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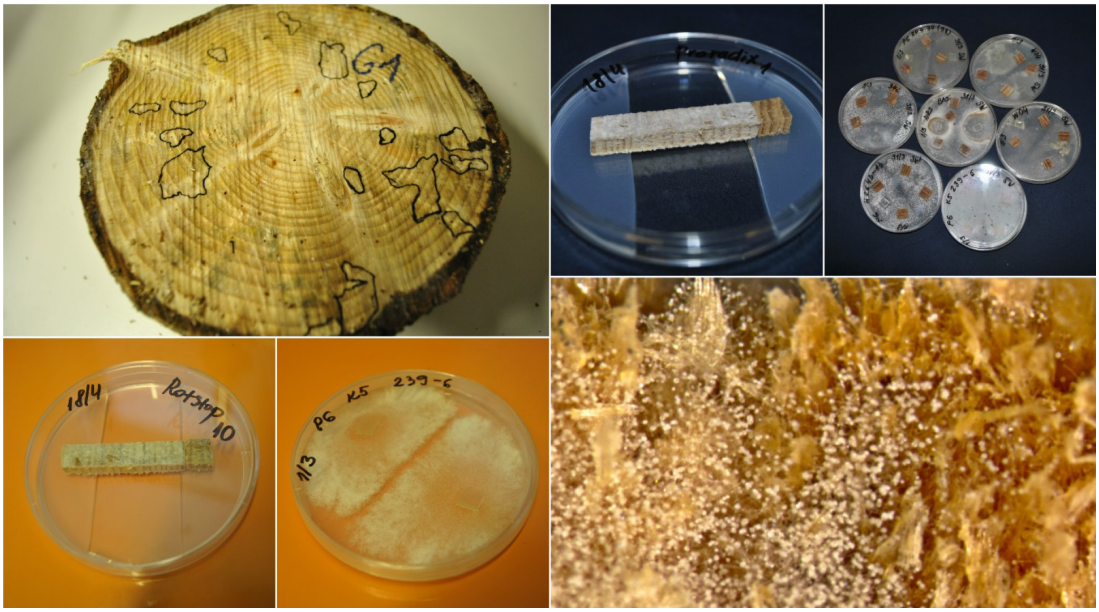

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Swedish University of Agricultural Sciences
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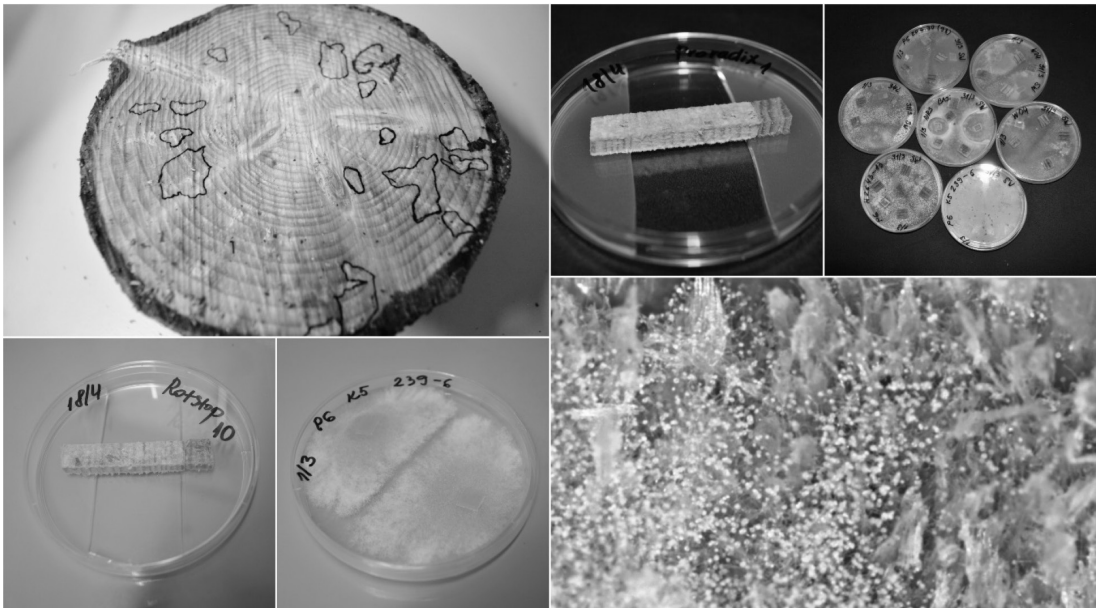

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ABSTRACT

Root rot caused by the fungal pathogen *Heterobasidion* spp. is one of the most spread and devastating conifer diseases in the Northern Hemisphere. There is increasing interest from the forestry sector in new biological agents and means of control for *Heterobasidion* spp., as root rot becomes an economically more important issue. More consistent and effective genotypes of *P. gigantea* isolates would benefit the forest industry as well as reduce ecological risks connected with using just one isolate for treatment of conifer stumps locally. Therefore, the aim of this study was to examine new biological protection agents on Norway spruce wood that could potentially be used commercially for stump protection against *Heterobasidion* spp.

The competitive ability against *H. parviporum* of ten different isolates of *P. gigantea*, including Rotstop S isolate and biological treatment Ox 24, containing a *Pseudomonas* spp. bacteria, were tested in laboratory conditions. In addition a small field study comparing Ox 24 and Rotstop S was evaluated.

Results of the current study revealed that *P. gigantea* isolate S2384_2_VI is significantly (p<0,05) superior to other 9 isolates by means of average growth rate per day on top of wood heavily infected by *H. parviporum.* Rotstop S together with PG342 isolate had the fastest performance of growth in the first two days of juxtaposition with *H. parviporum*. Wood colonized by *Pseudomonas* spp. bacteria *in vitro* is resistant to *H. parviporum* even after 25 days of close contact.

Breeding between the best isolates found in this study could be introduced in order to produce *P. gigantea* progenies that have higher antagonistic and biological control ability. *Pseudomonas* spp. showed remarkable results in resistance for *H. parviporum in vitro* and demonstrated apparent ability to protect stumps from primary infection with *Heterobasidion* spp. when compared to other treatment methods, yet it was only a pilot study of small scale. Therefore *Pseudomonas* spp. bacteria should be monitored in full scale field experiments before it can be considered as a potential alternative to *P. gigantea* in terms of biological stump control.

Keywords: *Heterobasidion* spp., *Phlebiopsis gigantea*, *Pseudomonas* spp., Rotstop, biological control.

Contents

1. INTRODUCTION

Root rot caused by the fungal pathogen *Heterobasidion* spp. is one of the most spread and devastating conifer diseases in the Northern Hemisphere (Hodges, 1969; Bendz-Hellgren *et al.*, 1998; Garbelotto & Gonthier, 2013). Economic losses associated with *Heterobasidion* spp. in Europe accounts for approximately 800 million € annually (Woodward *et al*., 1998). In Sweden incidence of infection doubled since 1970s and annual losses make up about 80 million € (Bendz-Hellgren *et al.*, 1998; Woodward *et al.*, 1998; Thor, 2005; Olsson, 2010). Infection rate of mature Norway spruce (*Picea abies* (L.) Karst.) in southern Scandinavia varies between 10 – 20% (Bendz-Hellgren *et al.*, 1998; Woodward *et al.*, 1998), but in Baltic countries is reported to be 20 – 50% and can reach 80% in heavily infected stands (Vasiliauskas, 2001; Korhonen & Holdenrieder, 2005; Arhipova *et al.*, 2011).

Heterobasidion spp. consists of five species that can be separated by geographical distribution and host preferences (Dalman *et al.*, 2010). In Sweden there are two *Heterobasidion* species that are naturally present in forest stands – *Heterobasidion annosum* (Fr.) Bref. s.s. and *Heterobasidion parviporum* (Niemelä & Korhonen, 1998). *H. annosum* is mainly present in the southern part of Sweden and preferably infects pines, but can also be found in spruce, birch and even alder wood, whereas *H. parviporum* is spread in the entire country and mainly affects Norway spruce trees (Hanso *et al.*, 1994; Korhonen *et al.*, 1998).

Root rot infection spreads in two ways: 1) primary by airborne basidiospores that are released from fruiting body when the average daily temperature is higher than +5 °C (Yde-Anersen, 1962; Kallio, 1970; Brandtberg *et al.*, 1996); 2) secondary by vegetative spread of mycelia through linked roots of trees (Rishbeth, 1951). Spores can infect only fresh wood surfaces – freshly cut stumps, wounds in stem and roots (Rishbeth, 1951), but as they travel hundreds of kilometers via wind dispersal, range of infection is very extensive (Korhonen & Stenlid, 1998). The average growth rate of *Heterobasidion* spp. in living spruce roots varies from 9,5 – 25 cm in a year (Pettersson *et al.*, 2003) and the fungus can remain vital in root systems for 62 years (Greig, 1976).

There are a number of methods used in practice to control the spread of *Heterobasidion* spp. in commercial forests: stump removal from stand, stump treatment with chemical and biological substances, forest regeneration with more resistant tree species, establishment of mixed stands etc. (Hodges, 1969; Greig, 1984; Kliejunas, 1989; Pratt, 1996; Thor & Stenlid, 2005; Oliva *et al.*, 2008). But so far stump treatment with chemical and biological substances is proven to be the most effective mean of protection, because it decreases the risk of infection up to 99% (Nicolotti & Gonthier, 2005; Thor & Stenlid, 2005).

Both chemical and biological substances for stump treatment show good performance of effectiveness (Thor, 1997; Pratt *et al.*, 1998; Johansson *et al.*, 2002; Oliva *et al.*, 2008), but due to possible environmental side effects of chemical substances, public is more supportive for usage of biological treatments (Thor *et al.*, 1997; Pratt *et al.*, 1998). In the last fifty years many fungal species have been tested for antagonism against *Heterobasidion* spp. (*Phlebiopsis gigantea*, *Trametes versicolor*, *Bjerkandera adusta*, *Fomitopsis pinicola*, *Hypholoma* spp., *Trichoderma* spp. and others) in order to find new biological pesticide

(Holdenrieder & Greig, 1998; Berglund *et al.*, 2005), yet currently only *Phlebiopsis gigantea* (Fr.) Jül. is used commercially as biological treatment for conifer stump protection (Pratt *et al.*, 2000; Drenkhan *et al.*, 2008; Ek, 2011; Kenigsvalde *et al.*, 2011).

P. gigantea is a strong antagonist that is able to limit development of *Heterobasidion* spp. in fresh stumps by outcompeting that fungus in the growth rate. These qualities of *P. gigantea* were found already in 1952 by english scientist Rishbeth (Rishbeth, 1952). In 1987, finnish scientists isolated *P. gigantea* from spruce that was effective in treatment of spruce stumps against *Heterobasidion* spp. Four years later this isolate got commercialized and named Rotstop® (Korhonen *et al.*, 1994), but in year 2004 a new product called Rostop® S which contained Swedish isolate became available on Swedish market (Berglund *et al*., 2005). At this moment Rotstop® S is the most popular biological treatment containing spores of *P. gigantea* used in the Nordic countries (Berglund & Rönnberg, 2004). Every year around 35 000 ha of forest is subjected for treatment with Rotstop® S isolate during thinnings in Sweden (Thor, 2003), which is estimated to be half of needed amount (Samuelsson & Örlander, 2001; Berglund, 2005).

