbales	29
Agaricales	30
Cantharellales	31
Venturiales	32
Ophiostomatales	33
Tubeufiales	34
Lichenostigmatales	35
Diaporthales	36

Symbiotaphrinales	37
Erythrobasidiales	38
Mytilinidiales	39
Chaetosphaeriales	40
Ustilaginales	41
Mucorales	42
Sordariales	43
Coniochaetales	44
Exobasidiales	45
Corticiales	46
Kriegeriales	47
Myriangiales	48
Hymenochaetales	49
Thelebolales	50
Dothideomycetes_ord_Incertae_sedis	51
Phacidiales	52
Rhytismatales	53
Others	54

Figure A2. *Histograms of associations depicted in the correspondence analysis of fungal community (Figure 11). Association (%) of stump treatments to dimension 1 (A), 2 (B) and 3 (C). Association (%) of orders to dimension 1 (D), 2 (E), 3 (F). The numbered orders are listed in Table A6. Samples groups abbreviations: wa-water; pr-Proradix®; cff-cell-free filtrate of* P. protegens *(strain DSMZ 13134); phl-*P. gigantea *(strain MUT 6212); ro-Rotstop®; and ur-urea.*




Cos2 of columns to Dim−1
Cos2 − Quality of representation
D
Cos2 of columns to Dim−2
Cos2 − Quality of representation
E
Cos2 of columns to Dim−3
Cos2 − Quality of representation
F

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