University of Natural Resources and Life Sciences, Vienna

Evaluating the persistence of *Phlebiopsis gigantea* in Norway spruce stumps after root rot protection treatment in a subalpine forest in Tyrol

Master thesis to obtain the academic grade

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Abstract

Heterobasidion parviporum is the most important root and butt rot pathogen of Norway spruce (*Picea abies*) in Europe. In subalpine spruce stands, this fungus is a hazard to the stability and therefore the protective function of the stands. The Federal Research and Training Centre for Forests, Natural Hazards and Landscape BFW conducted a project in a subalpine forest in East Tyrol, where the antagonist *Phlebiopsis gigantea* was tested against Heterobasidion using two different biological strains. The aim of the present master thesis was the evaluation of the persistence of the antagonistic fungus *P. gigantea* and its influence on the fungal biodiversity on experimental plots seven years after the application. Wood cores were taken from spruce stumps in three different depths using an increment borer. For the identification of the outgrowing fungi morphological and molecular detection methods were performed. The PCR with the fungal barcode primers ITS1/ITS4 detected *P. gigantea* two times, whereas *H. parviporum* could not be found. The cloning approach and the Blast search (Genbank) of the sequences indicated 34 different fungal species belonging to ascomycetes, basidiomycetes, hyphomycetes and unidentified taxa. The treatment with the commercial strain of *P. gigantea* (Rotstop®) decreased the fungal biodiversity, whereas the other strain of the fungus received from Poland did not. The antagonistic treatments showed good results, however, further studies with naturally occurring antagonists should be performed

Kurzfassung

Heterobasidion parviporum ist der wichtigste Wurzel- und Stammfäule- Erreger der Fichte (*Picea abies*) in Europa. In subalpinen Fichtenbeständen ist dieser Pilz eine Gefahr für die Stabilität und damit die Schutzfunktion des Waldes. Das Bundesforschungs- und Ausbildungszentrum für Wald, Naturgefahren und Landschaft (BFW) hat deswegen ein Projekt in einem subalpinen Wald in Osttirol durchgeführt, wo der Antagonist *Phlebiopsis gigantea* gegen Heterobasidion mit zwei verschiedenen biologischen Bekämpfungsmitteln getestet wurde. Das Ziel der vorliegenden Diplomarbeit war die Evaluierung der Persistenz von *P. gigantea* und deren Einfluss auf die pilzliche Biodiversität der Versuchsflächen sieben Jahre nach der Anwendung. Holzkerne wurden aus Fichtenstümpfen in drei verschiedenen Tiefen mit einem Zuwachsbohrer entnommen. Zur Identifizierung der entwachsenen Pilze wurden morphologische und molekulare Nachweisverfahren durchgeführt. Die PCR mit den pilzspezifischen Barcode Primer ITS1/ITS4 identifizierte *P. gigantea* zweimal, während *H. parviporum* nicht gefunden werden konnte. Die Klonierung und die Genbank – BLAST Suche mit den erhaltenen Sequenzen ergab 34 verschiedene Pilzarten, die zu den Ascomyceten, Basidiomyceten, Hyphomyceten und unindentifizierten Taxa gehören. Die Behandlung mit dem kommerziellen Präparat von *P. gigantea* (Rotstop®) reduzierte die Pilz-Biodiversität drastisch, während das andere Präparat aus Polen keinen Einfluss hatte. Die antagonistische Behandlung zeigte gute Ergebnisse, jedoch sollten weitere Studien mit natürlich vorkommenden Antagonisten durchgeführt werden, um eine biologische Bekämpfung gegen Heterobasidion in subalpinen Wäldern zu optimieren.

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1. Introduction

1.1 *Heterobasidion annosum* as main cause of root and butt rot on Norway spruce

Norway spruce, *Picea abies,* is economically the main important tree species in Central Europe. In subalpine forests, this tree species plays a major role as an avalanche- and erosion-control tool besides its ecological value (Oswald et al. 1998). Thus, in addition to bark beetles i.e. *Ips typographus,* pathogens can pose risk factor endangering the stability of forests (Altenkirch et al. 2002).

Among the pathogenic fungi, the root rot pathogen, *Heterobasidion annosum* s. l.*,* is an important damaging agent of conifers on the northern hemisphere (Woodward et al. 1998). The economic significance of this species is reflected by the annual wood loss for the European timber industry estimated to be about 790 million Euros per year (Woodward et al. 1998; Asiegbu et al. 2005).

The *H. annosum* complex consists of various types representing intersterility groups with different primary hosts and is distributed almost all over the world (Figure 1). In Europe the *H. annosum* complex consists of three different species (Niemäla and Korhonen 1998): the European P (=Pine) group, *H. annosum* (Fr.: Fr.) Bref. s. str., prefers pine species with Scots pine, *Pinus sylvestris* L, as the most important host; the F (=Fir) group, *H. abietinum* Niemelä & Korhonen, prefers fir species (*Abies* ssp.) in southern and central Europe; the S (=Spruce) group, *H. parviporum* Niemelä & Korhonen, prefers spruces, with the exception in north-eastern Europe, where the fungus attacks *Abies sibirica* Ledeb. (Korhonen et al. 1998).

Infection by *Heterobasidion* causes a root and butt rot by penetrating healthy trees in two different ways. The primary infection pathway takes place via the release of basidiospores, which can infect fresh wounds, roots and freshly cut stumps in an area of several kilometers (Rishbeth 1951; Kallio 1970; Korhonen).

The maximum spore discharge may occur the whole day and the highest density during summer with up to 1550 spores m^2/h in subalpine forests (Gonthier et al. 2005; Wood 1996; Kallio 1970; Sinclair 1964; Schmidt and Wood 1972; Rocket and Kramer 1974; Edmonds et al. 1984). The anamorphic stage of the fungus (conidia of *Spiniger meineckellus* Stalpers) can also be relevant for the infection pathway, but

the basidiospores continuously released by the basidiocarps provide the primary inoculum and replace the infection by conidia (Rishbeth 1952; Kuhlmann and Hendrix 1964; Redfern and Stenlid 1998).

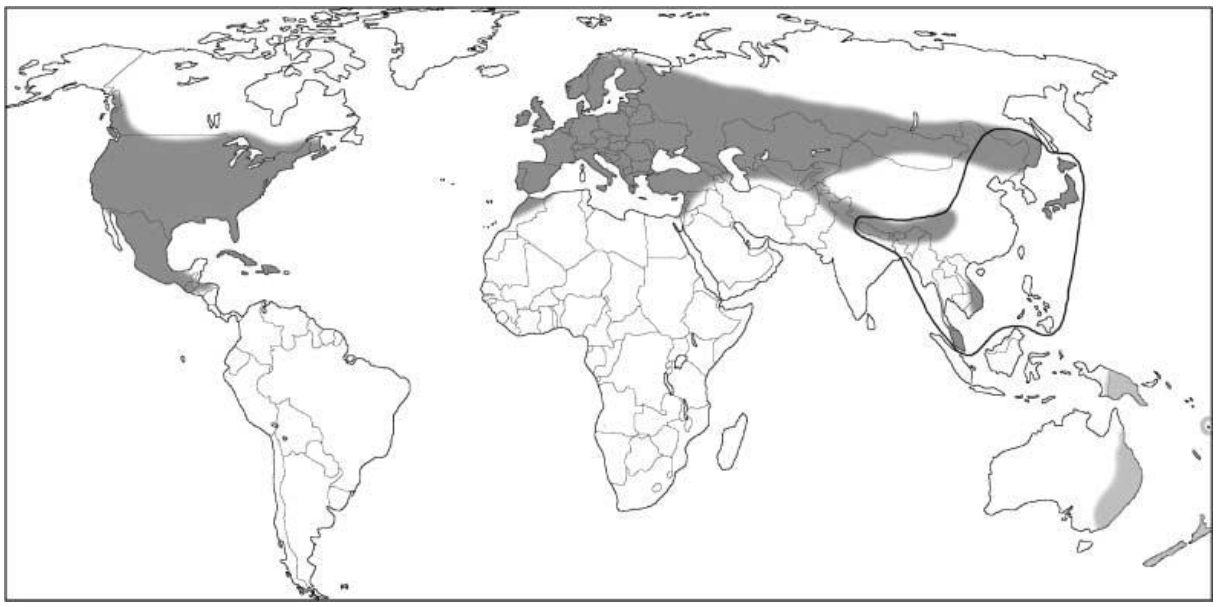

Figure 1: Distribution of the *H. annosum* complex (dark shaded areas) (K. Korhonen in Asiegbu et al. 2005).