Biological control methods are considered to be environmentally friendly, however there are risks associated with continuous use of the same treatment. Extensive distribution of one genotype can lower the rate of natural *P. gigantea* population and create a uniform population (Korhonen & Kauppila, 1987; Vainio *et al.*, 1998). Isolates that are now used in production of Rotstop® showed to be one of the most effective compared to other *P. gigantea* isoalates, however there were found local isolates that showed even higher efficacy (Berglund, 2005; Sun *et al.*, 2009b; Kenigsvalde *et al.*, 2015). And still there are studies that has shown quite low control efficacy of biological *P. gigantea* treatment (Berglund & Rönnberg, 2004; Berglund, 2005; Gunulf *et al.*, 2012). This is the reason why more research needs to be done to find new agents and means of stump protection against *Heterobasidion* spp.

There is an increasing interest from the forestry sector to find new agents and means of control for *Heterobasidion* spp., as root rot becomes more devastating and economically important issue. For more than 25 years *P. gigantea* has been used in coniferous forests in Northern Europe as biological treatment against *Heterobasidion* spp., but research showed that efficacy of this treatment varies a lot. More consistent and effective genotypes of *P. gigantea* isolates would benefit the forest industry as well as reduce ecological risks connected with using just one isolate for treatment of conifer stumps locally. Therefore it is of great importance to conduct sound laboratory analyses on new possible biocontrol agents that would be followed by field investigation. The biological treatment agent Ox 24 has been suggested as possible agent for stump protection against root rot. Normally it is used in agriculture for vegetable protection against different diseases like *Phytophthora* spp., *Rhizoctonia* spp., *Erwinia* spp. and others. The Ox 24 agent contains *Pseudomonas* spp. bacteria that provides protection of roots and an optimal water and nutrient uptake through various mechanisms of action. Application of this treatment activates resistance mechanisms of a plant and helps in competing with soil-borne pathogens, which could potentially work similarly also in wood cells, when competing with *Heterobasidion* spp. (Agneta Färlin, personal communication, 2016).

The aim of this study was, therefore, to examine new biological protection agents on Norway spruce wood samples that could potentially be used commercially for stump protection against *Heterobasidion* spp. and compare them with the existing treatment, Rotstop® S Gel, in order to make sound conclusions about their efficiency.

2. MATERIALS AND METHODS

This master thesis was carried out at the Southern Swedish Forest Research Centre in collaboration with Organox AB. The practical part of the work was done in both laboratory and field conditions starting from February till May of the year 2016.

2.1. In vitro experimental setup

Experimental setup in laboratory conditions was conducted in 3 stages: starting with cultivation of respective fungal strains and bacteria on nutrient agar, continuing with colonization of wood with chosen biological agents and *H. parviporum* and ending with examination of interactions between *H. parviporum* and the corresponding fungal strain of *P. gigantea* and *Pseudomonas* spp.

2.1.1. Biological agents subjected for study

Norway spruce sapwood samples were used in all experiments conducted in this study. *H. parviporum* strain Rb175 (Stenlid, 1987) was chosen from *Heterobasidion* species for infecting the wood. In order to compare protection abilities of antagonist fungus, 10 different isolates of *P. gigantea* (Table 1) were selected, including the Rotstop® S Gel isolate that is used for stump treatment in Sweden. Furthermore, the biological product – Ox 24, that is mainly used for agricultural purposes, was tested as a possible protective agent against *H. parviporum.*

Table 1. Fungal isolates of *P. gigantea* used for experiment

Isolate	Isolation date	Species	Location of isolation
S2384_2_VI	No details	*P. sylvestris* or *P. nigra* subsp. *Laricio* (mixed site)	Swaffham, England
PGM2643-13	No details	No details	England
PG16	No details	No details	England
PG28	No details	No details	England
PG3	No details	No details	England
PG342	1985	*Larix* spp.	Scotland
PGWO4	2000	*Pinus nigra* subsp. *Laricio*	England
PGK5239-6	2007	*Pinus nigra* subsp. *Laricio*	Thetford Forest, England
PGK5191-7	2007	*Pinus nigra* subsp. *Laricio*	Thetford Forest, England
Rotstop S	1997	*Picea abies*	Uppsala Råberg, Sweden

2.1.2. In vitro cultivation

In order to cultivate needed fungal strains, Hagem agar (Table 2) was firstly prepared, autoclaved and filled in Petri dishes. In every Petri dish approximately 12 ml of Hagem agar was filled, so it covered all the surface of dish. Filling of dishes was conducted under a laminar flow hood to avoid contaminations. When Hagem agar became solid, application of respective fungal strains could start. Inoculation of fungal strains on Petri dishes was done with a set of metal tools that were sterilized in flame between each isolate. The *H. parviporum* strain, Rb175, was used for cultivating the pathogen and 10 different strains of *P. gigantea* were cultivated as protective agents. Additionally the biological product Ox 24, containing *Pseudomonas* spp. bacteria, was applied on Petri dishes as powder, and left for colonization. 10 replicates for every biological agent were subjected for this experiment and cultivated in room temperature (~20 °C) for 2 weeks (Figure 1).

Table 2. Recipe for making 1 l of Hagem agar (ph = 5,5)

Components	Amount
Glucose	5,0 g
NH_4NO_3	0,5 g
KH_2PO_4	0,5 g
$MgSO_4$*7aq	0,5 g
Malt extract	5,0 g
Agar	20,0 g
Distilled water	1 l

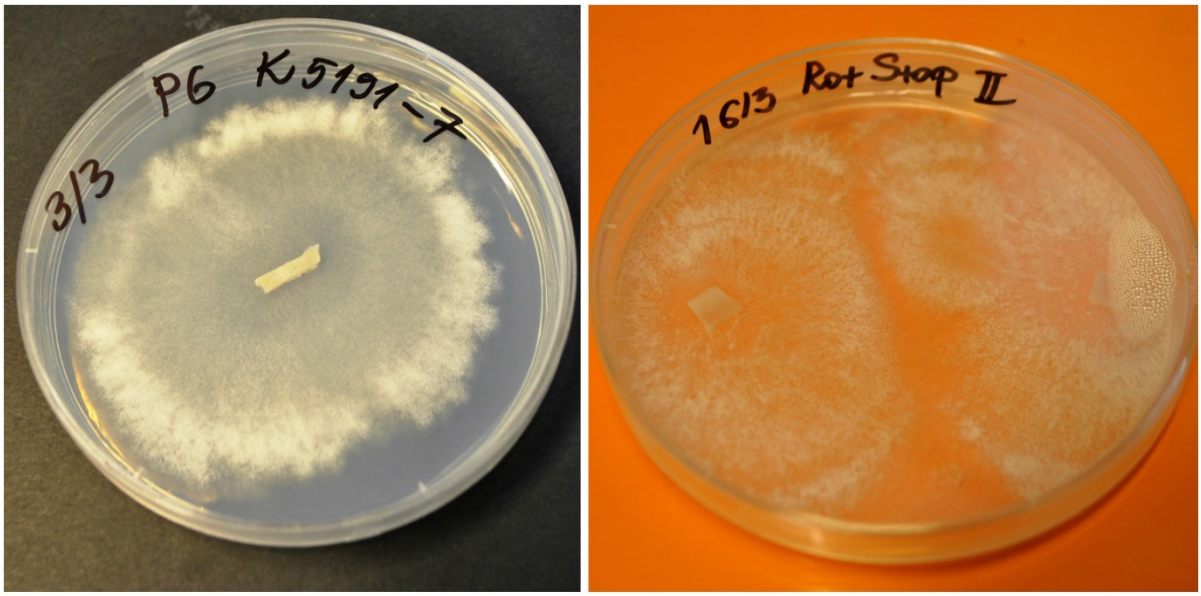

Figure 1. *P. gigantea* isolates after 2 weeks of growth on Hagem agar.

2.1.3. Wood inoculation

In order to mimic a natural substrate, we used fresh sapwood of spruce (felled 3 weeks prior to the start of experiment). From the wooden plank with thickness of 1 cm, there were cut short and long wood blocks. Long blocks were 5 cm long, short ones – 1 cm. After that, wood blocks were placed in aluminum foil and sterilized twice in an autoclave at 121 °C in liquid regime (Samils *et al.*, 2008). The blocks were then put on Petri dishes under a laminar flow hood to avoid contaminations. Long wood blocks were put on Petri dishes previously colonized by *H. parviporum* strain Rb175 and left for inoculation for 4 weeks (Figure 2). Short wood blocks were placed on Petri dishes previously colonized by 10 different strains of *P. gigantea* or *Pseudomonas* spp. bacteria and left for 2 weeks until thoroughly colonized (Figure 3). Long wood blocks were subjected for longer inoculation, because of the bigger size of inoculum and different rates of growth between *H. parviporum* and *P. gigantea*.

Figure 2. Long wood block application on Petri dishes fully colonized by *H. parviporum*.

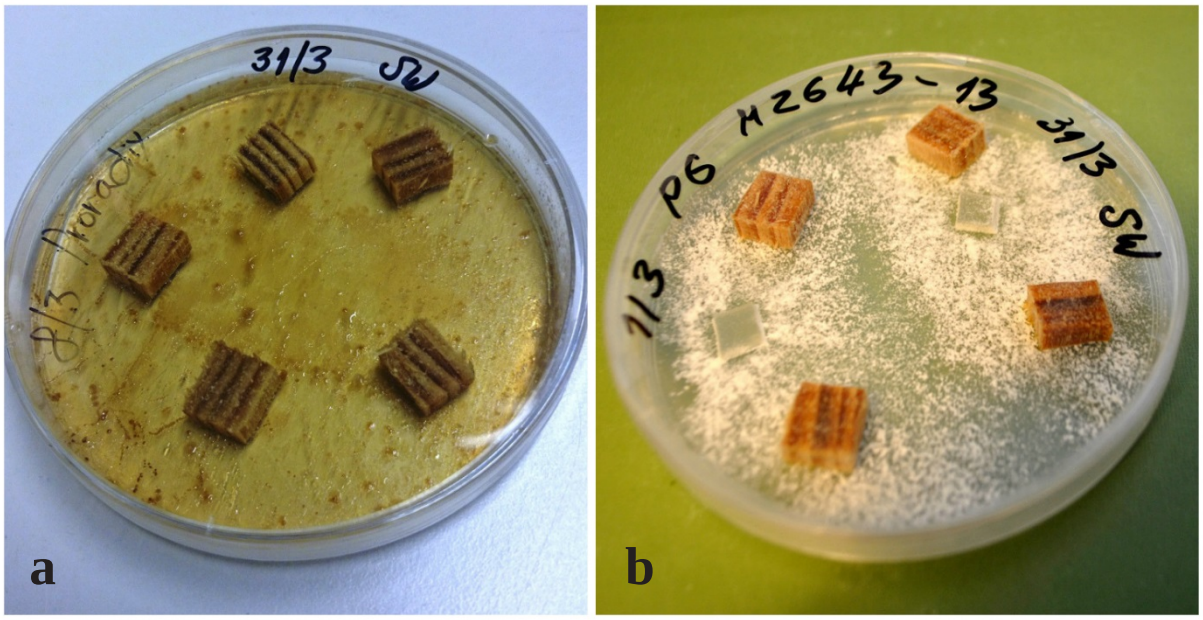

Figure 3. a) Small wood block colonization by *Pseudomonas* spp. bacteria; b) Small wood block colonization by *P. gigantea* isolate.

