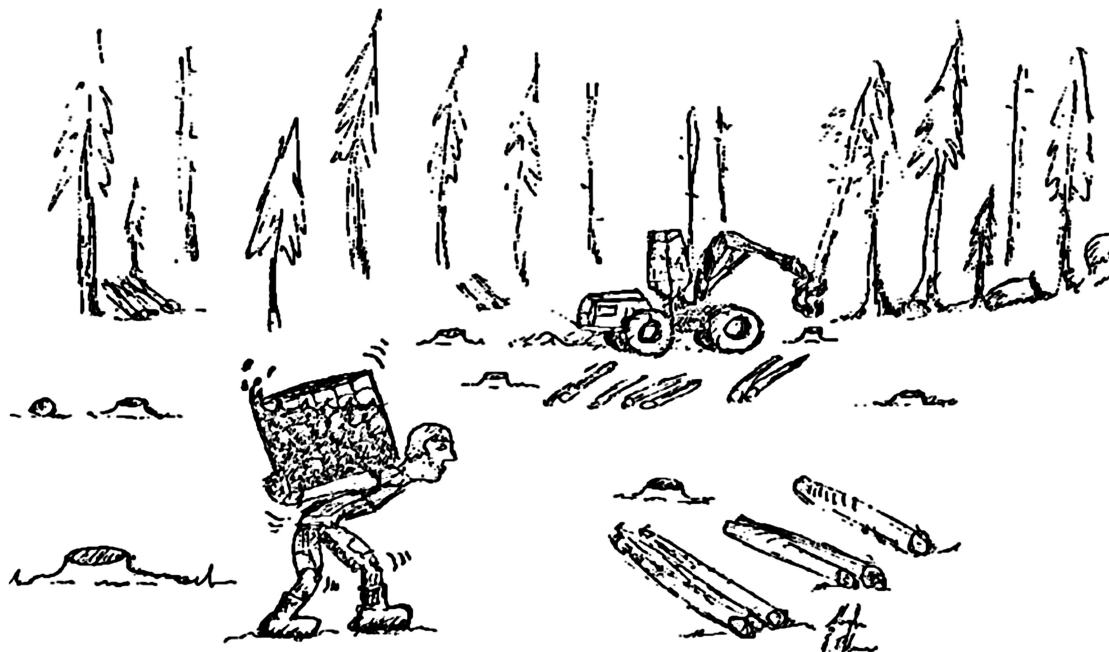


Stump Treatment with the Root Rot Antagonist *Phlebiopsis gigantea*:

- Sensitivity of *P. gigantea* Spores to High Pressure Stress
- Reduced Water Consumption for Stump Treatment



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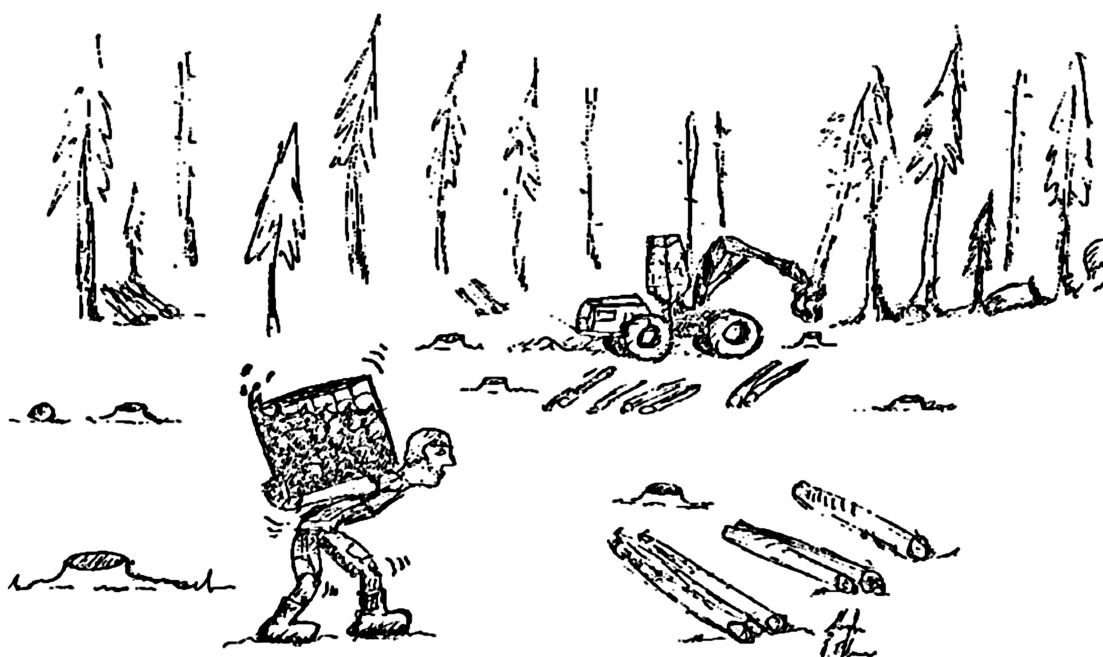
Master Thesis no. 211

Southern Swedish Forest Research Centre

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Summary

Stump treatment today requires large quantities of clean water mixed with the protective product Rotstop, to fully cover the stump surface. In order to reduce the water consumption without reducing the coverage of the stump surface, the pressure in the stump treatment device can be increased. Therefore, the *Phlebiopsis gigantea* (Fr.) Jül. suspension used as a biological control agent to prevent *Heterobasidion annosum* (Fr.) Bref. infection was pressure-tested. The pressurized suspensions were compared with the control suspensions and the survival was monitored by measuring oidia germination on agar medium. Pressures up to 150 bars (15 000 kPa) were tested and showed no negative effects on the survival of the *P. gigantea* spores.

Three *Phlebiopsis gigantea* suspensions, with the same quantity of *P. gigantea* spores but with different water amounts (the recommended water amount 1 l/m² stump surface, a reduced amount of 0.5 l/m² and an even more reduced amount of 0.25 l/m²), were compared with each other. This was done to investigate if it is possible to reduce the water consumption for stump treatment and thereby reduce stump treatment costs. The experiment was conducted on stem pieces (billets) of Norway spruce (*Picea abies* (L.) Karst.) in a laboratory, and the amount of *Heterobasidion* infection was screened. Results showed no difference between using 1, 0.5 or 0.25 l/m².

Keywords:

Stump treatment, *Heterobasidion annosum*, *Heterobasidion parviporum*, *Phlebiopsis gigantea*, *Picea abies*, Rotstop, pressure stress, water consumption.

Sammanfattning

Vid stubbehandling går det åt en stor mängd vatten. Om trycket i apparaturen ökas kan vattenförbrukningen minskas utan att täckningsgraden av stubbenytan försämras. I denna studie trycktestades *Phlebiopsis gigantea* (Fr.) som används vid biologisk stubbehandling för att förhindra angrepp av rottrötesvampen *Heterobasidion annosum* (Fr.) Bref. De trycksatta *P. gigantea* sporer jämfördes mot en kontroll utan tryck. Groningsförsök på agar-plattor visade att tryck upp till 150 bar (15 000 kPa) inte påverkade sporer.

Tre olika behandlingar jämfördes med varandra för att undersöka om det var möjligt att reducera vattenåtgången vid stubbehandling. En vanlig behandling med 1 l/m² stubbyta, en behandling med 0.5 l/m² och en behandling med 0.25 l/m² jämfördes med varandra. Mängden *P. gigantea* sporer var exakt den samma för de olika behandlingarna. Experimentet utfördes på stambitar av Gran (*Picea abies* (L.) Karst.) i ett laboratorium och mängden *Heterobasidion*-infektion mättes. Resultatet visade ingen skillnad mellan att använda 1, 0.5 eller 0.25 l/m².