The fungus grows from the stump surface down into the lateral roots and there the second pathway takes place by infection of the neighboring trees via root contact (Redfern and Stenlid 1998).

In managed forest systems, the fungus finds an excellent habitat for spreading and colonization as logging, damage from tree harvesting and skidding, provide wounds for infection. Since Rishbeth (1952) recognized that freshly cut stump surfaces play a major role in the epidemiology of *H. annosum*, many studies were conducted e.g. Thor and Stenlid 2005; Redfern and Stenlid 1998.

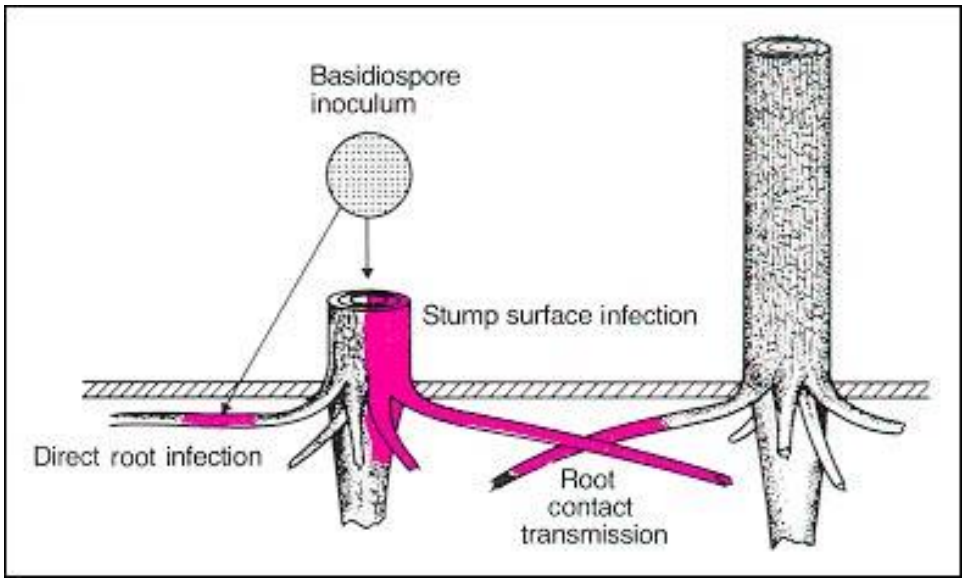

Figure 2: The different infection pathways of *H. annosum* sensu lato (dnr.wi.gov).

1.2 *Heterobasidion*- control by chemical and biological methods

Control of *H. annosum* has been practiced for many years. Since the infection pathway follows freshly cut stumps free from rot and moves on by root contacts to neighboring trees, the main objective is to protect healthy stumps against an infection by airborne basidiospores. This can be achieved by spraying or painting a spore suspension or chemical compound on the whole stump surface without contamination of the surrounding area (Pratt et al. 1998).

The chemical stump treatment is a preventive method and is practiced in conifer stands where *H. annosum* is missing (Pratt et al. 1998). Chemicals are for example: disodium octaboratetetrahydrate, propiconazole and urea (Olivia et al. 2008; Varese et al. 1999; Westlund and Nohrstedt 2000; Pratt et al. 1998). For example, Olivia et al. (2008) showed that urea treatment is a reliable and persistent protection method against *H. annosum*. However, chemical treatments can be a hazard for the environment, especially when automatically application systems via built-in nozzles in chainsaw and harvester head are used, who can't guarantee a treatment without contamination of the surrounding area (Pratt et al. 1998). Chemicals like borate and urea can cause acute damage to the ground-vegetation and also change soil chemistry or the fungal community of a forest (Westlund and Nohrstedt 2000;

Vasiliauskas et al. 2004). In order to prevent such ecological hazard, biological control methods are the only alternative in ecologically sensitive natural forests as the Central European Alpine spruce forests usually are.

Biological control agents like antagonists are a principal method to combat plant pathogens (Hayek 2004) and the interaction between both can be effected in different ways. The antagonist can (1) suppress and exclude the pathogen through the competition for space and nutrients, (2) produce antibiotics to kill the competitor, or (3) directly destroy the pathogen (Campbell 1989).

Such a competition between pathogen and antagonist has been used to control the infection of *H. annosum* in forests since Rishbeth (1950) provided first evidence of its effectiveness in the UK. Examples for fungi tested for competition against *H. annosum* are *Phlebiopsis gigantea* (Fr.: Fr.) S.S. Rattan et al., *Trichoderma harzianum* Rifai, *Hypholoma fasciculare* (Huds.: Fr.) P. Kumm., *Verticillium bulbillosum W. Gams & Malla* and others (Rishbeth 1963; Nicolotti and Varese 1996; Varese et al. 1999, 2003; Nicolotti et al. 1999; Roy et al. 2003). All showed good results to control the pathogen. However, *P. gigantea* is the most widely used antagonist and was also the first biological control agent to combat Heterobasidion (Hayek 2004). The ability of *P. gigantea* to colonize rapidly fresh wood surfaces and its strong antagonistic reactions against *Heterobasidion* were the main reasons for choosing it as nearly unique biological control agent against *Heterobasidion* (Drenkhan et al. 2008).

1.3 *Phlebiopsis gigantea* as antagonist

P. gigantea occurs as a white rot fungus colonizing dead wood and the rhizosphere of conifer forests (Asiegbu et al. 1996).The fungus grows in a flat manner on the surface with a pinkish-buff or marble grey color and forms large fruiting bodies on stumps, fallen trunks and log piles (Cram 2014; Holdenrieder and Greig 1998). Fructification with flat basidiocarps takes place within one year after infection and can continue for up to three years (Rishbeth 1963). The fruiting body produces basidiospores which become airborne and are distributed by wind. In the asexual lifecycle *P.gigantea* produces spores (oidia) that develop by segmentation of hyphae (Holdenrieder and Greig 1998).

The hyphae *of P. gigantea* can change the structure of neighboring *H. annosum* hyphae by penetration of cells, granulation and vacuolation of the cytoplasm and loss of opacity of the cells (Ikedingwu et al. 1970; Ikedingwu 1976). However, the most frequently cited reason for the efficiency of *P. gigantea* is the competition for resources and higher nutrition acquisition (Holdenrieder and Greig 1998; Adomas et al. 2006 and Asiegbu et al. 2005).

1.4 Molecular identification of fungi

In natural systems like forests the fungal species composition is usually assessed by the presence or absence of fruiting bodies in the field (Rajala et al. 2011; Renvall et al. 1991). However, fruiting bodies are formed only under certain environmental conditions and also identification of mycelia isolated from wood is difficult even for trained mycologists (Johannesson and Stenlid 1999). A molecular biology technique like sequencing of a particular gene-locus, which is assumed to be species specific, is an efficient alternative to identify fungal diversity in forests (Khaund and Joshi 2014). This method is called DNA barcoding (Schoch et al. 2012) and is commonly practiced nowadays. The two frequently used barcodes for the identification process of species are:

(1) The region of the mitochondrial gene encoding the cytochrome c oxidase subunit I (COI) as a universally used barcode for animals or plants also appropriate for some fungi (Seifert et al. 2007; Hebert et. al 2003).

(2) The internal transcribed spacer region ITS, containing two variable non-coding regions, which offer several advantages like high information content and ease of amplification, was designed as a barcode for fungi (Schoch et al. 2012)

Bergerow et al. (2010) pronounced that the COI region will never be a useful fungal primer posing problems in primer design. Thus the authors suggest the ITS region as the most useful barcode primer for the identification of fungi.

2. Aims of the Thesis

This master thesis was designed to evaluate the persistence of *Phlebiopsis gigantea* in a subalpine forest on an experimental plot established by the Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection. There freshly cut stumps had been treated against *Heterobasidion parviporum* with two biological control agents i.e. *P. gigantea* in 2006.

Three questions were posed:

How long is the persistence of *P. gigantea* after a root rot treatment in subalpine forests?

To answer these question wood cores were taken and morphological as well as molecular techniques were conducted to identify the fungus present in the cores.

What are the follow-up-colonizers in the wood stumps after *P. gigantea* application?

Besides morphological identification of the fungal isolates, DNA extraction from mycelia and wood was performed and PCR amplicons of the samples were sequenced to determine the different fungal species via DNA barcoding.

Can *H. parviporum* and its antagonist *P. gigantea* be detected in lower parts of the stump?

Cores were taken from different levels of the stump to include the role of the roots as infection source via molecular identification.