2.1.4. Juxtaposition of precolonized wood blocks

In order to observe interactions between our selected biocontrol agents and *H. parviporum*, fully colonized short and long wood blocks were juxtaposed on Petri dishes containing water agar (20 g of agar on 1 l of distilled water). These Petri dishes were prepared in a special way, so that central part was cut out, in order to promote mycelial growth in wood rather than on the agar surface. In each Petri dish firstly long wood block colonized by *H. parviporum* was placed over the central gap and then aligned with small wood block colonized by corresponding biocontrol agent (Figure 4). It was important to ensure that wood blocks were connected tight and their edges were in contact. This was set as a starting point for interactions between the biocontrol agents and *H. parviporum*. For each *P. gigantea* isolate and *Pseudomonas* spp. we used 10 replicates. Additionally 2 control Petri dishes were prepared for each agent, in order to see how well *H. parviporum* and respective control agents could colonize sterile wood. Petri dishes were placed in a ventilated cabinet at room temperature (~20 °C).

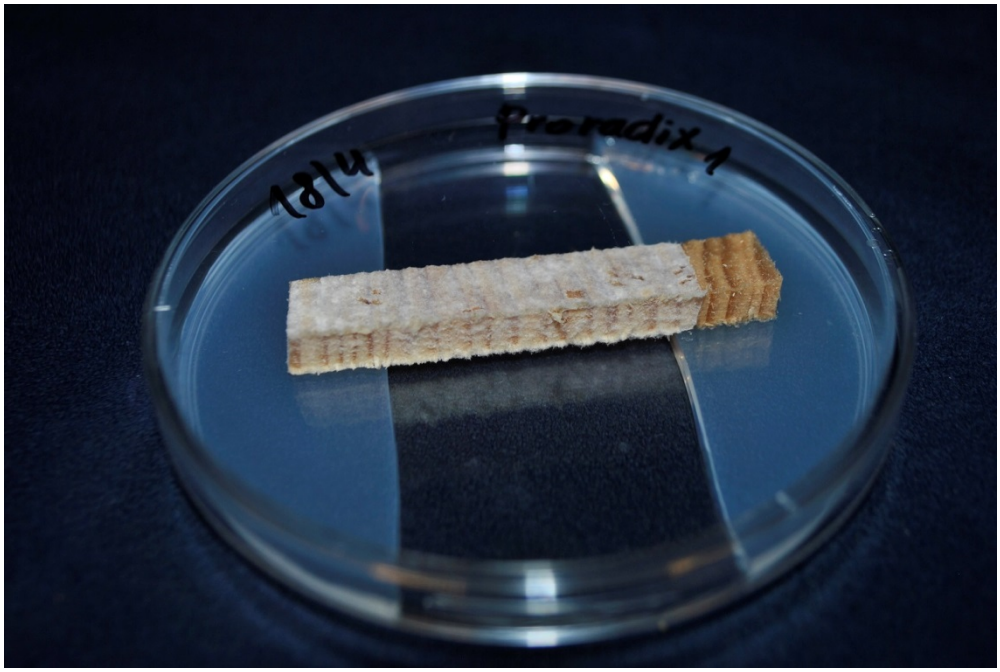

Figure 4. Long wood block fully colonized by *H. parviporum* aligned with a short wood block colonized by *Pseudomonas* spp.

2.1.5. Biological agents' growth rates and competitiveness against H. parviporum

Petri dishes containing wood blocks were subjected to examination 4 times. The first time the interaction between *H. parviporum* and biocontrol agent was examined was in the 2nd day after juxtaposition, then it was reexamined in the 4th, 7th and finally 9th day. Each examination series was conducted with the help of a dissecting microscope. *H. parviporum* and *P. gigantea* strains were identified by their rather different morphological features. *P. gigantea* hyphae is more fragile than *H. parviporum*. Another feature that helped in recognition of *H. parviporum* hyphae was the characteristic conidiophores. *P. gigantea* was easily visible by typical tufts it made on top of the wood colonized by *H. parviporum* (Figure 5). In these tufts there were no conidiophores, which is direct indicator that it is *P. gigantea*. The growth rate of *P. gigantea* strains was examined by measuring the furthest hyphae growing on top of *H. parviporum.* The length from the edge of long wood block till the endpoint of hyphae was measured with ruler and recorded (Figure 5). Then in every following examination, growth rate development of *P. gigantea* mycelia was observed and recorded. In addition the ingrowth of *H. parviporum* on the wood blocks inoculated with the corresponding biocontrol agent was also examined in order to evaluate the ability of the control agent to resist a pathogen.

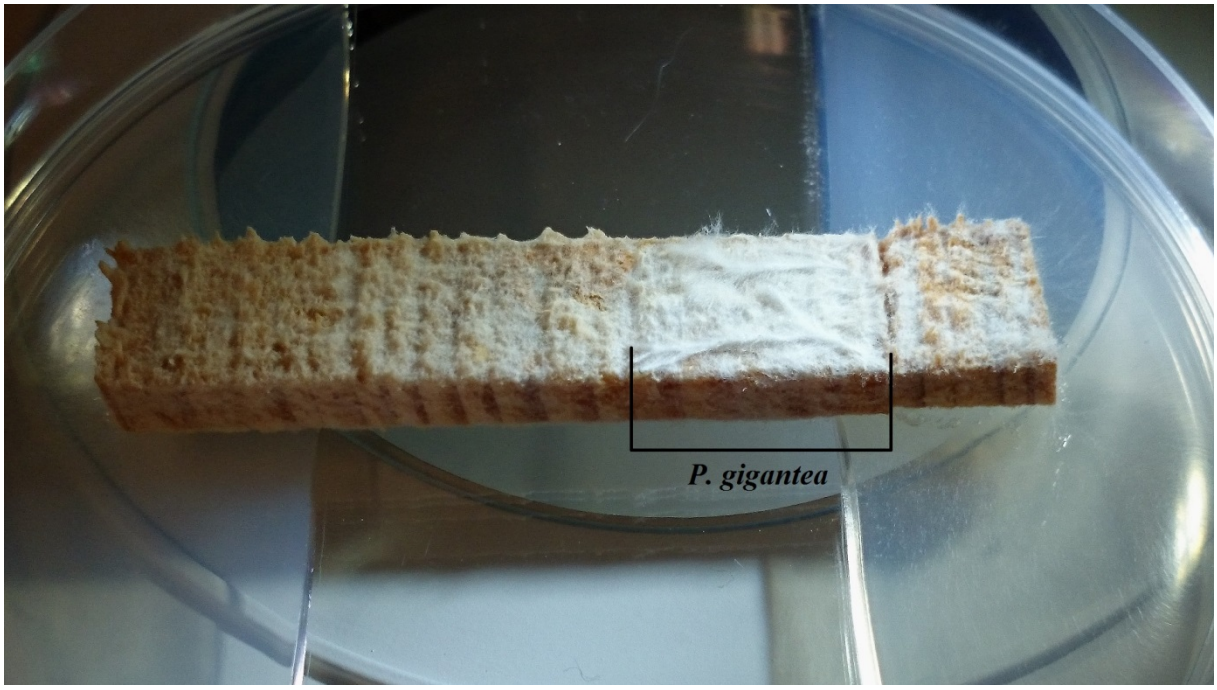

Figure 5. *P. gigantea* growth on top of wood block colonized by *H. parviporum*.

2.2. Field study

In September 2015 there was a thinning conducted in a 35 years old spruce and pine stand owned by Agneta Färlin in Grimslöv, Växjö. The coordinates of stand are N 56°43´06.7 and E 14°31´33.8. Site index is T24, management has been extensive and the stand has never been planted – only naturally regenerated. During this thinning operation 120 stumps were used for a field experiment. Three different types of treatments (Rotstop, Ox 24 and Experimental) were applied on stumps directly after felling the tree by the owner and approximately one fourth of stumps were left untreated for control purposes. Rotstop was applied by harvester at the moment of felling trees, following the general indications of manufacturer. Ox 24 containing *Pseudomonas* spp. bacteria was manually applied according to instructions of manufacturer, when applying it for agricultural purposes. Third treatment - experimental organic substance (further stated as “Experimental”) was applied in a manual way, but the specific contents of this treatment remained unknown to us, according to a confidentiality request from the provider. After treatment application, stumps were left for natural infection with *Heterobasidion* spp.

2.2.1. Collection of wooden discs

On 17th of February, after 6 months since thinning operation, wooden discs were collected for laboratory examination. Diameter of collected discs varied between 8 – 23 cm. Disc collection was carried with help of a chainsaw. Bark surface was disinfected with 70% ethanol solution before cutting was made in order to avoid risk of contaminations. At first a two cm thick upper disc was cut and discarded. After that a 5 cm thick disc was cut in a careful way, so that it did not touch the ground, which could induce contaminations. Wooden discs were marked with a water resistant marker, showing respective letter that

indicated the treatment method and an individual number, in order to track the side of the disc. Every disc was placed in marked plastic bag. In total 91 disc was collected and delivered to Southern Swedish Forest Research Centre in Alnarp and stored at 4 °C temperature before laboratory analysis.

2.2.2. Analysis of wooden discs

Before actual analysis of wooden discs they were incubated in room temperature (~20 °C) for 7-8 days. Both sides of the discs were examined under a dissecting microscope (Figure 6). A plastic grid was placed on top of the disc surface to provide easier examination of discs and ensure that all area was surveyed. When a group of conidiophores of *Heterobasidion* spp. was found, magnification was increased in order to detect the precise borders of infection. The borders of the infections were then marked with a water resistant marker and the infected area was measured using a special cm^2 grid.