Nyckelord:

Stubbehandling, *Heterobasidion annosum*, *Heterobasidion Parviporum*, *Phlebiopsis gigantea*, *Picea abies*, Rotstop, tryckstress, vattenkonsumtion.

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1 Introduction

1.1 Costs due to widespread infection of *Heterobasidion annosum*.

The amount of trees infected by root rot in final felling has doubled since the 1970s in the south of Sweden. Today, roughly one of five trees is subjected to root rot (Olsson, 2010). It is estimated that wood degradation caused by *Heterobasidion annosum* (Fr.) Bref. costs 120 million €/year (Witzell et al. 2009, Olsson, 2010). Annual loss for Europe is estimated to 790 million €/year. This makes *H. annosum* the most economically damaging fungus in the Northern Hemisphere (Hodges, 1969, Woodward et al. 1998).

The problem caused by *H. annosum* has not always been as severe as today. Traditionally trees were only harvested during the cold winter months in the Nordic countries. Harvesting while the temperature is below zero has been shown to considerably reduce the risk of spore infection by *H. annosum* (Kallio, 1970, Solheim, 1994, Möykkynen et al. 1988, Thor et al. 2005). Today the forest industry demands a continuous flow of raw material. Therefore, harvesting occurs nowadays all year around (Bendz-Hellgren et al. 1998), and has hence resulted in the higher infection levels.

1.2 The pathogenic fungus *Heterobasidion annosum*

The fungus has through the history been described with many scientific names e.g. *Polyporus annosus* Fr., *Trametes radiciperpa* (Hartig), *Formes annosus* (Fr) Karst., but it was Brefeld (1888) that named the fungus *Heterobasidion annosum* s.l. (Fr.) Bref. (Niemelä & Korhonen, 1998). In Europe there are three different species of *Heterobasidion annosum* s.l.:

- *Heterobasidion abietinum* s.s. Niemelä & Korhonen, previous known as the F-group.
- *Heterobasidion parviporum* Niemelä & Korhonen, previous known as the S-group.
- *Heterobasidion annosum* s.s (Fr.) Bref., previous known as the P-group.

These older group names (F, S and P) come from the species main host, namely: Fir (*Abies* spp.), Spruce (*Picea* spp.) and Pine (*Pinus* spp.) (Korhonen, 1978, Capretti *et al.* 1990). These three fungi species have similar morphological characteristics, but they differ in distribution. *Heterobasidion abietinum* is present in central and south east Europe while both *H. parviporum* and *H. annosum* sensu stricto occurs basically all over Europe, though *H. parviporum* stretches further north and *H. annosum* further south (Fig. 1). In Sweden, the last two species occur. *Heterobasidion parviporum* is the more common of the two and occurs in the whole country, whereas *H. annosum* mostly occurs in the southern part (Stenlid, 1987, Thor, 2005). *Heterobasidion parviporum* is mainly infecting Norway spruce (*Picea abies* (L.) Karst.) trees whereas *H. annosum* is considered to be more aggressive since it attacks several hosts e.g. spruce, pine and several broadleaved species (Korhonen, 1978, Stenlid *et al.* 1995, Korhonen & Stenlid, 1998).



Figure 1. Distribution of *Heterobasidion abietinum*, *Heterobasidion parviporum* and *Heterobasidion annosum* in Europe (map kindly provided by Kari Korhonen, 2012).

1.2.1 Biology of *Heterobasidion* spp.

Heterobasidion spp. forms perennial sporocarps or “fruiting bodies” on infected stumps and roots. Spores are produced and spread from these sporocarps when the average daily temperature exceeds +5 °C (Yde-Andersen, 1962, Kallio, 1970, Brandtberg et al. 1996). The normal route of infection is *via* basidiospores that land on freshly cut stumps or other open wood tissues. Mycelium grows and the fungus consumes wood tissues, in spruce the result appears as decaying or discolored wood in the center of the tree. The fungus can grow at a rate of 50 cm per year in dead stumps and it can spread to neighboring trees via root-to-root contacts (Rishbeth, 1951, 1957, Swedjemark & Stenlid, 1993, Swedjemark & Karlsson, 2004 Redfern & Stenlid, 1998).

In Scots pine trees the fungus attacks the cambium zone, causing growth losses and mortality (Stenlid & Redfern, 1998, Wang et al, 2012). In Norway spruce the fungus causes decay from the heartwood and out towards the sapwood, which leads to growth losses and reduced timber value. Severe decay can also increase the probabilities of stem breakage and uprooting (Bendz-Hellgren & Stenlid, 1995, 1997, Oliva et al 2008).

1.3 Control of *Heterobasidion* spp.

There are several ways to fight the disease. It can be done through silvicultural, chemical or biological control. Reducing the number of thinnings, winter logging and favoring of mixed forests are all silvicultural actions that reduce opportunities for *Heterobasidion* to enter and spread within the stand (Brandtberg et al. 1996). Stump removal is reducing already existing *Heterobasidion* in the stand.

Big efforts have been made to find chemical controls for stump-treatment. One problem has been to find an effective substance that is non-harmful to the environment. Many substances have been tested but only urea and borates provide enough protection to prevent infection (Brandtberg, 1996). There were other problems in addition to the environmental problems, e.g. urea is corrosive and therefore less suitable for the machinery than biocontrol preparations based on water (Pratt and Thor, 2001). Also, if urea and borates were applied in big amounts, then they could be damaging to the environment (Westlund and Nohrstedt, 2000).

Biological control has therefore received a greater attention. A biological control substance needs to work under a wide range of conditions, it must have minimal negative environmental effects, and it needs to be cheap, easy to produce and use (Pratt, 1999, Pratt & Thor, 2001).

1.3.1 Phlebiopsis gigantea as an antagonist against Heterobasidion spp.

In 1952, Rishbeth discovered the potential of *Phlebiopsis gigantea* (Fr.) Jülich as a biological control substance (Rishbeth, 1952). *Phlebiopsis gigantea* is a natural species found in the boreal forest. It is a common saprophytic fungus which grows on dead and decaying coniferous trees (Vainio, 2008). It causes white-rot just like *H. annosum*, though *H. annosum* is a pathogenic fungus which attacks both dead and living trees. *Phlebiopsis gigantea* has proven to be a strong competitor to *H. annosum* (Rishbeth, 1952, Korhonen et al. 1994, 1998).

In Finland, a heterokaryotic strain of *P. gigantea* was isolated from Norway spruce commercially referred as Rotstop (Korhonen et al. 1994). *Phlebiopsis gigantea* is grown and dried into powder. The powder is dissolved in water and sprayed onto stumps. The asexual oidia spore of *P. gigantea* germinates on the stump, the mycelium grows over and into the stump and outcompetes *H. annosum*. The substance made from the Finnish strain is commercially named Rotstop® and it has been tested in Finland, Sweden and Norway with good results on both *Picea abies* and *Pinus sylvestris* stumps (Korhonen et al. 1994, Oliva et al, 2010). Two products have also been made from a Swedish strain of *P. gigantea*; Rotstop S and Rotstop S Gel, where Rotstop S is a dry powder while Rotstop S Gel is a liquid, both contains living spores of *P. gigantea* and both are mixed with water to create the treatment suspension. None of the products are known to be harmful to the environment.