3. Material and Methods

3.1 Experimental Site and Preceding Antagonist Treatment by BFW

The study area was the Defereggen valley near to St. Veit in East Tyrol, Austria. The basic experiment by the Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), Department of Forest Protection was conducted in a protection forest with a stand age of 170-200 years and a tree distribution of 90% spruce and 10% larch. From the geological background the area is part of the East Alpine crystalline. Paragneiss and mica schist dominate as bedrock covered by a semi podsolic soil. The occurrence of *Heterobasidion annosum* was screened by cutting wood discs, which were incubated in the lab for production of the anamorphic stage *Spiniger meineckellus* and by an initial assessment of the presence of H. fruiting bodies on the site. The identification of the intersterility group of the *H. annosum* complex was performed by Prof. Paolo Gonthier (University of Turin) who confirmed the presence of the S-type (*H. parviporum*), and *Phlebiopsis gigantea* was chosen and tested as antagonist (Cech and Steyrer 2006).

On four different sample plots at an altitude between 1680m and 1950m a.s.l. applications of the antagonist were performed after a thinning event in 2006. The spruce stumps were treated with the commercial *P. gigantea* Rotstop® (Verdera) and another formulation of *P. gigantea* from Poland (Dr. Lakomy, University of Posznan). The suspension of Rotstop® was prepared according to the manual instructions, 1 l/1 g of powder. *P. gigantea* was applied by using 200g of the fungus-sawdust mixture and 5 l water. After the treatment the stumps were covered with soil and humus (Cech and Steyrer 2006).

3.2 Sample Collection and Culturing

Seven years later, in 2013, in total 215 wood cores were collected from all four experimental plots by using an increment borer (Table 1). In order to prevent contamination, the borer was cleaned in 70% ethanol before switching to a new stump surface. The samples were taken in three different depths of the spruce stump

(20 cm, 50 cm and 100 cm) and placed in a polythene bag. The cores had a length of up to 20 cm and a diameter of 5 mm. After collection, each sample was labeled and stored in a freezer at 4°C.

Table 1 Number of wood cores from three different layers of the stump.

Method/depth	20cm	50cm	100cm
Rotstop®	44	22	10
P.gigantea Poland	43	27	11
Control	40	17	4
Total	127	66	25

In the lab the wood cores were plated on malt extract agar (20 g/l malt extract, 15 g/l agar) 230 mg/l containing Thiabendazol in 1ml conc. lactic acid added after autoclaving (Holdenrieder et al. 1994). Every 14 days over a time period of 70 days the petri dishes were checked for mycelial growth. From the growing mycelia were isolated for producing a pure cultures grown were gained by transplanting them onto 2% malt extract agar.

3.3 Morphological identification of fungi

The morphological identification was concentrated on the detection of the two species *H. parviporum* and *P. gigantea*. The first step of the identification process was the classification of the color of mycelia. The pure cultures containing white mycelia were checked under a microscope considering the following different culture features: *H. parviporum* grows on malt extract agar first with white mycelium which later turns to a brown color. After a few days the fungus starts with the production of the characteristic asexual spores. These spores develop on club-shaped conidiophores with many characteristic sterigmata and pointed-oval blastospores (Holdenrieder 1982). *P. gigantea* also produces asexual spores (oidiospores, Figure 3) that develop from segmentation of the hyphae (Holdenrieder and Greig 1998).

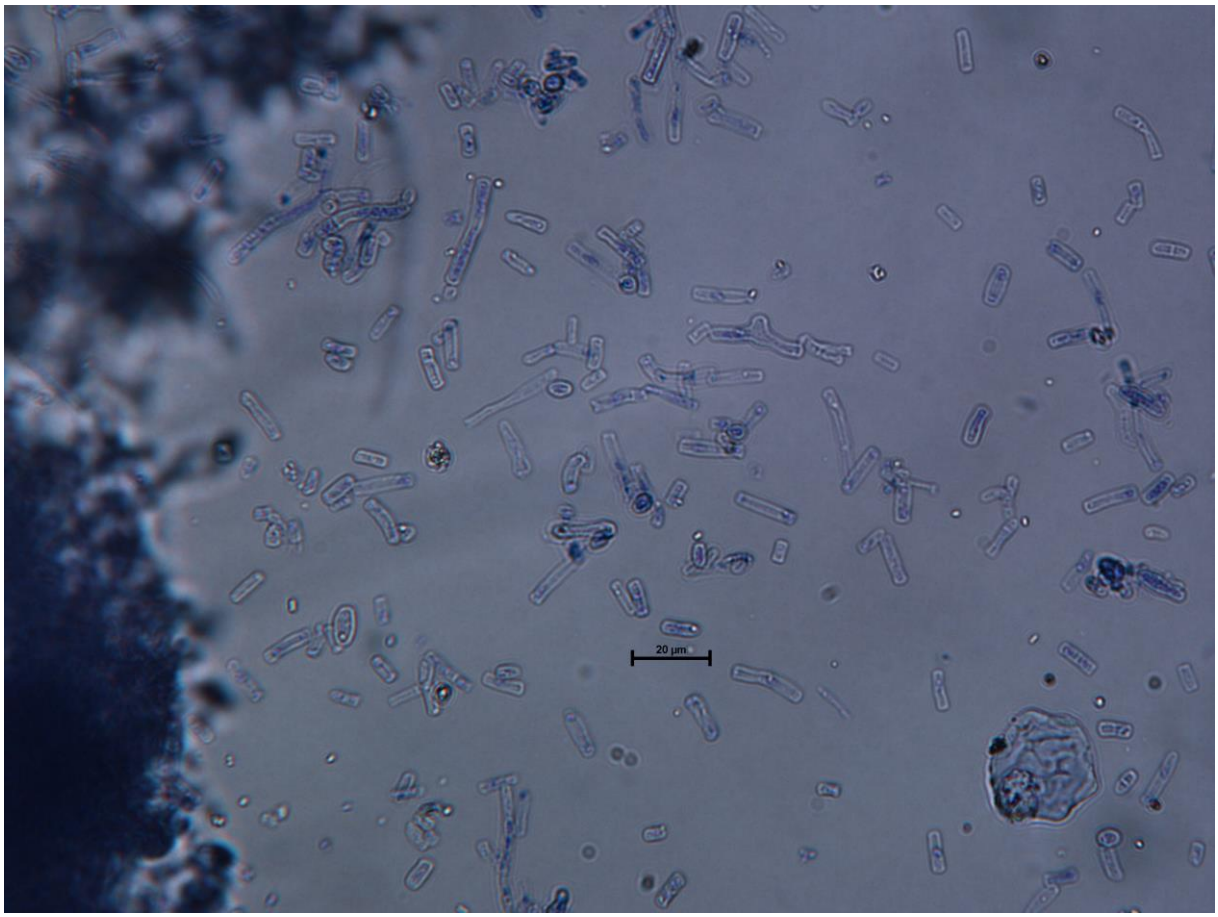

Figure 3: Oidiospores of *Phlebiopsis gigantea.*

3.4 Molecular identification of fungi

3.4.1 DNA extraction

Fungal DNA from wood cores and mycelia were extracted using the DNeasy® Plant Mini Kit (Qiagen). The wood cores were grinded by using liquid nitrogen and placed in a 2ml Eppendorf tube. A 5mm steel bead was added and in 3x 1 min steps, the samples were homogenized with a ball mill (MM2000, Retsch GmbH). 400 µl lysis solution and 4 µl RNase were added and incubated overnight at 400rpm at 55°C. For mycelia the homogenization step was only 2 x 1min using steel beads and the mixture was incubated for 10 min at 65°C. After incubation 130 µl neutralization buffer was added and some centrifugation steps were performed following the manufactory's protocol. The DNA in the remaining spin column was eluted with 100 µl elution solution and stored at 4°C.

3.4.2 PCR with general ITS primer

In order to distinguish the different fungi, the primer ITS1 and ITS4 (White et al. 1990) were used. The reactions were set up in 20 µl volumes containing, 1x NH_4 buffer, 2mM $MgCl_2$, 100 µM dNTPs, 0,3 µM of each primer, 0,1 U Taq polymerase and 1 µl of the template DNA. Amplification was performed in a 2720 thermal cycler (Applied Biosystems) with denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension 1 min at 72°C with 35 cycles. An initial cycle employed a 2 min denaturation at 94°C and a final cycle had an extension step of 72°C for 7 min. Agarose gel electrophoresis was performed to look up if the PCR succeeded. Gels with a 2% agarose concentration were used and DNA was visualized on a UV transilluminator. Sequencing of PCR products were performed by Eurofins (Ebersberg, Germany) and for the sequences of the chromatograms with single peaks a BLAST search in the Genbank was conducted.