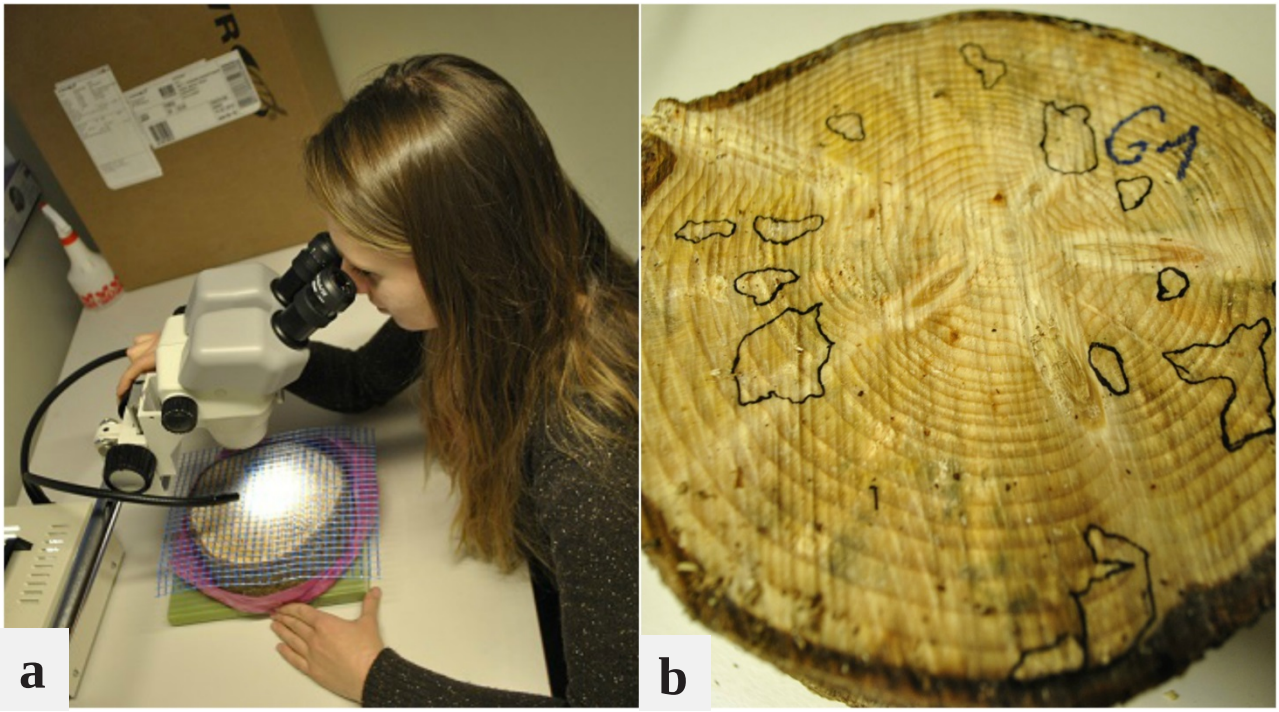

Figure 6. a) Examination of wooden disc under dissecting microscope; b) Detected area of *Heterobasidion* spp.

2.3. Calculations and statistical analysis

Microsoft Excel and IMB SPSS Statistics 20 softwares were used to analyze the data gained in this research. Means, standard deviations, standard errors, p-values and confidence intervals of the variations were calculated and compared.
A t-test was used in order to find out if there was a statistical significance between the tested parameters, mean values ± 2 standard errors of two or more independent sample groups which include the 95% confidence interval were compared. If the 95% confidence intervals

of two independent sample groups overlapped with each other, then the difference was not statistically significant.

Multiple statistical comparison of *P. gigantea* isolates was done by Post Hoc Tukey HSD test. With the help of this test we gained precise numerical information about statistical differences between isolates at certain point of time and results of this test are seen as letters in figures indicating level of significance.

3. RESULTS

3.1. Control agents' ability to resist a pathogen

During all the examination period in the laboratory, Petri dishes containing pathogen (*H. parviporum*) and respective control agent (*P. gigantea* and *Pseudomonas* spp.) were investigated in order to determine the control agent ability to resist a pathogen. All Petri dishes containing *P. gigantea* isolates could withstand the close contact with *H. parviporum* and did not get overgrown. There were occasions when some replicates of *P. gigantea* did not show any growth on top of wood infected by *H. parviporum*, but at the same time they could still resist the pathogen.

In case of Petri dishes that contained wood inoculated by *Pseudomonas* spp. and the pathogen, *H. parviporum* was not able to overgrow the wooden block inoculated with the bacteria (Figure 7). In the first part of the experiment we could observe unusual formations in the form of bubbles that appeared on the edge of some wooden block samples colonized by *H. parviporum*. As we could not find any evidence of research made in order to test the resistance ability of *Pseudomonas* spp. to *H. parviporum* in the literature, we assumed that this was the first case and requires special attention. That is why the Petri dishes containing this control agent were also checked after 25 days since the start of experiment. And wood colonized by *Pseudomonas* spp. was still resistant to *H. parviporum.*

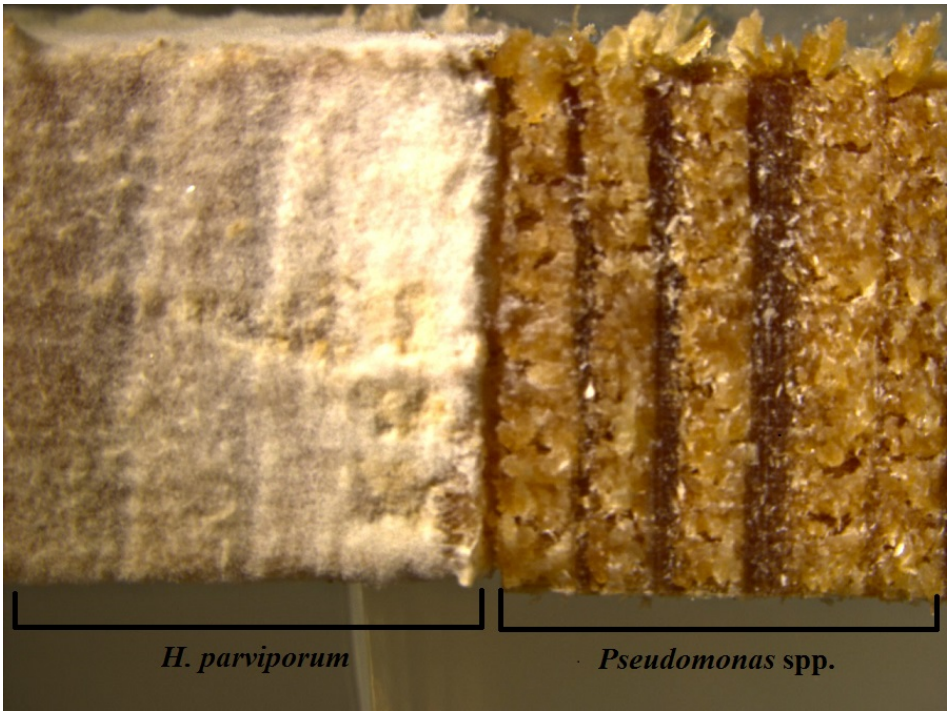

Figure 7. Resistance of wood block infected by *Pseudomonas* spp. to *H. parviporum*.

3.2. Comparison of *P. gigantea* isolates' growth *in vitro*

All 10 *P. gigantea* isolates that were subjected to laboratory analyzes showed big variation in their growth capacity over the wood block that was heavily infected by *H. parviporum* during 9 days of examination. Figure 7 shows the accumulative growth of respective isolates recorded over the period of inspection. As it can be observed, in the last day of examination the isolate S2384_2_VI was superior compared to others, because the average growth of the isolate in the last day of examination reached 14,15 mm. The next best isolates were PG16 (8,75 mm), Rotstop (8 mm) and PG342 (7,45 mm). Other tested isolates (except PGM2643-13) showed significantly lower growth rate in the 9th day of inspection (p<0,05) (Figure 11).

An important observation is that the isolates that showed the lowest growth rate at the end of the examination were already the weakest in the beginning of it (Figure 8). There were some isolates – PG28, PG3, PGWO4 and PGK5191-7, which had replicates that did not even start to grow on the wood block infected by *H. parviporum* which caused a reduction in the average accumulated growth for all of these isolates.

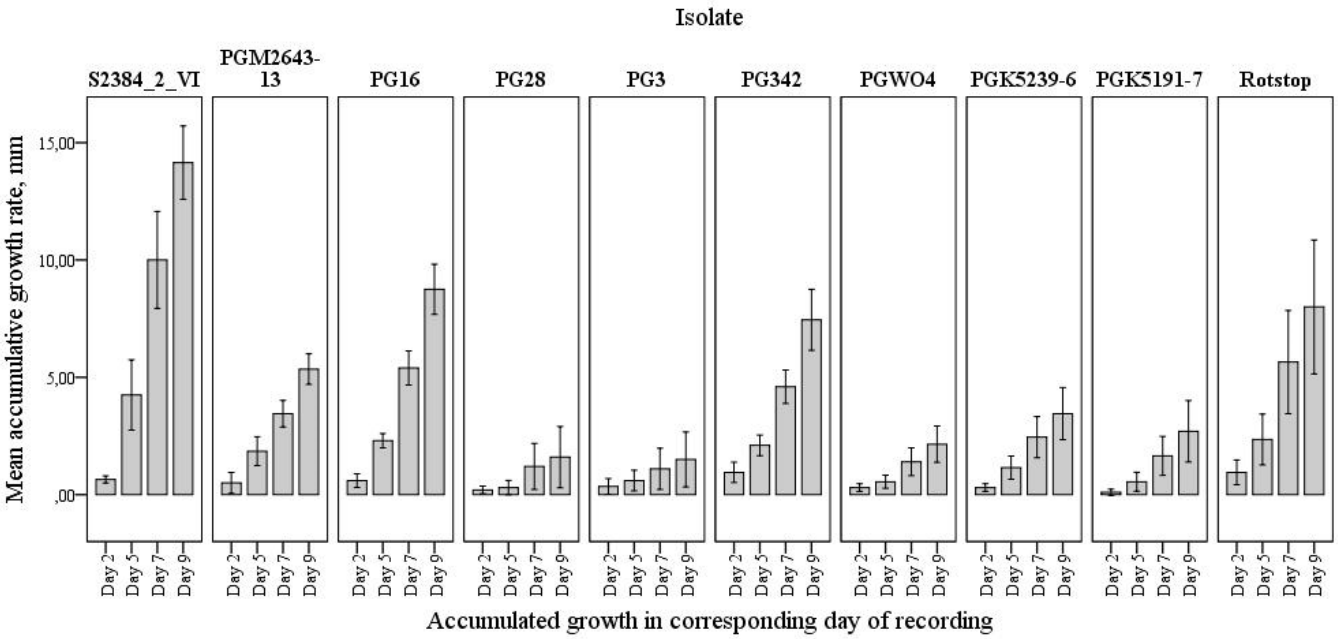

Figure 8. Accumulative growth of 10 *P. gigantea* isolates examined in the study. Bars showing isolate growth (mm) recorded in the respective day of examination. Error bars indicate ± 2 SE.

As the examination of *P. gigantea* isolate growth was done at four occasions, we could compare the periodical growth performance of isolates (Figure 9). In the first period (Day 0 – 2) the most rapid growth was observed for Rotstop and PG342 (0,95 mm), but the smallest recorded growth after 2 days was for isolate PGK5191-7 (0,1 mm).

In the second period (Day 2 – 4) most of the isolates had grown more than in the first period. Significant differences (p<0,05) between growth in first and second period were recorded for isolates PG16 and S2384_2_VI. For all of the 10 tested isolates growth in the third period (Day 4 – 7) was higher than in the second and isolate S2384_2_VI had the

highest growth among all the isolates in this period (5,75 mm). In the third period isolates were growing for 3 days compared to the other periods where they grew only 2 days. In the last period (Day 7 - 9), for 8 isolates the growth dropped when compared to the third period result, but it continued to exceed for isolates PG16 and PG342.