1.3.2 Practical use of the biological control agent Rotstop S Gel

In Sweden stumps are mechanically treated with the biological agent Rotstop S Gel during both thinning and final felling. Every year, 35 000 ha of thinning is treated (Thor, 2003). The biological control agent requires caution when handled. According to the Swedish retailer Interagro Skog AB, Rotstop S Gel must be stored cold when it is not used. It can be stored in a refrigerator, below +5 °C, for 6 months. Unopened it can be stored in a freezer for 12 months. At room temperature the durability is only 1

week. Prepared suspension should be used within 36 hours and Rotstop S Gel should never be exposed to temperatures above 40 °C.

During mechanized stump treatment there are several problems that can occur concerning the above restrictions. The water tank and the mechanical device that mixes the suspension with water can both be heated up by the sun or by the engine of the machine. The suspension is also heated up during pumping through the hoses from the tank to the harvester head. It is especially risky if the hoses are adjacent to hot hydraulic hoses.

1.3.3 Mechanical stump treatment

The stump treatment device (e.g. Droppen Dos mixing-system) is mounted on a single grip harvester. The system draws clean water from a large tank (100-200 l) and mixes it with Rotstop suspension from a smaller tank on the Droppen Dos system (Fig. 2) (Bo Axelsson, personal communication, October 25, 2012). The normal concentration is 1 l of water mixed with 1 ml of Rotstop S Gel i.e. the same as 1 g of dry Rotstop powder. Droppen Dos system situated in a locker that is normally placed in front of the cab, and the Rotstop suspension is pumped through hoses from the locker via the crane (either inside or outside the crane boom) to the harvester head. The total length of hoses can be up to 15 m, which corresponds to a liquid volume of 0.5 l (Thor et al. 1997). The spraying device is a through-the-bar system, where the suspension is sprayed through a number of small holes in the sword and onto the stump surface at the time of felling the trees (Pratt et al. 1998). The *P. gigantea* spores do suffer high pressure and impact-damage when they are sprayed through the holes in the sword and onto the stump surface (Bo Axelsson, personal communication, September 18, 2012). The study of Thor (1997) shows that *P. gigantea* do withstand the normal pressure (approximately 22 bars) of the spraying device, but in order to reduce the water amount for stump treatment and still achieve a full cover the stump surface, the spraying device must use a significantly higher pressure than today. This will be tested in this study.



Figure 2. Droppen Dos mixing-system (picture kindly provided by Bo Axelsson, 2012).

1.3.4 Costs concerning stump treatment.

One bottle of 200 ml Rotstop S Gel is sufficient for 200 l of water. Today's stump treatment uses approximately 1 l of suspension to 1 m² of stump area. Stump treatment cost 11-14 SEK/m³ for thinning and 3-6 SEK/m³ for final felling (Bo Axelsson, personal communication, September 18, 2012). From Thor et al, 2006, the cost for stump treatment was approximately 10 SEK/m³ in the first thinning, 8 SEK/m³ at second thinning, 6 SEK/m³ at third thinning and 3 SEK/m³ at final felling.

Transporting, filling and handling water is one of the main costs, another one is the Roststop suspension. At third thinning and final felling larger water amounts is required, in some cases the harvester needs to pause its logging in order to refill the water tank. Production stops are very costly and also annoying for the harvester drivers.

A goal is to reduce the water consumption from 1 l/m² to at least 0.5 l/m². With this reduction the harvester would only need to refill water once a day, at the same time as fuel. This would reduce the costs and the operations of transporting and handling water.

In order to achieve this goal of reducing the water consumption, the system must use higher pressure in order to achieve a full coverage of the stump surface. The holes in

the sword must be smaller in order to reduce spill and create higher pressure. A higher pressure stress could be problematic for the *P. gigantea* spores, they might lose vitality and this must therefore be tested first before testing stump treatment with lower water amounts.

1.3.5 Aim of study.

The aim of this study is to first test whether or not the *P. gigantea* spores could survive a higher pressure stress (up to 150 bars pressure).

The second part of the study aims at comparing a normal treatment using the recommended water amount of 1 l/m², with treatments using 0.5 and 0.25 l/m², and find out if there is any difference in efficacy of protection against *H. parviporum* infection.

1.3.6 Hypothesis.

1.) *Phlebiopsis gigantea* spores that have been subjected to high pressure stress (150 bars) do germinate equally well as spores which have not been subjected to any pressure stress. It is therefore possible to use the *P. gigantea* suspension in a renewed stump treatment device which uses higher pressure in order to reduce the water consumption.

2.) There is no significant difference in infection rate of *H. parviporum* between a normal treatment using the recommended water amount (1 l/m²) and treatments using a lower water amount (0.5 respective 0.25 l/m²).

2 Material and methods

2.1 Pressure test

2.1.1 Pressure test method

The inoculum for the pressure test was delivered from Interagro Skog AB. The Rotstop S Gel plastic bottles contained 50 ml of aqueous suspension with approximately 2-10 million living spores/g. Ten bottles were used for the experiment, where eight bottles were pressurized, two for each pressure level, and two bottles were untreated functioning as a control.

The pressure levels tested were 25, 50, 100 and 150 bars. First the caps were tightened hard before the pressure procedure started. Two bottles at the time were placed inside a hydraulic piston that was sealed and connected to a hydraulic engine. Hydraulic oil was pumped into the piston until the decided pressures were reached. In this way both bottles received exactly the same pressure. The bottles were compressed for one minute. Then each bottle were taken out of the piston and wiped clean with a tissue. None of the bottles were damaged or broken during the pressure procedure, though visible marks from the compression could be seen. The control bottles were bathed in hydraulic oil for one minute in order to receive as similar treatment as possible to the pressurized bottles. The bottles were kept in a refrigerator before and after the experiment.

2.1.2 Most Probable Number method

The Most Probable Number (MPN) method (Oblinger & Koburger, 1975), was used to determine the vitality of the pressurized *Phlebiopsis gigantea* spores. With the MPN-method it was possible to estimate the number of Colony Forming Units (CFU)/ml i.e. number of living spores/ml which can germinate. The method is especially applicable to fungi, e.g. *Phlebiopsis gigantea*, which form vague colonies which are difficult to count.

Samples from the Rotstop S Gel bottles were mixed with water and series of dilutions were prepared (the dilutions were expressed as g/ml: e.g. dilution 10^{-3} means 0.001 grams of Rotstop suspension in 1 ml of water). The following dilutions were made: 10^{-4} , $(10^{-4})/2$, $(10^{-4})/4$, $(10^{-4})/8$, $(10^{-4})/16$, $(10^{-4})/32$, $(10^{-4})/64$ and $(10^{-4})/128$. A sample from each dilution was pipetted onto petri-dishes containing Potato Dextrose Agar (PDA).

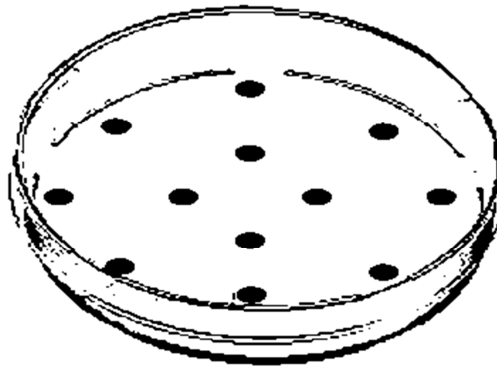


Figure 3. One PDA-plate with 12 drops of 10 μ l *Phlebiopsis gigantea* dilution.