3.5 Cloning

To determine samples with ambiguous sequence chromatograms cloning was performed. The working protocol is listed in appendix I. For cloning 0,8 µl of the fresh PCR product was mixed with 0,2 µl of the vector pTZ57R (Fermentas, Lithuania) (Figure 4), 0,3µl polyethylenglycol (PEG3350), 0,3 µl T4 buffer and 0,1 µl ligase. Afterwards the mixture was incubated at 15°C over night.

Transformation was performed with competent JM109 *Eschericha coli* cells. On each ligation-preparation, 50 µl of *E. coli* were pipetted, carefully vortexed and placed on ice for 20 min. Subsequently the samples were heated for 50 sec at 42°C in a water bath. After 2min on ice, 950 µl SOC-media were added and placed at 37°C for 60 min. The samples were centrifuged for 5 min at 4°C at 2500rpm. Supernatant was discarded and the remaining bacteria were pipetted on an agar media containing X-Gal, ampicillin and IPTG for Blue/White screening (Figure 5). The petri dishes were stored at 37°C over night. On the next day plates were checked for white bacteria colonies. These bacteria colonies were marked and transferred with a toothpick in a small tube containing LB broth and ampicillin (Sambrook et al. 1989). A PCR was done with M13 Primers (Appendix II). The agarose gel electrophoresis showed those

bacteria containing plasmids with PCR products. Purification and first generation sequencing was done by Eurofins.

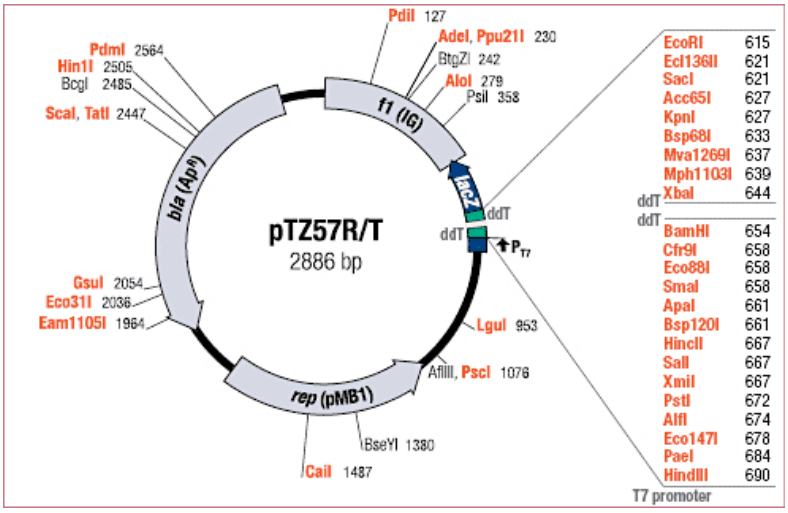

Figure 4: The vector used for cloning (source: thermoscientificbio.com).

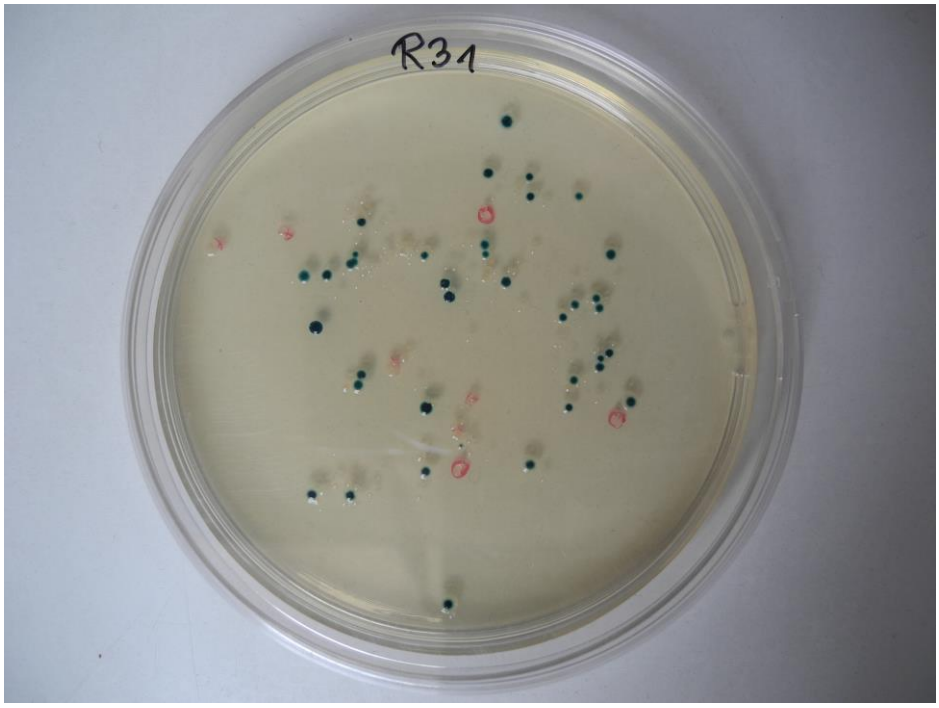

Figure 5: Cloning with blue and white colonies.

4. Results and discussion

4.1 Number of isolates and morphological detection

From the 215 wood cores taken from in total 127 spruce stumps, only 95 (44,2%) demonstrated mycelial growth. Holdenrieder et al. (1994) reported a similar isolation rate and showed that this rate depends on various factors: Taking wood cores with the increment borer produces heat which can lead to a sterilization of mycelium or spores (Holdenrieder et al. 1994). Furthermore environmental conditions play a crucial role in the development of fungi (Kües and Liu 2000). For example, changes in the natural environment can modify the micro-climatic conditions, especially in sun-exposed subalpine areas. The increased radiation in summer leads to an optimal habitat for dry and heat associated fungi like *Gloeophyllum sepiarium*, whereas other fungi would not grow, so that a shift in the fungal diversity may occur (Carlsson et al. 2014). In addition, the probability, that a spore reaches a suitable substrate is generally low and finally the chance of development decreases drastically when the spore is not adapted to environmental factors (Boddy et al. 2009). As an example UV radiation can decrease the vitality of a hyaline spore to 5%, whereas a pigmented spore can survive for a longer time and also maintains a higher vitality (Kallio 1973; Burnett 2003).

Table 2 Total number of wood cores listed by depth and treatment (red numbers indicate the cores with mycelial growth).

	20cm	**50cm**	**100cm**	**Total**	**Fruiting bodies**
Rotstop®	44/22	21/3	10/1	76/26	16
P.gigantea Poland	43/33	26/4	10/0	81/37	11
Control	40/30	17/3	4/0	61/33	15
Total	**127/85**	**64/9**	**24/1**	**215/95**	**42**

The stump surface of 42 samples showed only few fruiting bodies of *Gloeophyllum sepiarium* and *Armillaria sp.* (Figure 6).

The morphological method by searching the asexual spores could not detect the antagonist *P. gigantea* nor the pathogen *H. parviporum*. Also molecular methods were assessed to exclude the contamination with other fungi and detect the fungal competition in the wood.

Figure 6: stumps with fruiting bodies of *Gloeophyllum sepiarium* (left) and rhizomorphs of *Armillaria sp.* (right).

4.2 Molecular Analysis of the isolated fungi

4.2.1 PCR with fungal specific ITS primers

The plates with mycelia growth were screened for the fungal species with molecular methods. Therefore, the locus specific primer ITS1 and ITS 4 (White et al. 1990) were used. DNA was directly extracted from wood and mycelia.

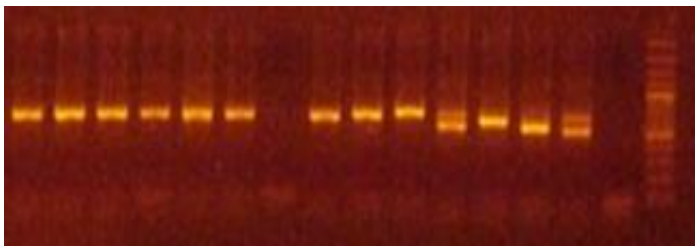

Figure 7: Figure 7: Agarose gel electrophoresis of the PCR with ITS1 and ITS 4 of fungal isolates.

From the 96 DNA extractions, 80 samples showed clear amplicons on the agarose gel electrophoresis indicating that the ITS primers were well suited for the work (Figure 7). The different layers of the wood showed various results. From the 20 cm layer, 75 showed clear bands, whereas from 50 cm four, and from 100 cm only one indicated a successful PCR amplification. Generally 16 samples (16.8 %) of the amplifications did not work. Here DNA concentration/quality of the samples might have been too low or other factors like annealing temperature might have been not optimal. In addition some sequence chromatograms showed no clear peaks when the DNA was extracted directly from the wood core (Figure 8). In this case cloning was performed to obtain clear sequences. The chromatograms with single peaks were analyzed for the fungal species via BLAST search in the NCBI Database.