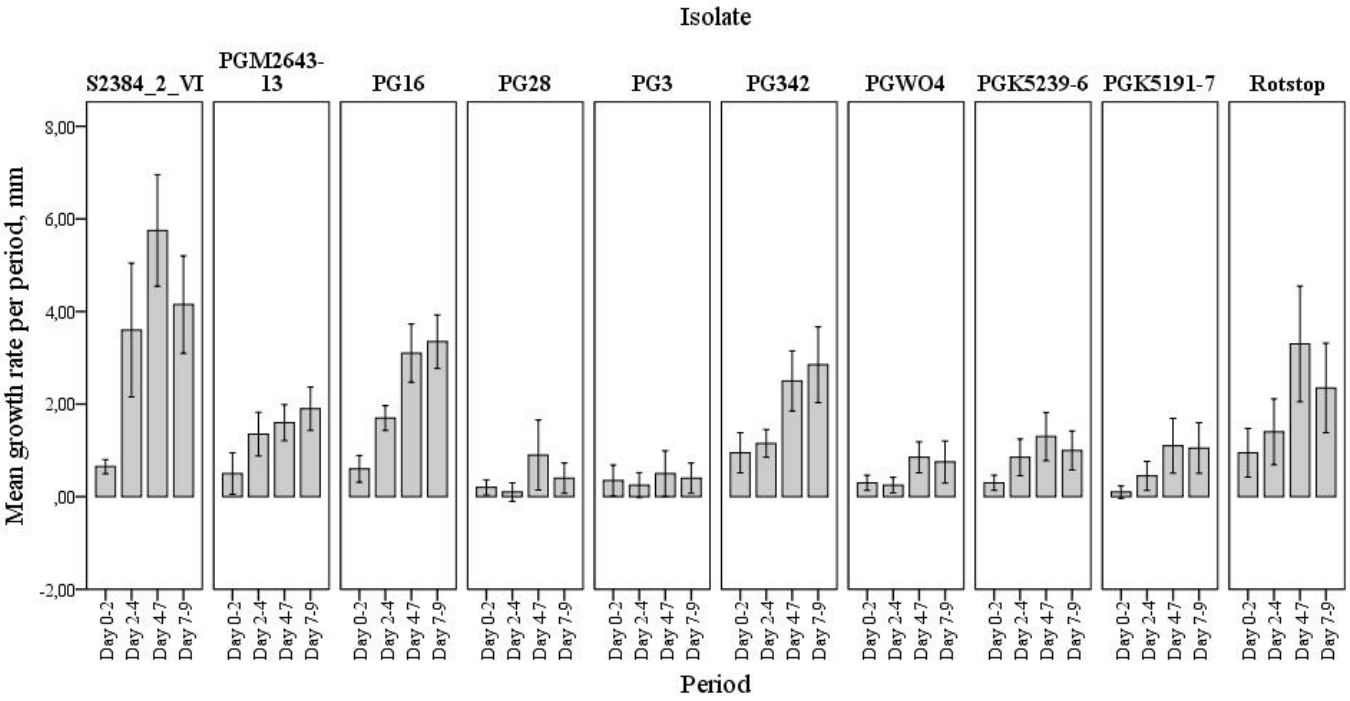

Figure 9. Periodical growth of 10 *P. gigantea* isolates examined in the study. Bars showing isolate growth (mm) between two measurements. Error bars indicate ± 2 SE.

Average isolate growth per day (mm) was calculated and showed significant differences between them (Figure 10). Isolate S2384_2_VI represented the highest average growth per day (1,57 mm) and it was significantly superior when compared to all the other isolates (p<0,05). Next best isolates in terms of average growth per day were PG16 (0,97 mm), Rotstop (0,89 mm) and PG342 (0,83 mm). There were no significant differences between these 3 isolates (p>0,05). The 3 isolates that showed the lowest growth score were PGWO4 (0,24 mm), PG28 (0,18 mm) and PG3 (0,17 mm). There were no significant differences between this group of isolates (p>0,05).

In figure 11 there can be observed relations between 10 tested isolates in terms of accumulative growth at 4 points of examination. At first point of examination (Day 2) Rotstop and isolate PG342 had significantly higher growth rates than isolates PG28 and PGK5191-7 (p<0,05). After 2 days situation changed and isolate S2384-2-VI had significantly higher accumulative growth (p<0,05) compared to all the other isolates and it continued to be like this throughout all examination. Rotstop and isolates PG16 and PG342 had significantly higher growth than PG28, PGWO4, PG3 and PGK5191-7 (p<0,05). At third point of examination (Day 7) Rotstop and isolate PG16 had significantly higher accumulative growth than PG28, PGK5239-6, PGWO4, PG3 and PGK5191-7. In the last day of examination (Day 9) Rotstop, PG16 and PG342 had significantly higher growth than PG28, PGK5239-6, PGWO4, PG3 and PGK5191-7.

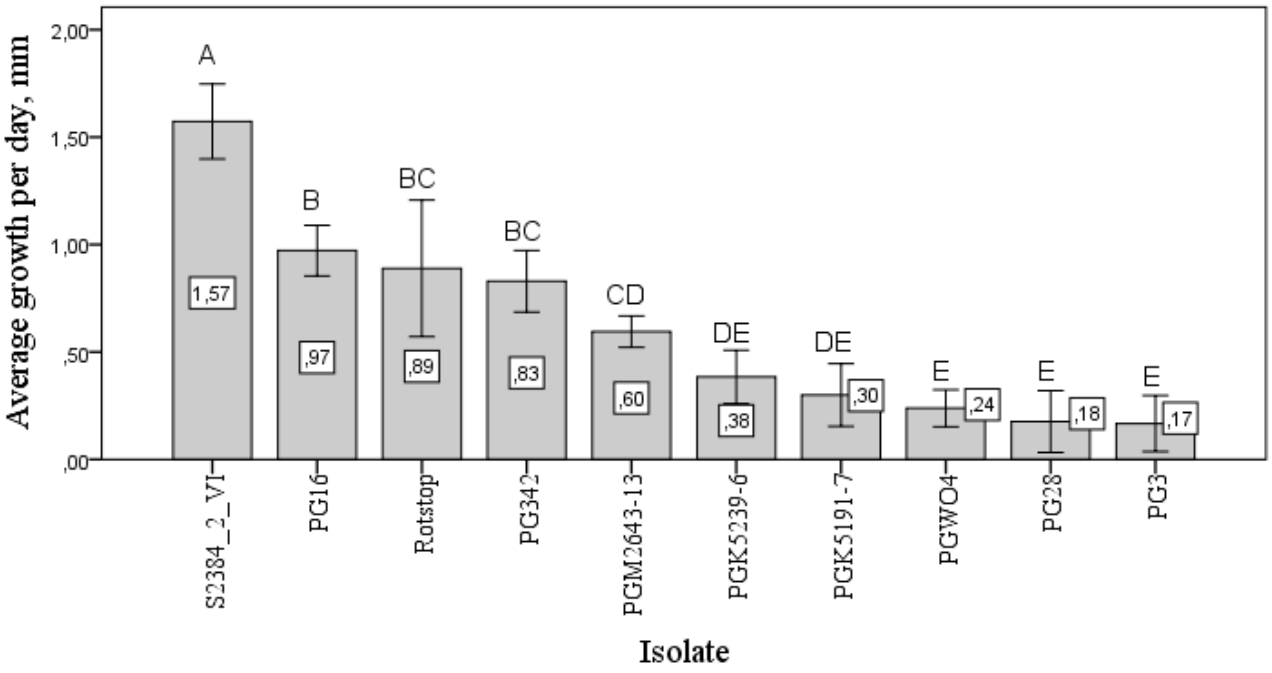

Figure 10. Average growth (mm) per day of *P. gigantea* isolates on top of wood heavily infected by *H. parviporum* over the whole period. Values that have the same letter are not significantly different at the p=0.05 level. Error bars indicate ± 2 SE.

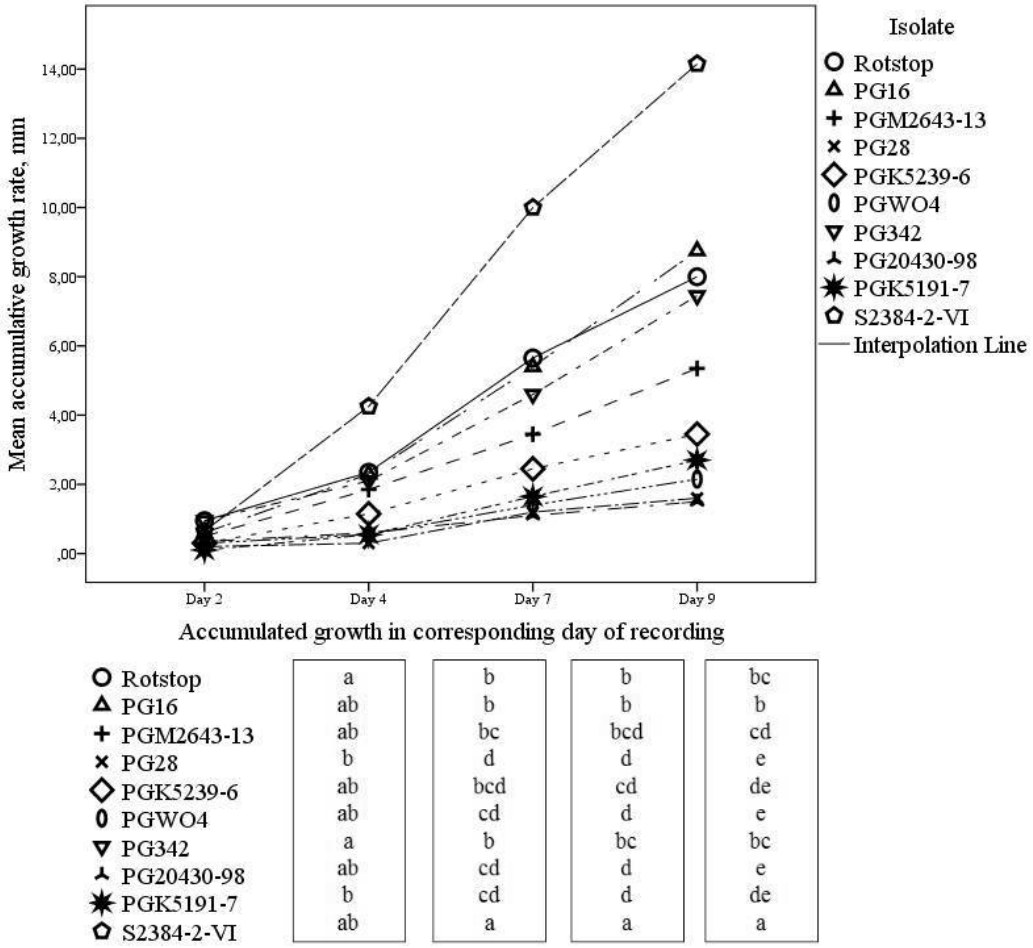

Figure 11. Comparison of mean accumulative growth rates (mm) of *P. gigantea* isolates in respective day of recording. Values that have the same letter are not significantly different at the p=0.05 level.