Two PDA-plates were made for each dilution and each dish was pipetted with 12 drops from the dilution (Fig. 3). This procedure was repeated for each pressure level (25, 50, 100 and 150 bars) tested in the experiment. The plates were incubated until visible growth could be observed on the spots (Fig. 4). The viability of the *P. gigantea* suspension for each pressure level were calculated on the basis of the number of positive and negative spots on each petri-dish. The higher the dilution the less spots were colonized.



Figure 4. Petri-dish showing 3 positive spots out of 12 (picture kindly provided by Pekka Seiskari, 2012).

2.2 Stump treatment using different amounts of water

2.2.1 Preparing billets and testing moisture content

Five spruce trees, with a diameter ranging from 20-25 cm at stump level, were felled in Trolleholm close to Eslöv (55°92'N 13°27'E). The trees were all about 25 years old and growing on abandoned agricultural land. The trees were cut down within 50 meters from each other and chosen due to its similarities in size and shape. For each tree, the first 1.5 m of the bottom log was cut and left in the forest. A 3 m log was then cut and divided into 1.5 m long pieces in order to facilitate transportation from the forest. The ends of the logs were sealed with duct tape and plastic bags. The logs were stored in a room at 4°C temperature for a month prior to setting up the experiment where the reduced water consumption for stump treatment was tested.

A disc from each log were analyzed to ensure that each tree was free from infection by *Heterobasidion* spp. Before the discs were cut, the bark was removed from the base of the log and the stem was sprayed with ethanol. First a disc was cut and wasted, then a second disc of 3 cm was cut and put in a plastic bag. The discs were stored for 11 days in room temperature before examination was done under a dissecting microscope. No conidiophores of *Heterobasidion* spp were found, so all logs were infection free and could therefore be used in the experiment.

Nine stem pieces (billets) were cut from 3 of the trees (Fig. 5). In total 27 billets were cut for the experiment, each billet was 30 cm. The bark and the saw blade are sprayed with 70% ethanol before each cut.

The billets were produced six days before setting up the experiment. Each billet was marked and sealed with a plastic bag and stored for 5 days in 4°C temperature in order to prevent mold or other fungal growth while experimental preparations were done. The billets were taken out one day in advance so they would become acclimatized to room temperature before starting the experiment.

The moisture content was measured in order to make sure that no big differences between the trees would affect the results. Of the five trees cut down only three of them were used in the experiment. A total of 36 wood samples were taken from these

trees, six samples from the upper-part and six from the lower-part of each log (three samples from the sap-wood and three from the heart-wood). All samples were weighed and dried for one week in an oven at 45 °C. The moisture content was calculated by taking “weight of water” divided by “fresh weight”.

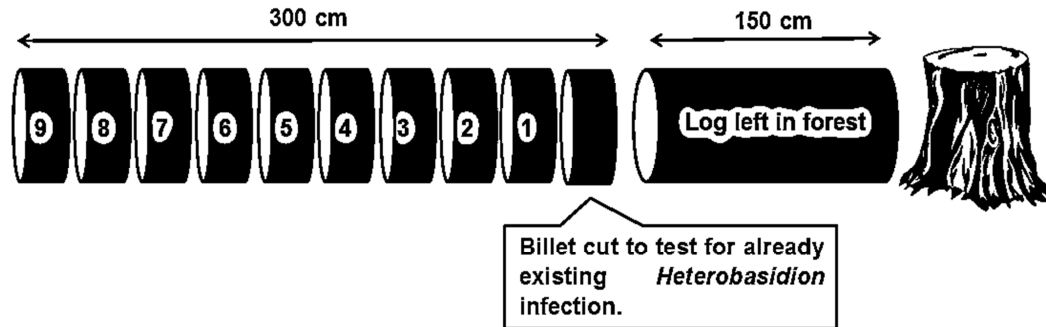


Figure 5: Number of billets made from each separate tree.

2.2.2 *Heterobasidion parviporum* suspension and the conidiospore density

A known individual of *H. parviporum* was used in the experiment, RB 175 (Swedjemark and Stenlid, 1997). The fungus was stored in the department of southern Swedish Forest Research Centre, in several sealed petri-dishes which were 4 years old. The fitness of the old RB 175 was tested by taking pieces of the fungus and growing them on petri-dishes with malt agar. Each petri-dish contained 12 ml of malt agar (5 g Glucose, 0.5 g NH_4NO_3 , 0.5 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7 \text{ aq}$, 5 g malt extract, 20 g agar and 1000 ml H_2O). The RB 175 fungus was growing and could therefore be used in the experiment. After 13 days of growth several pieces of fungi was taken from the petri-dishes and distributed to 27 new dishes. The RB 175 was growing for a month before the *H. parviporum* suspension could be made.

The conidiospore density per dish was estimated by taking one of the 27 dishes with growing *H. parviporum*, adding 10 ml of tap water and using a sterile spatula to gently brush the surface of the fungi. The 10 ml water (now containing spores of *H. parviporum*) was poured into a sterile bottle. This procedure was repeated three times so 30 ml of *H. parviporum* suspension was obtained. From the suspension a sample was taken and dilution series was made similar to the MPN-method. A sample from each dilution was spread on petri-dishes that were kept in room temperature until

visible growth could be observed on the dishes. The number of living spores in the original *H. parviporum* suspensions was calculated on the basis of the number of colonies found for each dilution. It was assumed that each of the 27 petri-dishes contained a similar amount of spores as the tested dish since the cultivation time was the same. Calculation showed that 25 dishes of *H. parviporum* would be needed to make one liter of *H. parviporum* suspension with a concentration of 50 spores/ml (a guideline value is to have 25 spores /cm² on the billet surface, and that would be obtained by spraying a 0.5 ml thick layer of the 50 spores/ml *H. parviporum* suspension over the billet surface).

To make one liter of *H. parviporum* suspension the above procedure was used, were 10 ml of tap water was added three times to each plate, a sterile spatula was used to gently brush on the surface and the liquid was poured into a sterile glass bottle. This procedure was repeated on 25 of the petri-dishes, which equals 0.75 l suspension. Then 0.25 l of tap water was added to make it one liter of suspension with the concentration of 50 spores/ml.

To double check that the spore amount in the *H. parviporum* suspension was right, a sample was taken from the suspension and another dilution series was made and the spore amount calculated.

2.2.3 Amount of Heterobasidion parviporum suspension sprayed onto each billet

The volume applied was adjusted to the diameter of each billet, i.e. 0.5 l/m² of *H. parviporum* suspension and it was applied using a spray bottle head and a measuring glass. The amount 0.5 l/m² is equal to a 0.5 mm thick layer of suspension on the billet surface (Table 1).

Table 1. Amount of liquid applied on the billet surface (the amount of treatment was rounded off to the closest 0.1 ml).

Radius of billets (cm)	Amount of liquid applied on the billet surface			
	Treatment 1	Treatment 2	Treatment 3	<i>H. Parviporum</i>
	1 l/m ² (ml)	0.5 l/m ² (ml)	0.25 l/m ² (ml)	Suspension 0.5 l/m ² (ml)
7	7.70	3.85	1.92	3.85
7.5	8.84	4.42	2.21	4.42
8	10.05	5.03	2.51	5.03
8.5	11.35	5.67	2.84	5.67
9	12.72	6.36	3.18	6.36
9.5	14.18	7.09	3.54	7.09
10	15.71	7.85	3.93	7.85

2.2.4 Experimental setup in the laboratory

In this experiment three *Phlebiopsis gigantea* suspensions using different water amounts were compared with each other.