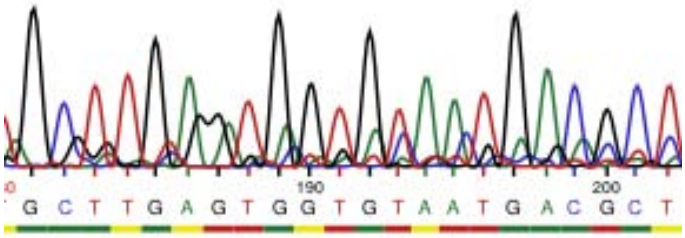

Figure 8: Sequence chromatogram showing ambiguous peaks.

4.2.2 Cloning

Eleven isolates of woody DNA showed ambiguous sequences (Figure 8) and thus cloning was performed. The blue/white selection showed only for six isolates clear colonies. Those white ones were picked and plasmids were screened for positive inserts via M13 PCR (Figure 9).

The sequencing of the PCR products indicated good chromatograms with single peaks. The identified species belonged to species *Candida sp*. (R18), *Lecytophora sp*. (R18, R43), *Phlebiopsis gigantea* (R31), *Rhodoturala sp*. (R43), *Scleroconidioma sphagnicola* (R60).

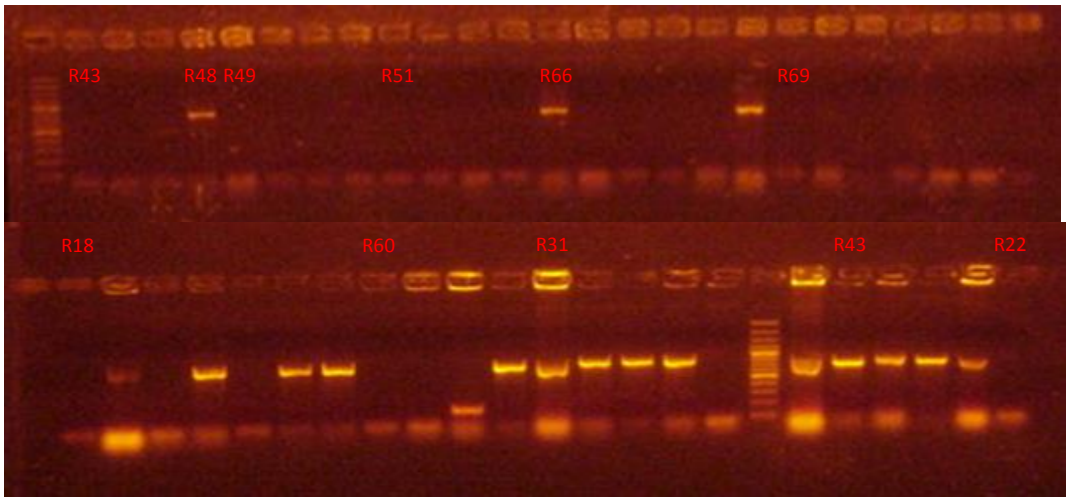

Figure 9: Agarose gel electrophoresis with M13 primer.

4.3 Detection of *P. gigantea*

Figure 10 shows the isolation success and molecular detection of the antagonist *P. gigantea*. One to two years after the stump treatment the Polish strain of the fungus could be found in 100% of the collected wood discs (Cech and Steyrer 2006). On the other hand, Rotstop® was detected only in 60% of the stumps after one year and surprisingly, this rate slightly increased to 78% one year later. In 2013 a total of two samples contained the fungus and in each treatment (Rotstop® and *P. gigantea* from Poland) one single strain was found. The control contained *P. gigantea* in the first three years (2006, 2007, 2008), but not anymore in 2013 (Cech and Steyrer 2006).

The studies conducted by Cech and Steyrer (2006) showed a high percentage of the antagonist *P. gigantea* one to two years after treatment, a phenomenon, which was observed by Vainio et al. (2001) as well. However, after six years, they detected the fungus in 53% of the treated Norway spruce stumps. Terhonen et al. (2013) showed using next generation sequencing that 13 years after a treatment *P. gigantea* was absent. In our experiment, a spread of the biocontrol agent to non-treated trees was observed only during the first years, which coincides with the assumptions by Menkis et al. (2012): however after seven years the fungus was absent.

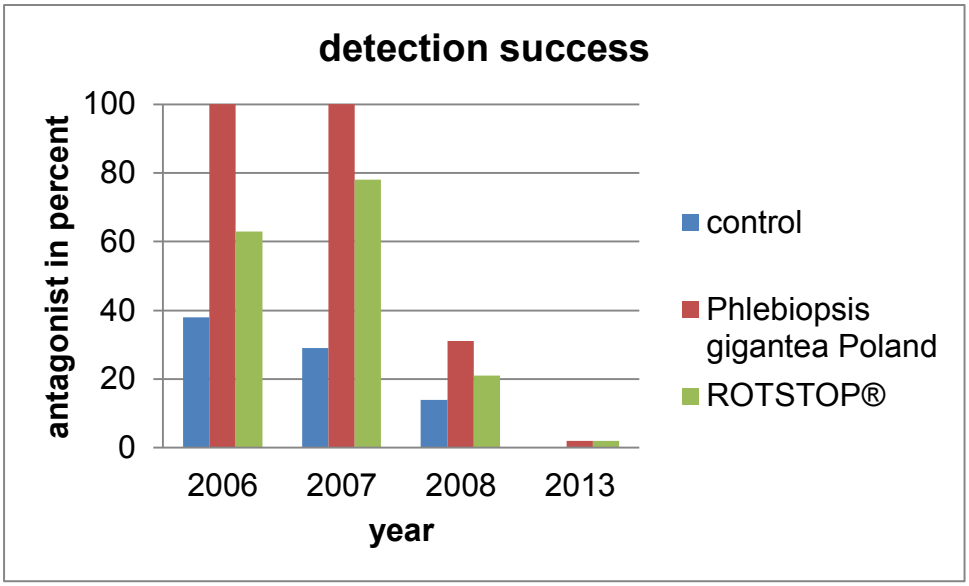

Figure 10: Detection success of the antagonist from wood.

4.4 Verification of the presence of *Heterobasidion parviporum*

H. parviporum could not be detected in any sample including the control. This might lead to the assumption that the infection rate by airborne spores at a higher altitude is very low in summer. By contrast, Nicolotti and Gonthier (2005) showed that in the Western Alps both a high spore intensity and a high infection frequency of *H. parviporum* in summer was recorded. This was explained by the environmental conditions and the regional selective cutting practices in summer which are favoring infection by this species (Nicolotti and Gonthier 2005). Moreover, Gonthier (2001; et al. 2005) found that even the highest inoculum density of *Heterobasidion* spores appears in summer and he concluded that *H. parviporum* is well adapted to the weather conditions at high altitudes. By contrast, though Cech and Steyrer (2006) demonstrated that *H. parviporum* was present in the experimental site, they found only few fruiting bodies and isolated the asexual *Spiniger meineckellus* in low frequency. This finding is likely to be a consequence of several environmental factors as nutrition, pH-value and temperature (Johannesson and Stenlid 1999; Piri 1998; Courtious 1972; Zweck and Hüttermann 1980; Gibbs 2002). A further problem is the mycelial competition of pathogen and antagonist. *H. parviporum* generally grows faster into the wood than *P. gigantea* (Sun et al. 2009; Rönnberg and Cleary 2012;

Holdenrieder and Greig 1998) expanding to lower parts of the stump and the roots, remaining there alive for several years (Piri 1996). When both species colonize a stump similarly, *H. parviporum* colonizes deeper layers of the stump quicker than *P. gigantea* without being suppressed by the latter (Berglund and Rönnberg 2004). In addition, Holdenrieder (1982) showed that *Heterobasidion* is generally more competitive in deeper layers of the wood than closer to the stump surface. This observation was the reason, why in the present experiment cores were taken from 50-100cm depth to detect the spread of the fungus there: however, neither the pathogen nor *Phlebiopsis* could be found. Regarding the spread of the antagonist in roots, Tubby et al. (2008) came to the result, that this is less effective in spruce than in pine. For the actual experiment, the possibility that the pathogen is present in roots, cannot be excluded, since only a few samples were taken and the isolation success was only 15%. However, no fruiting bodies could be detected on the roots and also no rot could be found in the deeper parts, which indicates that no colonization of these parts had occurred.