Figure 12 shows the differences between isolates in the periodical growth. Isolates S2384_2_VI and Rotstop have rather similar pattern, if excluding the fact that the last one is not so steep in growth in the starting phase. Peak of growth for most of the isolates is in the third period (Day 4 – 7).

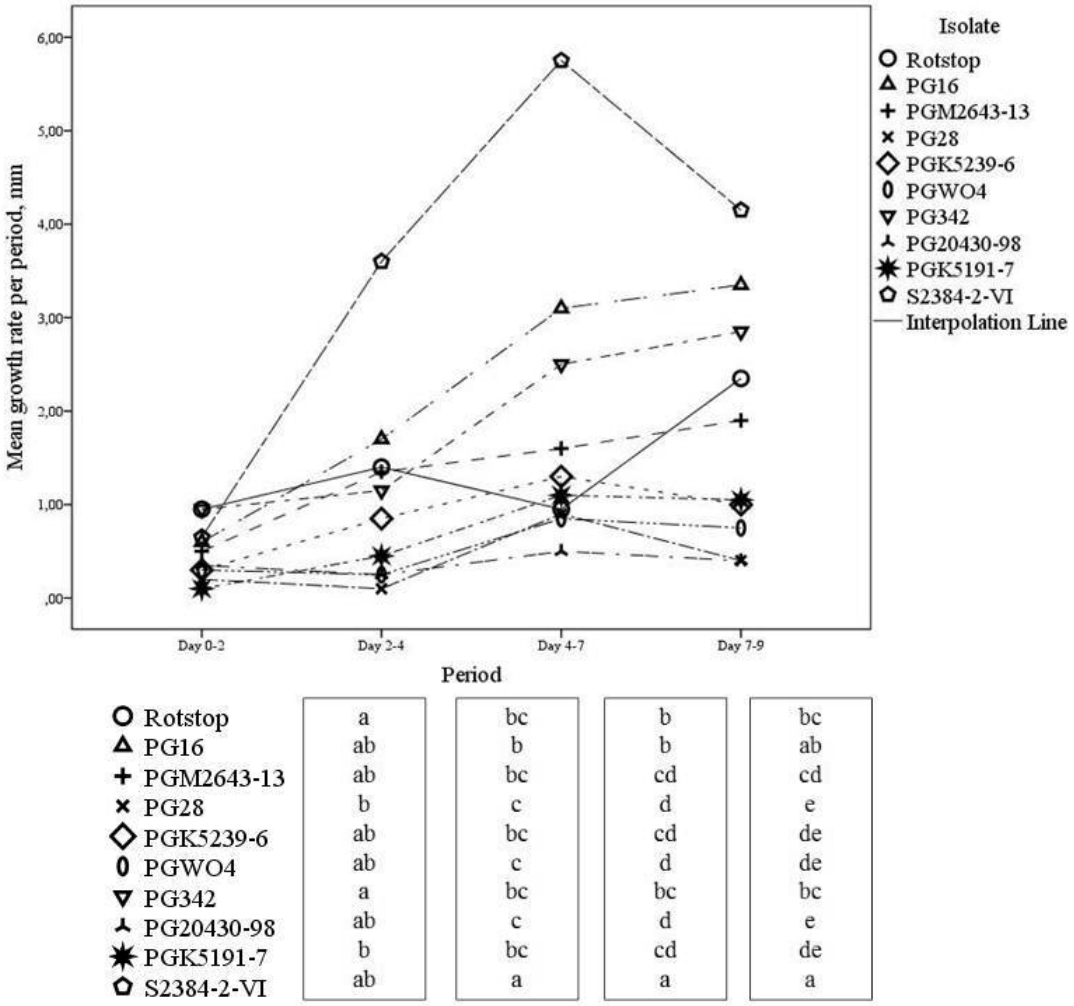

Figure 12. Comparison of mean growth rates (mm) of *P. gigantea* isolates in respective period of growth. Values that have the same letter are not significantly different at the p=0.05 level.

Along with the measurements of isolate growth on top of wood infected by *H. parviporum* (*in vitro* experiment), the isolate growth on top of sterile wood (Control) was also measured and compared (Figure 13). Mean growth rate per day was calculated for first 2 days, 4 days, 7 days and 9 days. When comparing results of mean growth per day between experiment and control, there were found considerable differences at all 4 points of examination. Whilst in the control the highest growth score per day in the beginning of experiment was 4 mm, in the experiment best score per day was just 0.5 mm. The mean growth rate of isolates in control after 7 days was 4.91 mm/day.

The length of long wood block, that needed to be overgrown by respective fungal isolate, was 50 mm and the best result that was reached out of this length in the end of experiment was 14.15 mm for isolate S2384_2_VI. But in control already in day 7 isolate PG342 reached the end of 50 mm long wooden block. In the last day of examination 7 isolates reached the end of block in control.

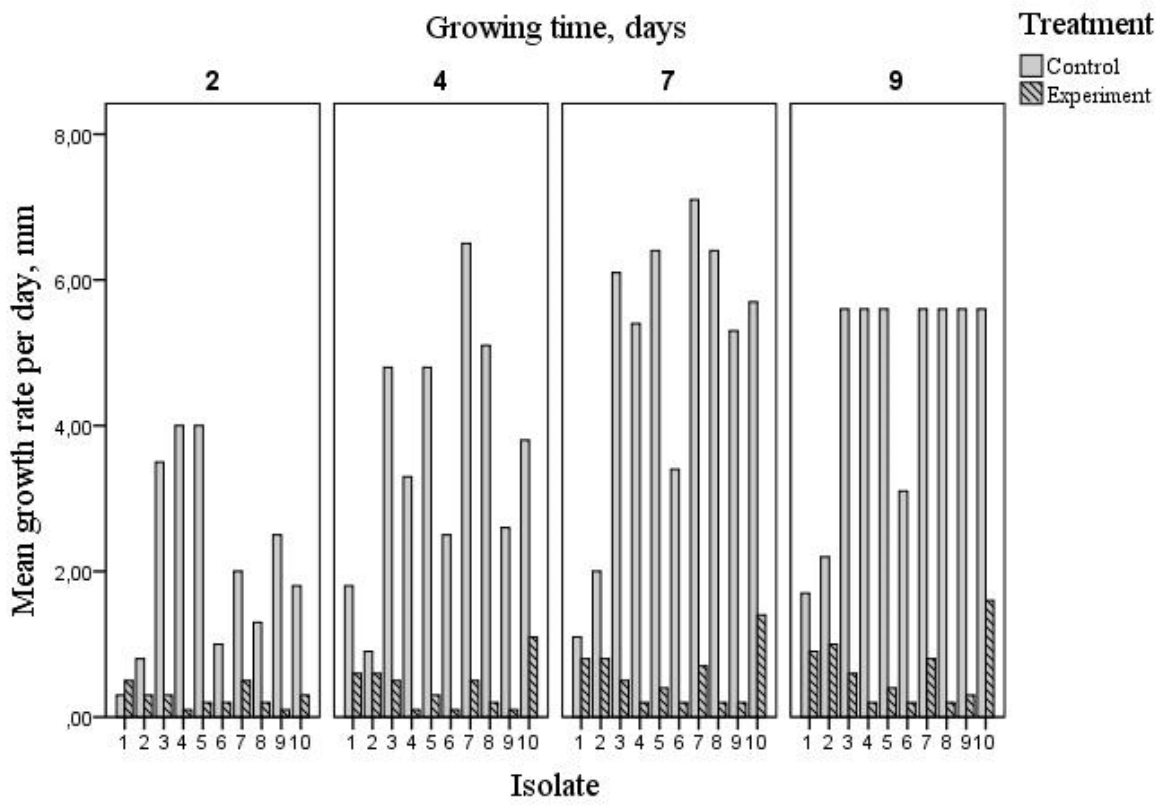

Figure 13. Comparison of *P. gigantea* isolate mean growth rate on sterilized wood (Control) and wood heavily infected with *H. parviporum* (Experiment). Isolate 1 – Rotstop, 2 – PG16, 3 – PGM2643-13, 4 – PG26, 5 – PGK5239-6, 6 – PGWO4, 7 – PG342, 8 – PG3, 9 – PGK5191-7, 10 – S2384-2-VI.

3.3. Efficacy of stump treatment in the field pilot study

Results of the pilot study, where different stump treatment methods were used on 91 Norway spruce tree, are shown in table 3. The pilot study reveals that infection rate with *Heterobasidion* spp. was the highest for stumps treated by experimental substance, 43%, respectively. Stumps that were left as control, were infected in 35% of cases. Infection rate was lower, when using Rotstop and the biological substance containing *Pseudomonas* spp., 17% and 13% of infection rate, respectively (Table 3). Thus Rotstop treatment reduced the total infected area by 83% and *Pseudomonas* spp. by 87%.

Table 3. Comparison of stump treatment methods used in pilot study

Treatment	N of samp-les	Mean Ø of samples	N of infected stumps	Infection frequency, %	Average relative infected area of all discs, %	Average relative infected area of infected discs, %	Efficacy, %
Pseudo-monas spp.	24	11,36	3	13%	0,24%	1,87% ±1,59%	87%
Rotstop	23	11,2	4	17%	0,31%	1,78%±1,25 %	83%
Control	23	10,9	8	35%	1,93%	5,53%±3,66 %	NA
Experi-mental	21	12,02	9	43%	0,79%	2,60%±1,53 %	57%

When analyzing all of the discs infected by *Heterobasidion* spp. for a certain treatment method (Table 3) the biggest relative infected area was found on control discs (5,53%), but the smallest on stumps treated by Rotstop (1,78%), following *Pseudomonas* spp. (1,87%). But when analyzing relative infected area on all discs treated by respective method, then the smallest infected area per disc is for stumps treated by *Pseudomonas* spp. (0,24%).