The 27 billets were divided into six boxes of four and five each, two boxes for each treatment. Treatment 1 used the recommended water amount 1 l/m², treatment 2 and treatment 3 used 0.5 l/m² respective 0.25 l/m² of water. The amount of Rotstop was the same for each treatment, only the amount of water varied i.e. the concentration of Rotstop in treatment 2 was twice as high as in treatment 1, and four times as high in treatment 3 compared to treatment 1.

The upper surface of each billet was divided in two symmetrical halves with a line (Fig. 6). One half was covered while the other half was sprayed with Rotstop suspension using a measuring glass with the right amount of suspension and a hand sprayer head. The “non-treated” half was functioning as a control. By having treatment and control on the same billet the effect of different moisture content and density between billets was eliminated. After waiting 20-30 minutes for the Rotstop suspension to dry, both halves of the surface were sprayed with *H. parviporum* suspension.

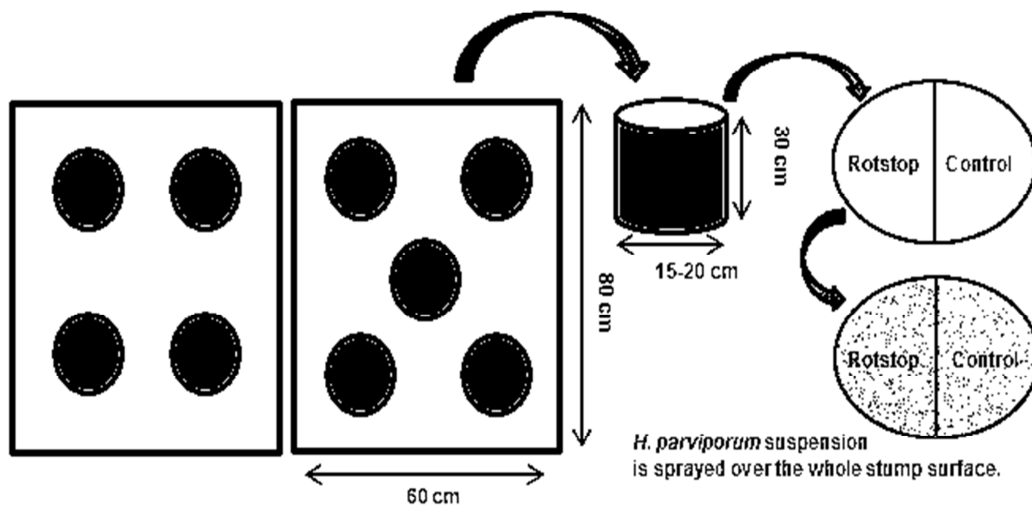


Figure 6: Treatment setup with number of billets per treatment and stump surface subdivision.

The boxes were kept in half shade for six weeks in a laboratory in the Hortucum building in Alnarp. Some wet sand covered the bottom of the boxes in order to keep the billets from drying out. The sand was kept moist by pouring water on the sand when needed (Fig. 7).

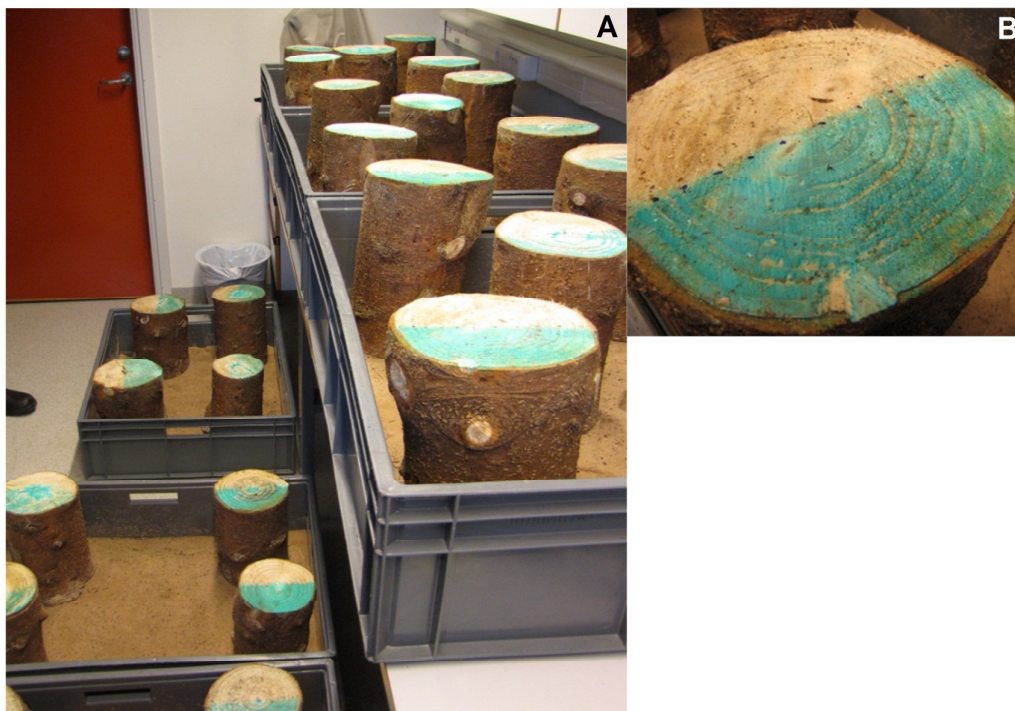


Figure 7. The treatment setup consisted of 3 boxes of 5 billets each on the desk and 3 boxes of 4 billets each on the floor (A). Every slice was half treated with Rotstop suspension (blue staining in B), the other half is functioning as control (uncoloured half in B).

2.2.5 Analyzing wood discs

After six weeks of growth in room temperature, a disc was cut from each billet. First the bark was removed from the billet with the help of a sterile knife. The bark stripped area was sprayed with ethanol, an electric chainsaw (Stihl MSE 140 C-BQ) was used to cut the discs. First a 1 cm thin disk was cut and wasted. A second disc of 5 cm was cut and put in a plastic bag. The discs were kept in a cold storage room for two weeks, and then incubated for 9 days in room temperature before the analyses started. All discs were striped with a marker pen and investigated on both sides (i.e. at 1 and 6 cm from the top of the billet) for the presence of the *H. parviporum* in its conidial stage. A dissecting microscope with a magnification ratio of 15-40x was used (Fig. 8). Infections of *H. parviporum* were recognized by the mycelia morphology and its conidiophores. Infected areas were marked and the number of colonies were counted for the treated half and the control half separately. A grid of 1 cm² squares on transparent film was put on the surface and the infected area (number of cm²) for each half was estimated.

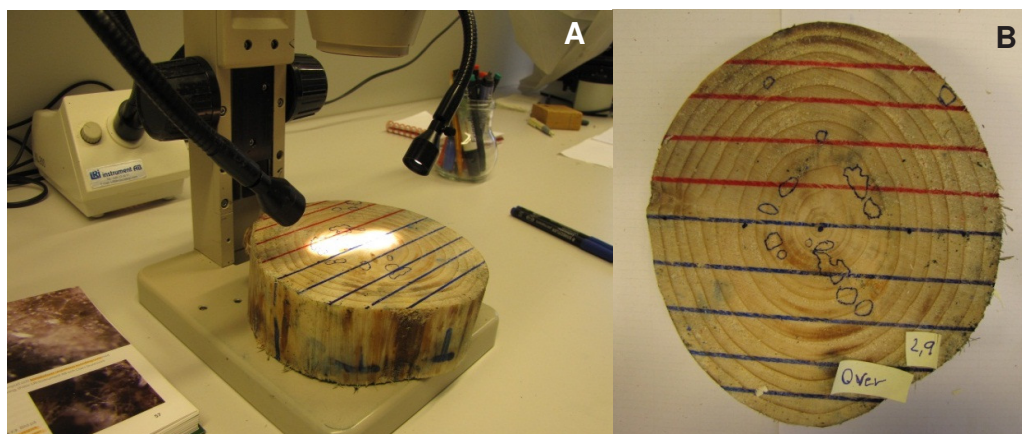


Figure 8: A) Examination of disk under the dissection microscope. B) Top side of disc nr 9 in the second treatment, blue stripes marks the area which was treated with Rotstop and red stripes mark the control area.