The infection rate of *Heterobasidion* is strongly dependent on many factors. First of all, climatic parameters, like high annual rainfall and high air temperature can decrease the incidence of infection by a negative influence on the sporulation of the fungus (Puddu et al. 2003; Redfern and Stenlid 1998). In addition to the climate, radiation, especially at a high altitude, may influence the airborne spore infection at a high level. Also the initial infection intensity (colony size) can influence the survival of *Heterobasidion* (Morrison et al.1986; Bendz-Hellgren and Stenlid 1998). Furthermore diameter, age of the infected tree, virulence, competition with other fungi and defense mechanisms of the host can also affect the process of colonization by *H. parviporum* (Korhonen and Stenlid 1998; Hüttermann and Woodward 1998; Asiegbu et al. 2005; Paludan 1966 and Holdenrieder and Greig 1998).

4.5 Follow up colonizers

4.5.1 Fungal species identified by molecular methods

The fungi identified from 20 cm depth comprise 33 species differentiated into 20,5% basidiomycetes, 8,6% ascomycetes,0,9% hyphomycetes and 4,5% unidentified fungi (Table 2 and Figure 11)

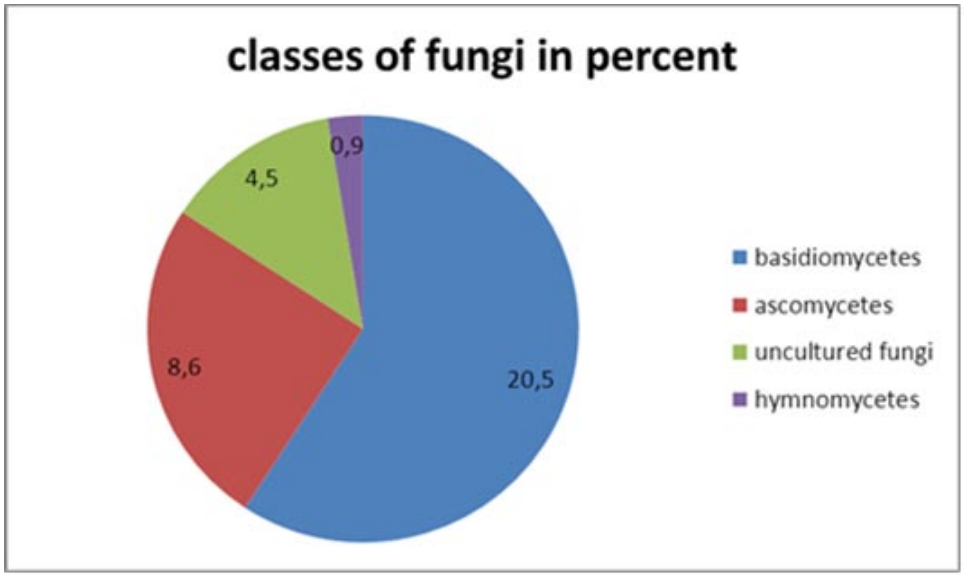

Figure 11: classes of identified fungi from 20cm deep wood cores.

The saprophytic fungus *Gloeophyllum sepiarium* was the most frequently detected species and this could be explained by the changed climatic factors caused by the clear-cuts. The occurrence of many wood inhabiting fungi depends on features like micro-climate and stage of decomposition of the wood (Rayner and Boddy 1988, Niemelä et al. 1995; Renvall 1995). The dark brown hyphae of this genus are resistant to radiation, drought and higher temperatures (Holdenrieder 1982; Carlsson et al. 2014). Furthermore, the fungus demonstrates a strong antifungal activity (Nakajima et al. 1976; Holdenrieder 1982).

Table 3 different identified fungal species from 20cm deep wood.

Species	Class	Treatment		
		P.gigantea	Rotsto®	C
Unspecified fungi	Unknown	2	6	2
Columnocystis abietina (Pers.) Pouzar	B	2	-	-
Entomocorticium sp.	B	1	-	-
Fomes fomentarius (L.: Fr.) J.J. Kickx	B	1	-	3
Fomitopsis pinicola (Sw.: Fr.) P. Karst.	B	1	-	1
Hyphoderma sp.	B	1	2	1
Lecythophora sp.	A	1	-	-
Lipomyces sp.	A	1	-	-
Gloeophyllum sepiarium (Wulfen: Fr.) P. Karst.	B	8	4	1
Peniophora sp.	B	1	-	-
Phialocephala fortinii	A	1	-	-
Phialocephala lagerbergii	A	1	-	1
Phlebiopsis gigantea (Fr.) Jülich, Persoonia	B	1	1	-
Resinicium bicolor (Alb. & Schwein.) Parmasto	B	2	-	-
Stereum sanguinolentum (Alb. & Schwein.) Fr.	B	2	1	-
Rhinocladiella sp.	A	1	1	2
Rhodotorula sp.	B	-	2	-
Pholiota sp.	B	-	1	-
Antrodia serialis (Fr.) Donk	B	1	-	1
Penicillium sp.	B	-	-	1
Ceriporia lacerata Maek., Suhara & R. Kondo	B	-	-	1
Rhinocladiella atrovirens Nannf.	A	-	-	1
Geomyces sp.	A	-	-	1
Alternaria alternata (Fr.) Keissl.	A	-	-	1
Gymnopilus penetrans (Fr.) Murrill	B	-	-	1
Candida sp.	A	1	-	-
Schizopora flavipora (Berk. & M.A. Curtis ex Cooke) Ryvarden	B	1	-	-
Pichia fermentans Lodder	A	1	-	-
Scleroconidiomata sphagnicola Tsuneda, Currah & Thormann	H	2	-	-
Cladosporium herbarum Link	A	-	-	1
Kuraisha sp.	A	-	-	1
Phlebia radiata Fr.	B	-	-	2
Talaromyces sp.	A	-	-	1
Total		33	18	24

A- ascomycetes, B- basidiomycetes, H- hyphomycetes, C-control

The white rot fungus *Fomes fomentarius* could be identified via ITS- sequencing on the spruce stumps. This fungus normally occurs on hardwood, for example beech but it is sometimes also found on coniferous hosts (Suvorov 1967). Furthermore, the presence of some birches around the site could explain the presence of the fungus.

Fomitopsis pinicola and *Resinicium bicolor* could also be identified as wood colonizers. Both are known to occur commonly on spruce stumps and were also tested for antagonism against Heterobasidion (Holdenrieder and Greig 1998). *R. bicolor* recently showed that it can reduce the incidence of Heterobasidion root rot in nature, whereas *F. pinicola* only in vitro indicated antifungal activities (Holdenrieder and Greig 1998; Kirby et al. 1990; Holdenrieder 1982)

Normally ascomycetes are the primary colonizers of wood followed by filamentous basidiomycetes like *Fomitopsis pinicola* or *Resinicium bicolor*. However, yeasts, which belong also to the basidiomyctes appear frequently after one year (Terhonen et al 2013). Over time the yeasts disappeared and, in this study after seven years only *Candida sp.* could be detected, which matches with the study of Terhonen et al. (2013).

Ascomycetes are common colonizers of plant tissues in subalpine areas. *Phialocephala fortinii* for example is an endophyte of roots in spruce with the ability to form ectomycorrhiza (Fernando and Currah 1996; Wilcox and Wang 1987).

Some further fungi are known to be associated with bark beetles: *Kuraisha capsulata* and *Talaromyces* ssp. are associated with *Ips typographus* (Giordano et al. 2012; Leufvén and Nehls 1986), which is common in subalpine forests in Tyrol.

From the wood cores of the deeper layers only four fungi could be detected: *Diplomitoporus lindbladii* (50cm) and three fungi of the genera *Cladosporium sp.* (50cm), *Entomocorticium sp.* (100cm), and *Rhinocladiella sp.* (50cm).

4.5.2 Fungal diversity per treatment

The composition of fungi in forests is an important ecological factor. The saprophytic genera providing nutrient ressources and the decomposing effects are necessary for an intact enviromental system.