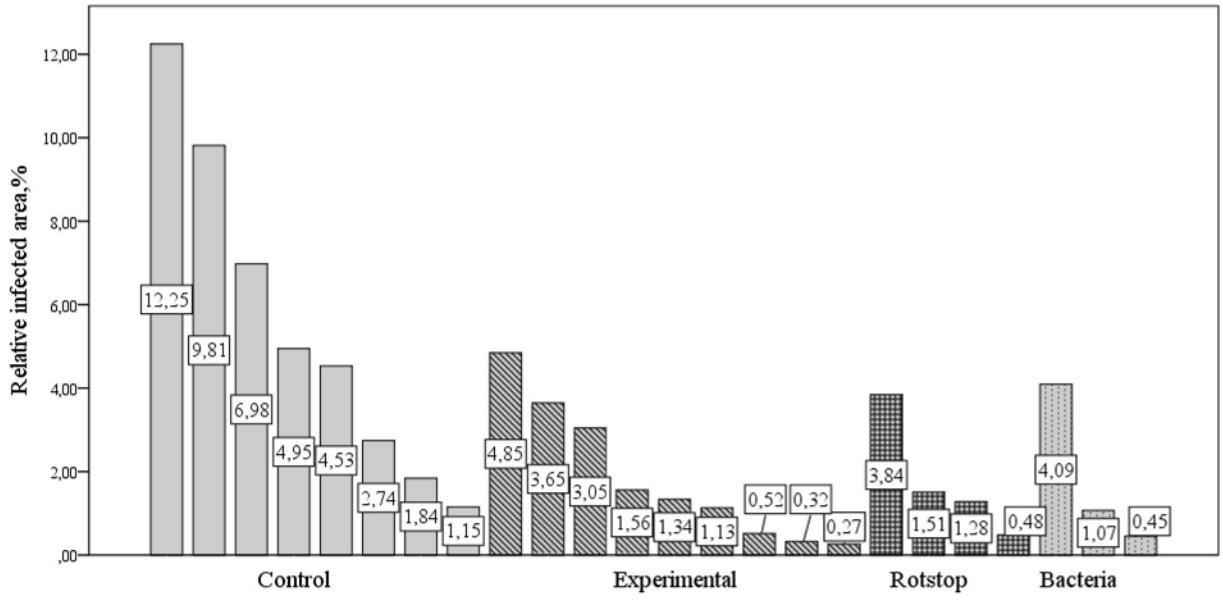

Figure 14. Relative infected area by *Heterobasidion* spp. per stump treatment method. Bars per treatment method indicate all discs from study that were found to be infected.

The biggest amount of discs infected per treatment method was found for the experimental treatment – 9 out of 21 that were subjected to this treatment type. In control, 8 discs out of 23 were infected by *Heterobasidion* spp. In the Rotstop treatment 4 discs out of 23 were infected by *Heterobasidion* spp., but the smallest amount of infected discs was found for stumps treated by *Pseudomonas* spp. – 3 discs out of 24 (Figure 14).

4. DISCUSSION

The *in vitro* and pilot study results on new protective agents for biocontrol of *Heterobasidion* spp. revealed that, despite a long research history on the pathogen and the possible control agents, there still are fungal and bacterial agents that could potentially work better as commercial protective agents being used currently. *P. gigantea* isolate S2384_2_VI showed significantly superior growth compared to the other 9 tested isolates including Rotstop S strain, when growing on top of wood heavily colonized by *H. parviporum*. But more surprising was the ability of wood colonized by *Pseudomonas* spp. bacteria to resist *H. parviporum* even after 25 days of close contact. This could be a start of a fairly new biocontrol agent in stump treatment practice.

Unexpected results were obtained, when testing ability of *Pseudomonas* spp. to resist pathogen. It is worth mentioning that this bacteria have not been tested as a potential biological control agent for *Heterobasidion* spp. in wood before, which makes this study unique. During 9 days of laboratory experiment, where pathogen was juxtaposed to wood colonized by *Pseudomonas* spp., mycelia of *H. parviporum* could not get on surface of *Pseudomonas* spp. We observed some attempt of *H. parviporum* mycelia to grow on top of small wood block inoculated by *Pseudomonas* spp., but it could only hang above the wood, not getting any deeper. Being intrigued by this result, we decided to do a check-up of these Petri dishes again after 2 weeks. Surprisingly, after 25 days of examination, *H. parviporum* was still unable to overgrow *Pseudomonas* spp., which demonstrates the previously undiscovered abilities of this bacteria to protect wood from pathogens, although it has been reported that some strains of *Pseudomonas* spp. are involved in suppression of fungal root pathogens and parasitic nematodes in plants (Timper *et al.*, 2009).

According to the accumulative growth results of 10 *P. gigantea* isolates, we could clearly divide isolates in two groups, because five of the isolates (S2384_2_VI, PG16, Rotstop, PG342, PGM2643-13) excelled at growth and the other five (PG28, PGK5239-6, PGWO4, PG3, PGK5191-7) showed significantly lower rates of growth (p<0,05). In the last day of examination isolate S2384_2_VI represented the highest rate of growth and significantly differed from the other 4 best isolates (p<0,05).

Ability to colonize wooden surface rapidly, is one of the most important traits of *P. gigantea*, when speaking about pathogen control, because *P. gigantea* is competing for a substrate with the pathogen. Consequently, growth rate of isolates in the beginning is more important than ability to expand later (Berglund & Rönnberg, 2004; Berglund *et al.*, 2005; Sun *et al.*, 2009b). Thus the performance of Rotstop and PG342 in the beginning of experiment should be considered as important trait, when choosing isolates for future tests. When analyzing performance of *P. gigantea* isolates period wise, substantial differences between respective periods and isolates could be found. In the first period (Day 0 - 2) Rotstop and PG342 showed the best growth performance from all tested *P. gigantea* isolates and was significantly faster than isolates PG28 and PGK5191-7. In the next period (Day 2 – 4) isolate S2384_2_VI took the leading position and kept it till the end of experiment, having significantly higher growth rate (p<0,05).

When *P. gigantea* is subjected for growth on top of wood that is pre-colonized by *H. parviporum*, the growth of *P. gigantea* is considerably suppressed. However, *P. gigantea* isolate S2384_2_VI demonstrated the highest mean growth rate over *H. parviporum* colonized wood (1.57 mm/day) and significantly differed from the other isolates. Samils *et al.* (2008) reported that in their study there was a wide distribution of *P. gigantea* strains to overgrow *H. parviporum*. The results ranged between 2.1 and 5.0 mm/day and mean growth was estimated to be 3.2 mm/day. Our results showed much slower growth and the reason for that could be rate of *H. parviporum* infection in wood that needs to be overgrown by *P. gigantea*. Wooden blocks that were subjected for *H. parviporum* infection were left in Petri dishes longer for their inoculation than the blocks subjected for colonization with *P. gigantea*. When wooden blocks colonized with both antagonists were juxtaposed, it was clearly visible that *H. parviporum* already started to form fruiting bodies on some of the replicates, which is the sign that wood is heavily infected. In the study of Sun *et al.* (2009) they were testing 64 *P. gigantea* isolates and their variation in biocontrol of Norway spruce against *Heterobasidion* spp. Mean growth rate over wood infected by *Heterobasidion* spp. varied between 0.2 and 2.3 mm/day. Growth was faster against *H. parviporum* than *H. annosum*, 1.7 mm/day and 1.2 mm/day, respectively. These results go in line with ours, with the only difference that none of the isolates tested in our study reached the growth of 2.3 mm/day, but it is very probable that if we would increase the number of tested isolates, some of them could be faster in growth than our best one - S2384_2_VI. What applies to growth on sterile wood (Control), there we gained noticeably faster growth results. The average growth of *P. gigantea* isolates after 7 days of experiment was 4.91 mm/day. Average growth was calculated after 7 days, because in the last day of examination most of the isolates already reached the maximum of 50 mm they could grow over. In the study of Samils *et al.* (2008) growth rate over uncolonized wood was 8.7 mm/day, but Sun *et al.* (2009) reported that growth rate in spruce wood varied from 0.1 to 3.9 mm/day. Our results are somewhat in the middle of these findings, but one possible reason for these variations is the temperature that could differ in different laboratories.

Biological treatment resistance to pathogen is essential when trying to tackle primary infection of *Heterobasidion* spp. In our study we were testing *P. gigantea* capacity of resisting competitive interaction with *H. parviporum* by evaluating mycelial overgrowth. As a matter of fact all wooden blocks colonized by *P. gigantea* isolates could resist competition with *H. parviporum* and our findings are similar to those of Lakomy *et al.*(1998). But important thing to mention is that we used only one individual tree for our laboratory tests and using various trees of the same species could alter the results we gained, because of genetic differences in wood. We could observe some cases when the relationship between both antagonists was neutral, meaning that none of them did actually overgrow the other. And it could be explained by the fact that in presence of competitors, need to defend previously colonized area is more crucial than occupying new uncolonized surfaces (Rayner & Boddy, 1988). But in most of the cases *P. gigantea* overgrew *H. parviporum* already in the first days of experiment. Higher aggressive colonization of *P. gigantea* probably is due to the ability to overcome toxic effect of different phenolic compounds that can be found in conifer woody tissues and easier access to host's nutrients (Mgbeahuruike *et al.*, 2011).

In the pilot study that was conducted in Grimslöv, we compared three different types of stump treatment and control, including *Pseudomonas* spp. bacteria and Rotstop, previously

described in the laboratory study. The biggest infection rate (43%) with *Heterobasidion* spp. was found on stumps treated by experimental substance, stumps that were not treated with any substance (Control) were infected in 35% of cases. Relatively low rate of infection was for both Rotstop and *Pseudomonas* spp. treated stumps, 17% and 13%, respectively. Judging by the relative infected area of wooden disc, in untreated stumps (Control) we could find the biggest areas occupied by *Heterobasidion* spp., but stumps treated by Rotstop and *Pseudomonas* spp. again showed the best protection results. In the study of Dimitri *et al.* (1971) they found that 6 months after tree felling *Heterobasidion* spp. was present in ~ 20% of stumps. Berglund *et al.* (2005) found that infection rate in Norway spruce control stumps in Southern Sweden after thinning was on average 88%. Big differences in the infection rate with *Heterobasidion* spp. can mainly be explained by the fact that infection background of stands varies a lot. Kenigsvalde *et al.* (2015) reports, that in the study held in Latvia the efficacy of Rotstop in spruce stumps varied from 36% to 100%. Big variation on efficacy of Rotstop has been reported also in other studies from Sweden (Thor, 2005; Rönnberg *et al.*, 2006), which leads to a conclusion that there are many factors that influence the performance of *P. gigantea* as control agent and which need to be taken into account when presenting results. As this was meant to be just a small trial in field, showing tendencies, we do not aim for calling these field results completely representative, however, they need to be seen as encouragement for making a bigger study.

Laboratory test results just partly display the abilities of biological agents in control of *Heterobasidion* spp. Moving study to field conditions could reveal other aspects worth of consideration when choosing the best agents. Unstable weather conditions and competition are factors that will determine viability of certain biological agents in the field. Studies of other researchers have proven that *P. gigantea* growth *in vitro* is much more rapid compared to growth under field conditions (Sun *et al.*, 2009b), therefore results gained in laboratory conditions should not be overestimated.

Most *P. gigantea* isolates that were tested in the current study were isolated in United Kingdom and had host species different from Norway spruce, however, for getting optimal results of protection, *P. gigantea* isolates need to be well adapted to the host and to the site (Berglund *et al.*, 2005), so further behavior of isolates in forest ecosystem would be crucial to observe. There was an isolate of *P. gigantea* that stood out from the others examined in our study - S2384_2_VI. But at the same time the Rotstop isolate had the most rapid growth in the very beginning of the study and of course is already known for the good adaptation in field conditions. Idea of combining these isolates with an aim of getting the best parental traits is possible to realize, as many other researchers had already worked with this matter (Vainio *et al.*, 1998; Grillo *et al.*, 2005; Sun *et al.*, 2009a; Mgbeahuruike *et al.*, 2011). Furthermore, the study of Sun *et al.* (2009a) showed that progeny strains were better than parental strains, when comparing essential traits of *P. gigantea.* The breeding method described in the study of Sun *et al.* (2009a) seems to be a promising approach for biocontrol agent selection in the future and it is an advantage to have a number of isolates available for use in the biological control products, either as replacements for isolates that fail or for making mixtures (Pratt *et al.*, 2000).