2.3 Calculations and statistics

2.3.1 Pressure test

Microsoft Excel and the statistical software Minitab® were used to analyze the data from the pressure test. Due to the variability in the count data, the data was log-transformed in order to reduce the influence of outliers and extreme values (Zar, 1984).

2.3.2 Treatments using different water amounts

Differences between control and Rotstop treatments, and differences between the Rotstop treatments high, medium and low water usage, were expressed by calculating the relative infected area (i.e. area of *H. parviporum* divided by the total disc area, times 100), the efficacy, (difference in infected area between control side and treatment side of the disc divided by the infected area on the control side, times 100), the number of colonies/disc and the size of colonies/disc.

The experiment had a hierarchical design since both control and treatments were applied in slices that belong to a set of trees. In order to consider the link between slices and trees, both 'tree' and 'slice' nested to 'tree' were included in the design. The third factor, treatment was regarded as crossed and included four levels: the three Rotstop treatments 'high' 'medium' and 'low' water usage (1, 0.5 and 0.25 l/m²) as well as 'control' (without Rotstop). Means were compared with Tukey Honest Significant Difference (HSD) method for multiple comparisons. All three protection treatments were compared with the control with the Dunnett method. The analysis used a General Linear Model (GLM) procedure in Minitab® 16.1.0 software.

3 Results

3.1 Pressure test

All the samples that were taken from the pressurized Rotstop S Gel bottles showed similar mycelium growth independently of the pressure they were exposed to (Table 2).

Table 2. Number of spots on the petri-dishes which showed visible growth of *Phlebiopsis gigantea*.

Dilutions	Number of spots with growing <i>Phlebiopsis gigantea</i> ¹				
	Pressure				
	0	25	50	100	150
10 ⁻⁴	23	24	24	22	24
(10 ⁻⁴)/2	14	21	20	13	12
(10 ⁻⁴)/4	9	8	12	6	6
(10 ⁻⁴)/8	2	7	15	0	2
(10 ⁻⁴)/16	0	6	3	0	2
(10 ⁻⁴)/32	0	4	0	0	0
(10 ⁻⁴)/64	0	3	0	0	0
(10 ⁻⁴)/128	0	0	0	0	0

¹The maximum number of spots for each dilution was 24.

Analyses showed no significant difference in the number of colony forming units per gram (CFU/g) for the different pressures ($p=0.215$). The Rotstop S Gel do normally contain 2-10 million CFU/g. In this experiment the control, which received no pressure, contained roughly 2 million CFU/g, while the Rotstop which received the highest pressure (150 bars) contained approximately 1.2 million CFU/g (Fig. 9).

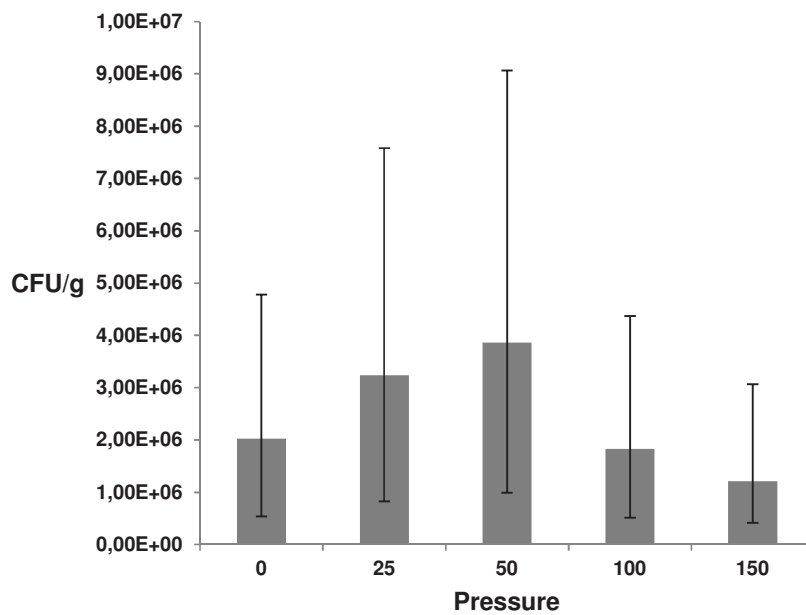


Figure 9. Number of colony forming units per gram (CFU/g). The error bars show the maximum and the minimum CFU/g of the tested pressure.

3.2 Reduced water consumption for stump treatment

There was a significant difference in sap-wood moisture content ($p=0.033$) between the three different trees used in experiment. The heart-wood moisture content was also significantly different ($p=0.010$) (Table 3).

Table 3. Moisture content of the *Picea abies* trees used in the experiment.

Tree number	Average moisture content	
	Sap-wood (%)	Heart-wood (%)
2	69 ^a	27 ^b
5	68 ^{ab}	30 ^b
3	65 ^b	42 ^a

In this experiment both parameters ‘tree’ and ‘slice’ (which were cut from the trees), were significantly different ($p=0.000$) for each of the dependent variables, i.e. relative infected area, number of colonies and size of colonies (Table 4).

Table 4. Summary of the statistical analysis for relative infected area, number of colonies and size of colonies.

	Relative infected area ¹			Number of colonies ¹			Size of colonies ¹		
	df ²	F ³	P ⁴	df ²	F ³	P ⁴	df ²	F ³	P ⁴
Tree	2	20.90	0.000	2	14.18	0.000	1	5.89	0.000
Slice(tree)	24	7.65	0.000	24	6.17	0.000	2	5.30	0.000
Treatment ⁵	3	4.58	0.011	3	3.03	0.049	2	2.02	0.138

¹The dependent variable was square root transformed.

²Degrees of Freedom.

³F statistic (F-value).

⁴P-value.

⁵Treatment includes: control, high, medium and low water usage.

The relative infected area for treatment 1, which used the recommended water amount 1 l/m², was significantly different from the control ($p=0.011$). Treatment 2 and 3, which used 0.5 l/m² respective 0.25 l/m² of water, did not differ from the control or treatment 1 (Table 5). The infected area was highest for the control treatment where 6.25 % of the area was infected by *H. parviporum*. Treatment 1 (using 1 l/m²) had the lowest infection level (2.56). Figure 10 and 11 show the original values and the square root transformed values of the relative infected area.

Table 5. Relative infected area¹

Treatment	N	Mean ² (%)	Grouping ³
Control	27	6.25	A
LOW (0.25 l/m ²)	9	3.61	AB
MEDIUM (0.5 l/m ²)	9	4.41	AB
HIGH (1 l/m ²)	9	2.56	B

¹The relative infected area was square root transformed.

²The mean values were squared to show the % of infected area.

³Grouping was done by Tukey HSD method ($p < 0.05$).