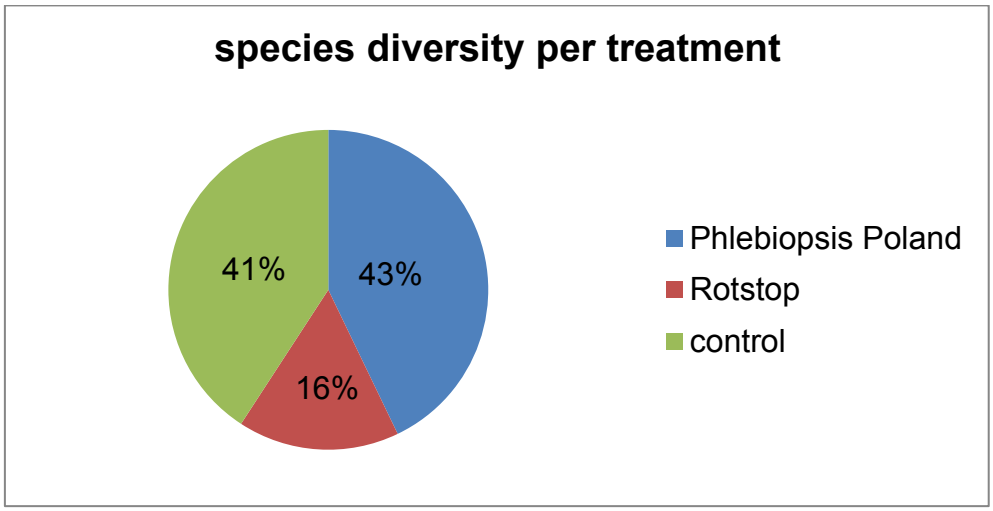

Figure 12: Fungal diversity in percent listed by the root rot treatment

Various studies described changes of the fungal community by using *P. gigantea* as a biological agent against *Heterobasidion* (Vasiliauskas et al. 2004). On the other hand Holdenrieder (1982) stated that the biological control with antagonists does not affect the naturally occurring composers.

In this study Rotstop® treatment decreased drastically the fungal diversity to 16% of all detected species, whilst the treatment with the other strain of *Phlebiopsis* had no influence on the fungal biodiversity (Figure 12). Our results indicate that a treatment with the commercial product Rotstop® may decrease the fungal biodiversity, which was also confirmed by other studies (Vasiliauskas et al. 2004, 2005; Vainio et al. 2005). However, the molecular detection technique investigated in this study covers only the tolerant fast-growing fungi, which occur in a higher DNA concentration, whereas the slow-growing or uncultivable fungi undoubtedly break away from detection (Hyde and Soytong 2008). Furthermore, the medium for cultivation contains

the fungicide thiabendazol, which has no effect on basidiomycetes, but inhibit other classes, what makes it difficult to give a clear statement about the diversity. Furthermore, the ITS region is not species specific for some fungal groups and have also a range of biases (Schoch et al. 2012; Cräutlein et al. 2011). In the species-rich Ascomycetes (*Cladosporium, Fusarium* and *Penicillium*) for example, the internal transcribed spacer region often shows a variation, what makes it difficult to identify the fungus on species level (Seifert 2009; Schubert et al. 2007; Skouboe et al. 1999; O`Donnell and Cigelnik 1997). In addition Bellemain et al. (2010) described different biases like length biases, taxonomic biases and primer mismatch biases which could be introduced during PCR. She also showed that the ITS1-F primer amplifies plant sequences after removing the fungal sequence what can lead to errors. Therefore, high-throughput sequencing like 454-pyrosequencing direct from the wood is a new alternative to cover the whole diversity. Terhonen et al. (2013) investigated this new technique and detected a total of 53.117 fungal sequences, whereas in this project only 75 sequences could be obtained by sequencing and cloning. However, to detect the whole diversity a mix of molecular and morphological techniques has to be combined.

5. Conclusions

In the present study *Heterobasidion parviporum* could not be detected seven years after root rot treatment indicating that the antagonistic fungus *Phlebiopsis gigantea* is a good control agent in subalpine spruce forests. However, the persistence of the antagonist and the competition success against saprotrophic fungi was relatively low and the persistence decreased drastically even during the first three years after the treatment. For deeper layers of the spruce stump, a competition between pathogen and antagonist could not be detected. Nevertheless for this crucial infection pathway more experiments have to be carried out and more concentration has been given on the pathogen spread via root contact.

Several studies indicated that *P. gigantea* is a better competitor in pine stumps than in Norway spruce (Holdenrieder 1982). Therefore, experimental plots should be established to track other naturally occurring competitors against *Heterobasidion* in subalpine spruce forests in Austria. The Basidiomycetes of subalpine spruce forests like *Fomitopsis pinicola* or *Resinicium bicolor,* which have been described also as antagonists against *H. annosum* as well as *Gloeophyllum sepiarium* or some species of *Trichoderma* could be alternative biological control agents.

The fungal diversity is another factor in the system of natural competition. The root rot treatment with the commercial strain of *P. gigantea* Rotstop® showed a decrease in the biodiversity of the other fungal species, whereas the *P. gigantea* agent from Poland had no influence. However, the introduction of a non-natural occurring agent isolated from a foreign region has to be considered as critical. Therefore, the production of an Austrian strain of *P. gigantea* isolated from spruce seems to be the best alternative as biocontrol agent.

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Appendix I DNA extraction (DNeasy® Plant Mini Kit, Qiagen)

- Homogenize samples with steel beads in an Eppendorf vial (1.5 ml).
- Add 400 µl buffer AP1 and 4µl RNase A. Vortex and incubate for 10 min at 65°C for mycelia and one day at 55° C. Invert the tube 2-3 times during incubation.
- Add 130 µl Buffer P3. Mix and incubate for 5 min on ice
- Centrifuge the lysate for 5 min at 20.000 x g (14.000 rpm)
- Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20.000 x g.
- Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.
- Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.
- Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at 6000 x g. Discard the flow-through
- Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20.000 x g. Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through
- Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
- Add 50 µl Buffer AE for elution. Incubate for 5 min at room temperature (15-25°C). Centrifuge for 1 min at 6000 x g. Repeat this step.

Appendix II Cloning

Day 1

- mix fresh PCR product with 1,4 µl H2O, 0,1 µl ptZ57R, 0,3 µl PEG3350, 0,3µl T4 Buffer, 0,1 µl T4 ligase
- add 0,8 µl DNA
- incubate over night

Day 2

- thaw 35 µl competent cells per reaction on ice
- pre-cool ligation reaction mixtures on ice in 0.5 ml reaction tubes
- add competent cells to the ligations
- incubate on ice for 20 min
- heat shock bacterial suspensions in a 42 °C hot water bath for 50 sec
- put reactions back on ice for 1-2 min immediately
- add 300 µl of SOC medium were added to each tube
- incubate at 37 °C for 1-2 hrs
- prepare LB-Amp plates in the meantime: plate 40 µl X-Gal (20 mg/ml) and 40 µl IPTG (24 mg/ml) on each plate with a Drigalski spatula
- plate transformation reactions on the plates
- incubate upside down over night at 37 °C

Day 3

- transfer 0.5 ml up to 2 ml of overnight E. coli cultures into 1.5 ml reaction tubes.
- tip with a sterile toothpick
- transfer into Eppendorf tubes (containing master mix for PCR) and a cap-o-test vial containing 3 ml LB broth containing 50 µg/ml ampicillin.
- vials were incubated at 37 °C overnight under vigorous shaking (180 – 200 rpm).

Day 4

- transfer 0.5 ml up to 2 ml of overnight E. coli cultures into 1.5 ml reaction tubes.
- pellet cells by centrifugation: 10.000 rpm, 4 min
- discard supernatant and re-suspend pellets in 100 µl resuspension solution
- add 1µl RNase
- incubate for 2-5 min
- add 200 µl NaOH-SDS
- vortex at 1.400 rpm
- add 150 µl ice cold Kac-solution, vortex 10 sec
- put samples 5 min on ice
- centrifuge 5 min on 4°C at 15.000 rpm
- pipette supernatant in a new tube
- add 900 µl EtOH and vortex carefully
- incubate for 2 min and centrifuge for 5 min at 4°C on 15.000 rpm
- discard flow-through and dry the tube
- add cold 70% EtOH vortex carefully and centrifuge for 5 min at 4°C on 15.000 rpm
- discard ethanol and air-dry pellets for approximately 2 hours
- re-suspend pellet in 10 mM Tris

LB broth (Sambrook et al. 1989)

Bacto-tryptone	10 g
Bacto-yeast Extract	5g
NaCl	10g
Agar	15g

Adjust the pH to 7 with 5N NaOH and volume to 1 l

Appendix III

Figure 13: Fruiting body of *Heterobasidion parviporum* (Picture by Thomas Cech)

Figure 14: *Heterobasidion parviporum* in pure culture (Picture by Thomas Cech)