The *Pseudomonas* spp. bacteria ability to resist *H. parviporum in vitro* as well as the stump protection efficacy observed in the pilot study are among the most surprising findings of our study yet requires deeper understanding of these attributes. Apart from *P. gigantea,* many

other fungus have been investigated as possible biological control agents for *Heterobasidion* spp. - *Resinicium bicolor, Hypholoma fasciculare, Trichoderma harzianum* and others (Holmer & Stenlid, 1997; Holdenrieder & Greig, 1998; Lakomy *et al.*, 1998; Berglund *et al*., 2005; Lehtijärvi *et al*., 2011), but unlike fungi, bacteria is a totally different type of organisms that have not been tested for control abilities in treating stumps against *Heterobasidion* spp. In the laboratory study we observed that wood that was inoculated by *Pseudomonas* spp. was undoubtedly resistant to *H. parviporum*, but deeper microbiological research is needed in order to answer the questions: how exactly the mechanism of wood inoculation works; how fast and deep bacteria gets into the wood; is it important to have full coverage of stump with *Pseudomonas* spp. treatment in order to have thorough protection etc. Clearly more research will be needed before *Pseudomonas* spp. can be considered as potential alternative to *P. gigantea* in terms of a biological stump control.

The significance of biological control treatments for limiting spread of *Heterobasidion* spp. in the future is apparent. Pathogens develop resistance to chemical treatments much faster than for biological control agents (Samils *et al.*, 2008), which makes them more preferable for using in control of *Heterobasidion* spp. Other convincing factor is the forest certification systems that encourage forest owners to use biological treatments instead of chemical. Likewise, increasing concerns of society about the use of chemical pesticides in forests, will give a constant “green light” for production of new biological protection agents in future. Nevertheless, production of new biological control products requires certain expertise and precaution. To be of commercial interest, biological control agents must also be easy to apply on the stump surface. It needs to be highly effective in order to be economically justified. Finally, potential biological control agents need to be thoroughly examined, before they can be used in the practical forestry and their possible influence on non-target organisms should be considered in order to avoid undesirable shifts in biodiversity (Lehtijärvi *et al*., 2011).

In conclusion, the results of the current study revealed that *P. gigantea* isolate S2384_2_VI is significantly (p<0,05) superior to the other 9 isolates by means of average growth rate per day on top of wood heavily infected by *H. parviporum.* Rotstop S and PG342 isolate has the fastest performance of growth in the first two days of juxtaposition with *H. parviporum*. Wood colonized by *Pseudomonas* spp. bacteria *in vitro* is resistant to *H. parviporum* even after 25 days of close contact. These findings demonstrate that there are isolates of *P. gigantea* that possibly could be more effective than the ones that are commercially used now. Careful seeking and selection of the best isolates is important for assuring effective and economically justified *Heterobasidion* spp. control in the future. Breeding between the best isolates found in this study could be introduced in order to produce *P. gigantea* progenies that have higher antagonistic and biological control ability. *Pseudomonas* spp. bacteria showed remarkable results in resistance for *H. parviporum in vitro,* but little is known about pathogen control capabilities in field conditions and possible influence on non-target organisms. Therefore *Pseudomonas* spp. bacteria should be monitored in long term experiments before it can be considered as potential alternative to *P. gigantea* in terms of biological stump control.

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In the late August of year 2014, when I first arrived to Alnarp in order to become a part of Euroforester program, I had no idea that I could stay in Sweden for the next study year as well and even do my master thesis there. But luckily life is full of surprises and you never know where the road will take you. Important thing is not being afraid to take a chance, when life offers you one. There were many people sharing this road to master thesis with me to whom I would like to express my sincerest gratitude and appreciation.

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APPENDIX

Table 1. Multiple statistical comparison of *P. gigantea* isolates, Post Hoc Tukey HSD test, Dependent Variable: growth rate per period, mm

Period	Isolate	Isolate	Mean Differen -ce (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
0-2	Rotstop	PG16	.350	.222	.855	-.369	1.069
		PGM2643-13	.450		.580	-.269	1.169
		PG28	.750		.034	.031	1.469
		PGK5239-6	.650		.112	-.069	1.369
		PGWO4	.650		.112	-.069	1.369
		PG342	.000		1.000	-.719	.719
		PG3	.600		.187	-.119	1.319
		PGK5191-7	.850		.008	.131	1.569
		S2384_2_VI	.300		.938	-.419	1.019
2-4	Rotstop	PG16	-.300	.411	.999	-1.632	1.032
		PGM2643-13	.050		1.000	-1.282	1.382
		PG28	1.300		.062	-.032	2.632
		PGK5239-6	.550		.941	-.782	1.882
		PGWO4	1.150		.152	-.182	2.482
		PG342	.250		1.000	-1.082	1.582
		PG3	1.150		.152	-.182	2.482
		PGK5191-7	.950		.391	-.382	2.282
		S2384_2_VI	-2.200		.000	-3.532	-.868
4-7	Rotstop	PG16	-2.150	.461	.000	-3.645	-.655
		PGM2643-13	-.650		.921	-2.145	.845
		PG28	.050		1.000	-1.445	1.545
		PGK5239-6	-.350		.999	-1.845	1.145
		PGWO4	.100		1.000	-1.395	1.595
		PG342	-1.550		.036	-3.045	-.055
		PG3	.450		.993	-1.045	1.945
		PGK5191-7	-.150		1.000	-1.645	1.345
		S2384_2_VI	-4.800		.000	-6.295	-3.305

7-9	Rotstop	PG16	-1.000	.456	.470	-2.480	.480
		PGM2643-13	.450		.992	-1.030	1.930
		PG28	1.950		.002	.470	3.430
		PGK5239-6	1.350		.105	-.130	2.830
		PGWO4	1.600		.023	.120	3.080
		PG342	-.500		.984	-1.980	.980
		PG3	1.950		.002	.470	3.430
		PGK5191-7	1.300		.136	-.180	2.780
		S2384_2_VI	-1.800		.006	-3.280	-.320

Table 2. Discs infected by *Heterobasidion* spp. Results of pilot study on different stump treatments

Treatment	Disc	Diameter, cm	Total area of disc, cm^2	Infection with *Heterobasidion*, cm^2		Relative infected area, %
				Top	Bottom	
Pseudomonas spp.	B5	9.45	70.1	0	0.75	1.1.%
	B24	14.5	165.05	0.75	0	0.5%
	B16	11.5	103.82	1	3.25	4.1%
Rotstop	R16	9.55	71.59	0.25	2.5	3.8%
	R5	14.1	156.07	0.25	0.5	0.5%
	R15	13.2	136.78	0.75	1	1.3%
	R1	13	132.67	0	2	1.5%
Control	G19	9.3	67.89	0	1.25	1.8%
	G14	11.25	99.35	3	6.75	9.8%
	G16	11.7	107.46	3.75	3.75	7.0%
	G13	11.3	100.24	0.5	2.25	2.7%
	G1	15.55	189.81	16.5	6.75	12.2%
	G2	19.3	292.40	7	6.25	4.5%
	G8	11.9	111.16	2.25	3.25	4.9%
	G21	9.1	65.01	0.75	0	1.2%
Experimental	M13	15.5	188.60	0.5	0	0.3%
	M2	16.45	212.42	1.5	6.25	3.6%
	M11	18.25	261.45	3.5	0	1.3%
	M5	14	153.86	0.25	0.25	0.3%
	M3	12.1	114.93	0	3.5	3.0%
	M1	11.95	112.10	0.5	1.25	1.6%
	M14	11.1	96.72	0.5	0	0.5%
	M4	9.5	70.85	0.5	0.3	1.1%
	M6	8.5	56.72	2.75	0	4.8%

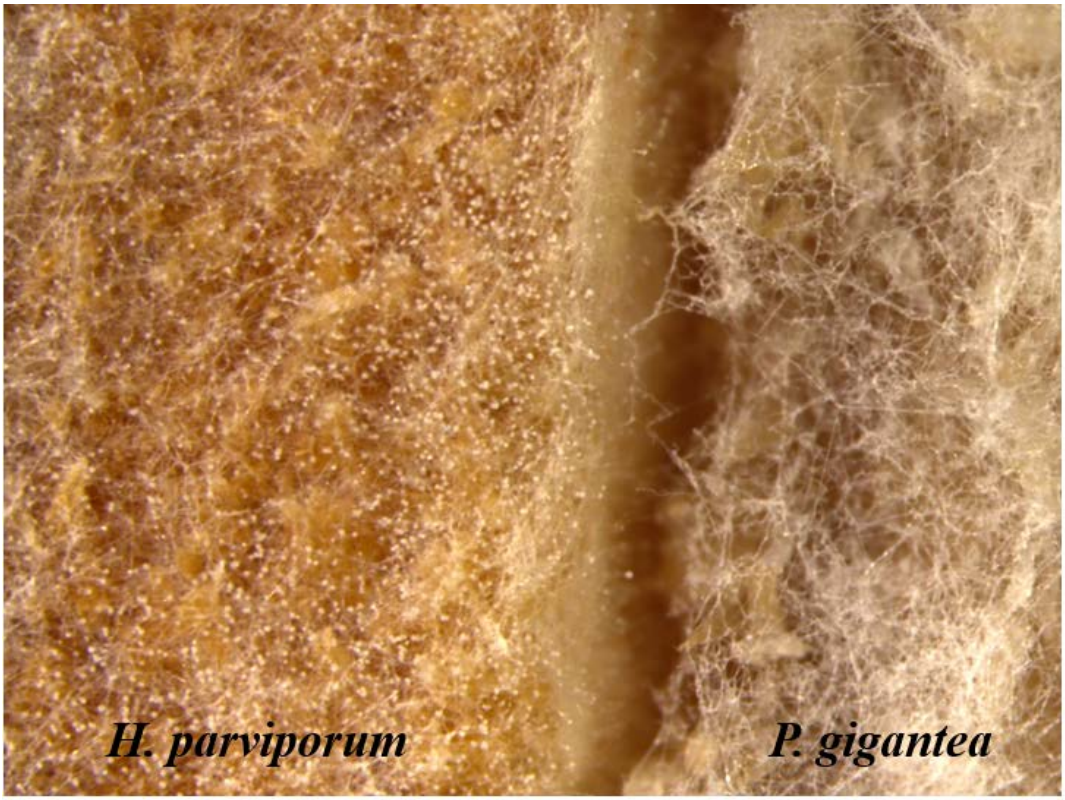

Figure 1. Resistance of *P. gigantea* isolate to *H. parviporum* after 9 days of experiment.

Figure 2. Resistance of *Pseudomonas* spp. bacteria to *H. parviporum* after 9 days of experiment.