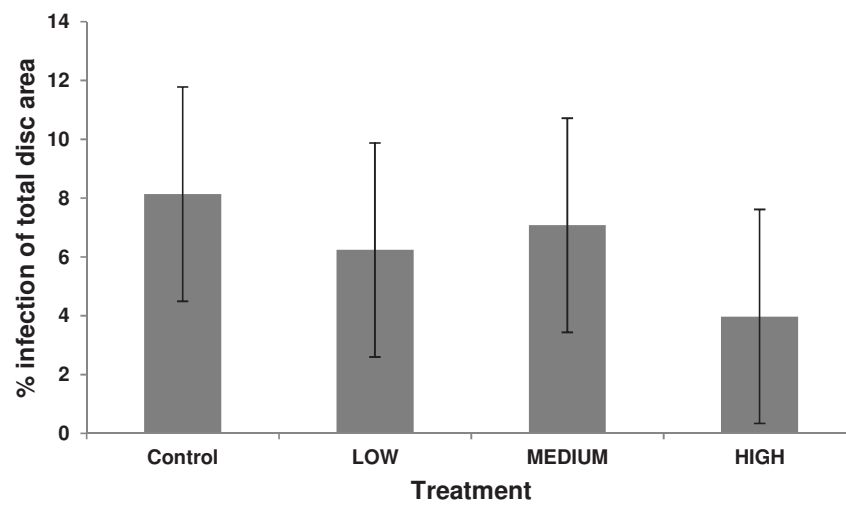


Figure 10. Relative infected area

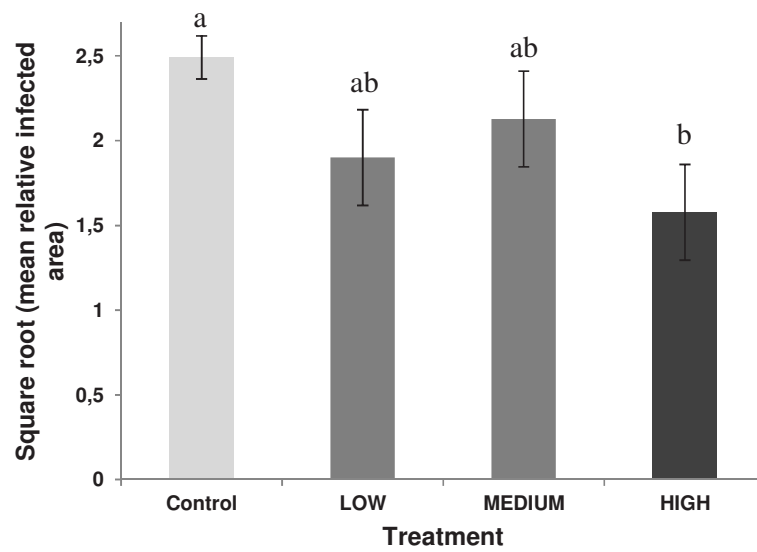


Figure 11. Square root transformed relative infected area. Means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

There was no significant difference ($p=0.276$) in efficacy level between the different treatments.

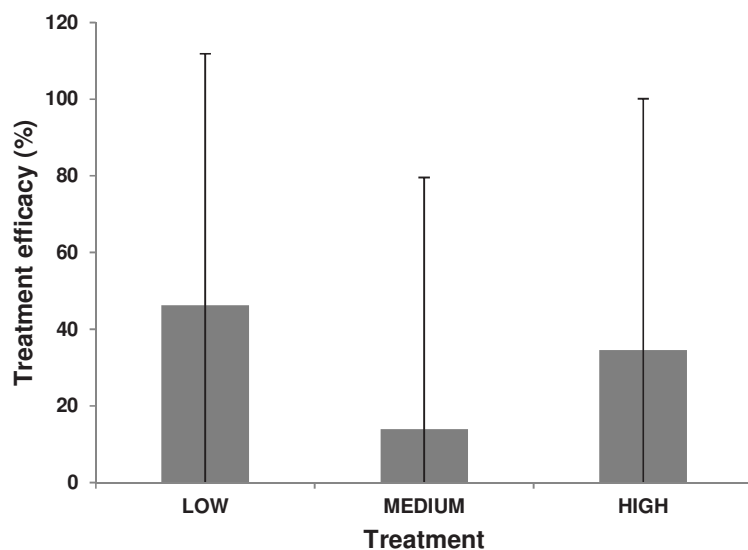


Figure 12. The efficacy (%)

The square root transformed values showed a significant difference in the number of colonies ($p=0.049$), where the control showed the highest colony number and the high water treatment usage showed the lowest values. Though neither the Tukey HSD method nor the Dunnnett multiple comparison method (which both are stricter than the normal F-test), were able to detect any differences in grouping. Figure 13 shows the non-transformed mean values of the number of colonies.

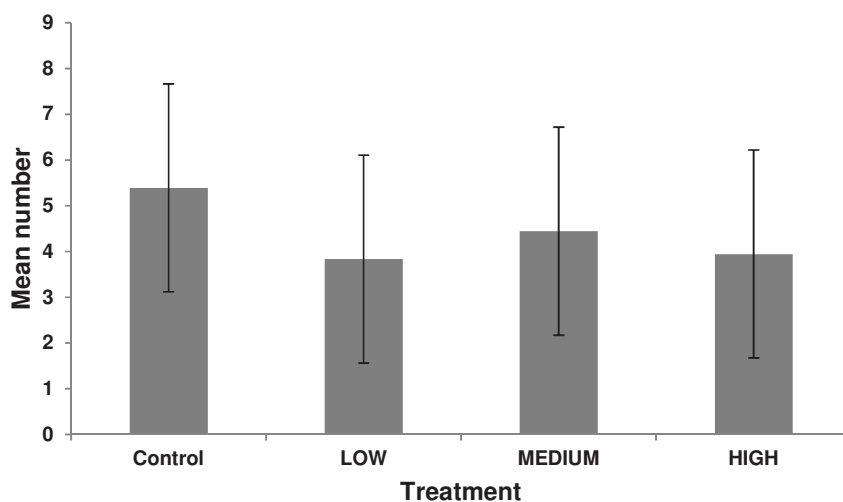


Figure 13. Number of *H. parviporum* colonies

The square root transformed values showed no significant difference ($p=0.138$) in size of colonies between treatments and control. Figure 14 shows the non-transformed mean values of the size of colonies.

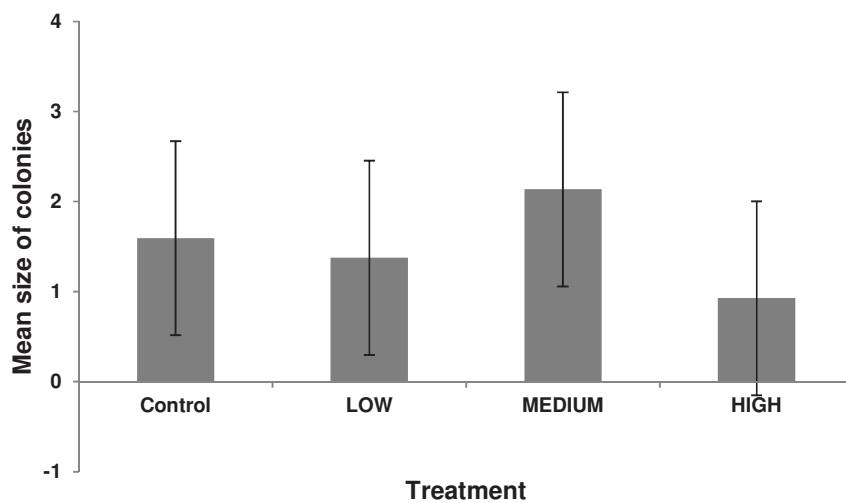


Figure 14. Size of *H. parviporum* colonies

4 Discussion

This study has shown that Rotstop bottles containing *Phlebiopsis gigantea* spores can withstand a pressure stress of 150 bars for at least one minute. This indicates that the *P. gigantea* spores can tolerate a high pressure stress long enough for it to work in a new stump treatment device which will use a higher pressure than today. The research thereby confirms the first hypothesis that *P. gigantea* spores do germinate equally well independently of the pressure stress they were subjected to.