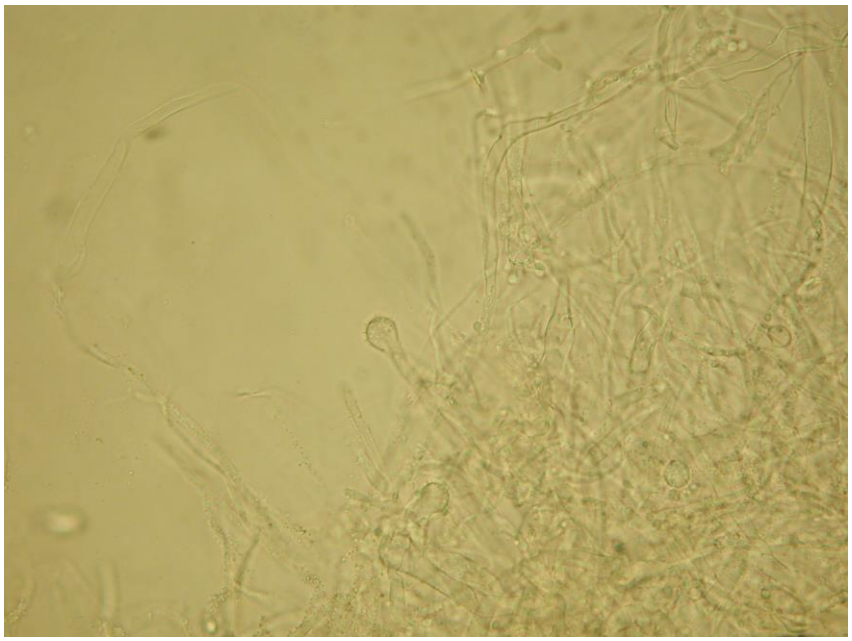

Figure 15: *Spiniger meineckellus* Stalpers (Picture by Thomas Cech)

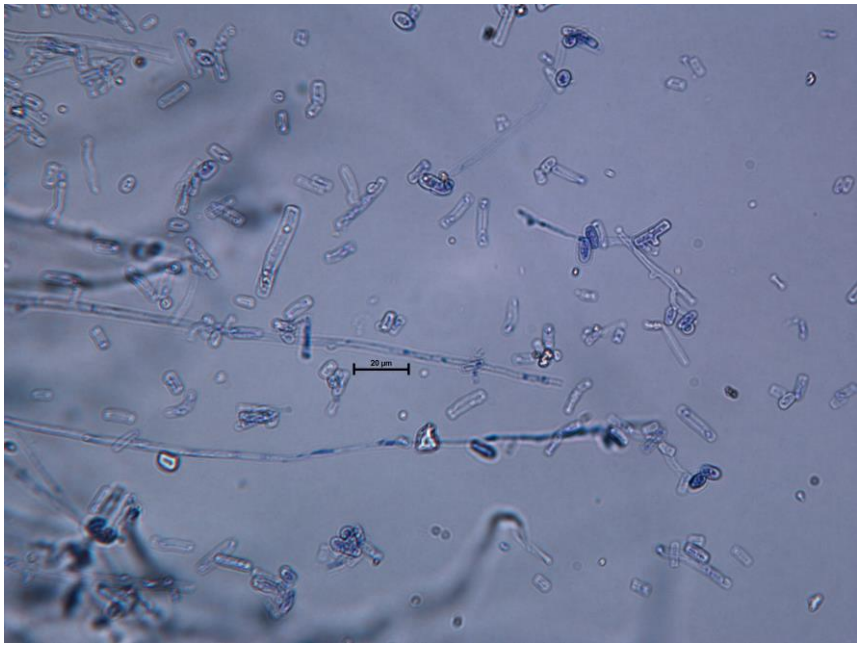

Figure 16: Oidiospores of *Phlebiopsis gigantea*

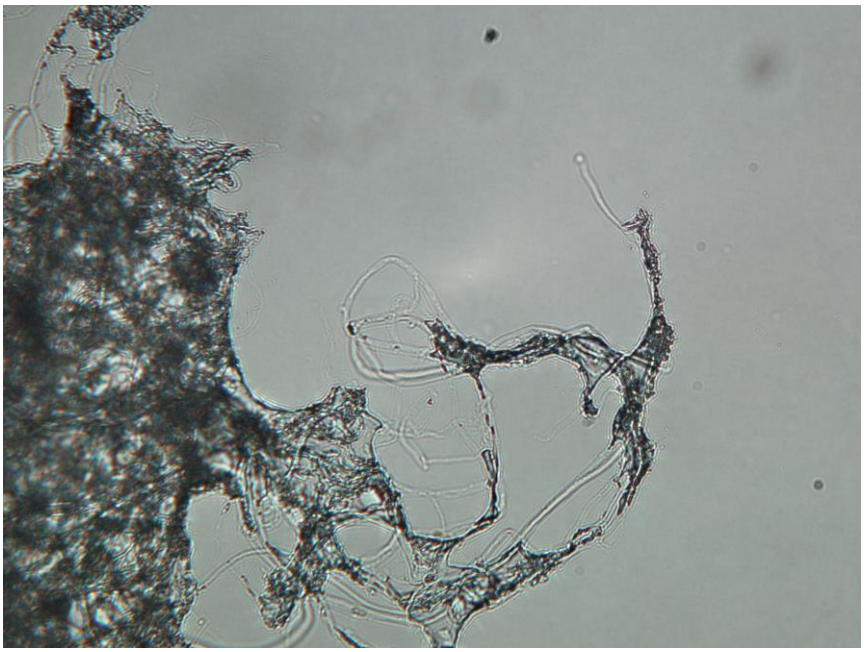

Figure 17: Mycelia of *Fomes fomentarius* in pure culture on 2% malt extract agar.

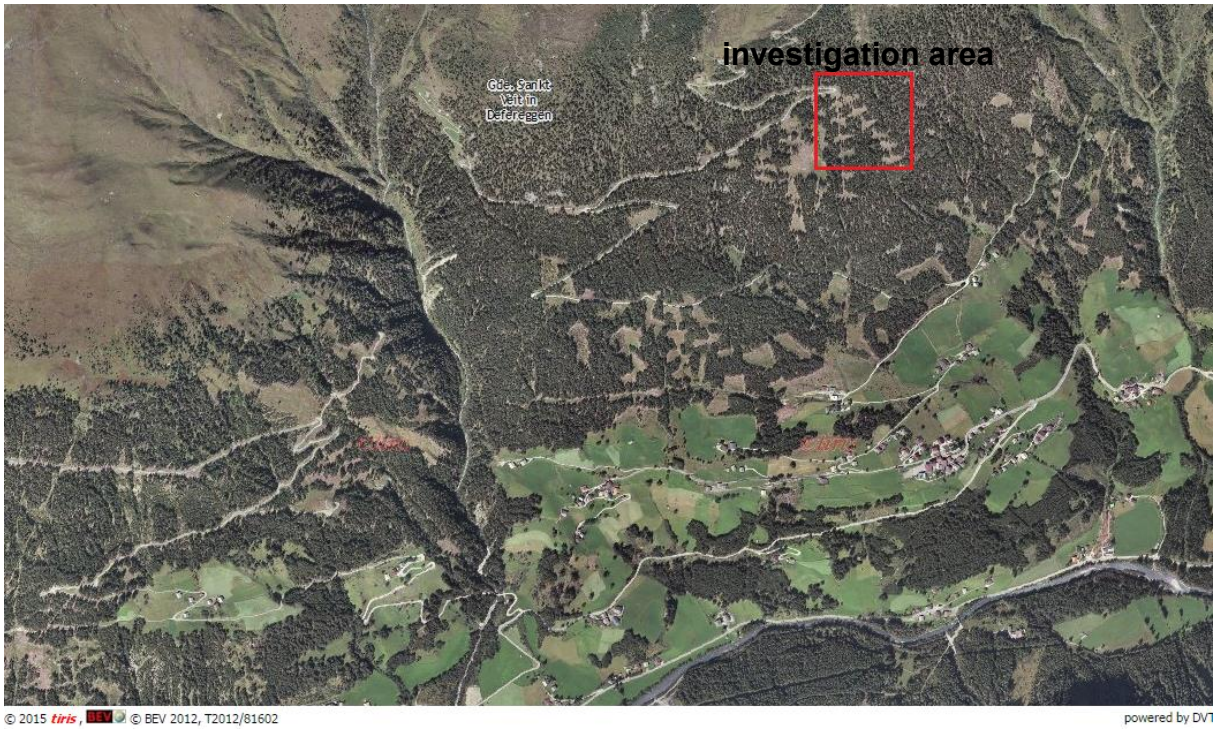

Figure 18: Sample site in the Defereggen valley (tirisMap, https://www.tirol.gv.at/statistik-budget/tiris/)