In the study of Thor (1997), it was indicated that *P. gigantea* can withstand a pressure stress of 22 bars. Higher pressure stress than 22 bars has not been tested until now, and the effects of how pressure is affecting the spores is still poorly known and poorly investigated, even after this study. Though this study has expanded the pressure stress range up to 150 bars, which is nearly 7 times the pressure that Thor used in his study. However, one could discuss if the pressure on the individual *P. gigantea* spore really came to be 150 bars. Even though the bottles with Rotstop S Gel were put in a hydraulic piston and were pressurized with 150 bars for one minute, one could still argue that the pressure received by the individual spore were lower than 150 bars, because; (1) the Rotstop bottles contained air (approximately one-sixth of the bottle volume was air). Air is compressible and if the air in the bottle were compressed, then one could argue how much of the pressure was left to compress the Rotstop suspension. (2) Compressing liquids is possible but might require a much higher pressure than 150 bars, so how much pressure the individual *P. gigantea* spore received is hard to say. Another effect of compressing liquids is that the viscosity i.e. the resistance of the fluid to flow, increases as the density increases. This is because the atoms are forced closer together, and as a result the temperature increases. Though, in this case the bottles were not warm when they were taken out of the piston.

Bo Axelsson reasoned that if a bottle of Rotstop could make it through the pressure procedure without being broken, then the Rotstop suspension would have been enough compressed or at least received similar forces as it would in a mechanical stump treatment device which uses a higher pressure. Therefore it will work in a

stump treatment device that uses a higher pressure than today (personal communication, September 18, 2012).

With higher pressure comes also higher temperature. The temperature was not measured during this study and there is a chance that the compressed Rotstop bottles increased in temperature during the one minute compression.

From several studies it is known that decay causing fungus, e.g. *P. gigantea*, is sensitive to high temperatures (Gooding et al. 1966, Palmer & Payne 1986, Thor et al. 1997). These studies have shown that *P. gigantea* oidia do have higher spore germination at temperatures of 30°C compared to 20°C. Thor (1997) showed that *P. gigantea* could tolerate a temperature of 35°C, but temperatures of 40°C were deadly for the fungus and could only be withstood for a short period of time.

For mechanical stump treatment procedures there are two reasons for worrying about the temperature: (1) the *P. gigantea* suspension in the tank can be heated up by the sun or the engine and (2) the suspension can be heated up by being pumped through the hoses (Thor et al. 1997). The length and the dimension of the hoses are influencing the temperature of the suspension and especially if there are adjacent hydraulic hoses in contact with the suspension transporting hoses.

The second part of the study showed that there was a difference in relative infected area between Rotstop treatment 1, using the highest water amount (1l/ m²), and the control. Though, there was no difference found between Rotstop treatment using the high water amount (1l/ m²) and treatments using the lower water amounts (0.5 and 0.25 l/ m²). Same result applies for the efficacy, the number of colonies and the size of *H. parviporum* colonies, no differences between the different Rotstop treatments (1, 0.5 and 0.25 l/ m²). Though there were a lower number of colonies for treatment 1 (1l/ m²) compared to the control.

These results indicates that the amount of water appears to have some kind of germination effect on the *P. gigantea* spores, otherwise the Rotstop treatment using the high water amount (1l/ m²) would not have been different to the control when the treatments using the lower water amounts were not different to the control. This

positive germination effect connected to the high water amount (1 l/m^2) might be an obstacle that prevents the possibility of reducing the water consumption without losing protective effect of *P. gigantea*. However, this experiment may not have been big enough to say with certainty that the water amount is not reducible for stump treatment. Analysis show rather small differences between the treated side and the control side of the billet. A possible explanation might be that there were too few samples (nine per treatment) in relation to the normally large variation in this kind of research (Berglund & Rönnberg, 2004). Rotstop treatments normally do have protective effect against *H. parviporum* infection (Rishbeth, 1952, Korhonen et al. 1994, 1998, Berglund & Rönnberg, 2004). Some studies show that Rotstop have a control efficacy of nearly 100% (Korhonen et al. 1994, 1998) while other studies show lower control efficacy and questions the effectiveness of *P. gigantea* (Berglund, 2005, Gunulf et al. 2012). During the experiment of reducing water consumption for stump treatment, no viability tests of the *P. gigantea* suspensions were done. This could explain the poor result of the *P. gigantea* protection against *H. parviporum* infection. Though, the *P. gigantea* was from a standard commercial batch and the prescriptions given by the manufacturer were strictly followed.

Evaluation of this experiment was done by using relative infected area, efficacy, number of colonies and size of colonies all together. To only focus on one of these measurements might be misleading (Redfern 1982). This study indicates that Rotstop treatment using high water amount (1 l/m^2) do have a higher protective effect against *H. parviporum* infection. Though there were no differences found between the different treatments using high, medium and low water amount.

The trees used in the experiment were all about the same age and similar in size and shape to one another. Still the heart-wood and the sap-wood moisture content were significantly different between the different trees. What possible effect this could have for the experiment is unknown. It is debated what kind of effect the moisture content have on the probability of *H. parviporum* and *P. gigantea* infection. Bendz-Hellgren & Stenlid (1998) indicated that increased moisture content in the sapwood can have a negative effect on the probability of infection. Redfern (1993) measured the moisture content of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) stumps and pointed to an optimum level for colonization of 30-70% of saturation. The moisture

content in this experiment was therefore advantageous for infection. Though, it is not likely that the large variation in this study is due to the difference in moisture content between the trees.

In this study, the aim was to inoculate each billets with 25 *H. parviporum* spores/cm², which can be considered as a reasonable inoculation density. Though, it is probably higher than the natural spore densities in the natural forest (Gunulf et al, 2012). Estimation of the conidiospore density was done two times. The first estimation was performed before the *H. parviporum* suspension was made. A calculation from the analyses showed that 25 petri-dishes of *H. parviporum* were needed to make one liter of *H. parviporum* suspension. This calculation was based on the expectation that all of the 25 dishes contained similar spore amounts to the analyzed petri-dish since the cultivation time was the same. The second estimation of the conidiospore density was a sample taken from the *H. parviporum* suspension. It showed that the spore amount was approximately 7.5 spores/cm², which is lower than the guideline value of 25 spores /cm², but still thought to be a good enough inoculation density. In any case, the inoculations showed plenty of *H. parviporum* infections on the billets and it is not likely that the large variation in this study is due to what inoculation density were used.

In conclusion, this study shows that there were no differences in using high, medium or low water amounts for stump treatment in order to protect against *H. parviporum* infection. Though, result also indicates that there was some positive germination effect connected to using the high amount of water (1 l/m²) for stump treatment since this treatment was the only treatment which differed from the control. This study was done in a laboratory environment, but it is likely that the reduced water consumption will also work practically in the field and that the harvester can use a higher pressure in the spraying device without reducing the vitality of the *P. gigantea* spores. Future studies should be conducted in the field to mechanically test the reduced water consumption at stump treatment and to find out more about possible germination effects of water on the *P. gigantea* spores.

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