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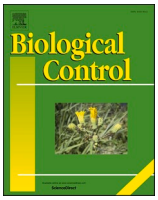

Preventing mycelial spread of *Heterobasidion annosum* in young Scots pine stands using fungal and viral biocontrol agents

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HIGHLIGHTS

- New method to control Heterobasidion root rot in infested pine stands.
- *P. gigantea*-treated stumps around disease centres limited the spread of the pathogen.
- Heterobasidion partitivirus HetPV13-an1 enhanced the control effect.

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ABSTRACT

Heterobasidion annosum is one of the most important causal agents of root rot of pines in Europe. The timing of cuttings to wintertime or stump treatment by control agents in summertime are used to prevent the spread of the fungus to new forest sites via aerial spores. However, there are no efficient control treatments for an already established infection except for changing the tree species to a resistant one, which often is not possible. In this study, we tested whether treating stumps around *Heterobasidion* disease centres by the biocontrol fungus *Phlebiopsis gigantea* could reduce the spread of the pathogen. In addition, we tested whether the infection of *H. annosum* by a debilitating mycovirus, HetPV13-an1, would affect the control efficacy. The results showed that the enlargement of disease centres was reduced by *P. gigantea*, and that this biocontrol effect was enhanced by the virus application. Furthermore, the results showed that the growth rate of *P. gigantea* varies not only between root systems, but also among different roots of a single stump.

1. Introduction

The *Heterobasidion* species are the most important causal agents of root and butt rot of coniferous trees in northern temperate regions. Damage caused by *Heterobasidion* sp. is associated with intensive forest management with the main infection route of the fungus into previously healthy forests being via freshly cut stumps. To prevent primary stump infections, loggings should be carried out when *Heterobasidion* spores are not released, i.e., when the temperature is below zero degrees, or the stumps should be protected against infection with a control agent during the sporulation time of the pathogen (Kallio, 1970). Stump treatment with urea or a biological control agent is an effective prophylactic method, reducing spore infections of stumps by 90–95% (Korhonen et al., 1994).

The major weakness of disease management by currently available methods is the lack of measures to prevent the spread of disease to adjacent trees once the fungus has advanced to the root system. If the tree species cannot be replaced by a resistant one after final felling, or decayed stumps including their root system cannot be removed from the site (Piri and Hamberg, 2015), Heterobasidion root rot continues to spread to the subsequent tree generation (Korhonen et al., 1998).

Of the two *Heterobasidion* species occurring in Finland, *Heterobasidion annosum* sensu stricto (Fr.) Bref. is specialized to Scots pine (*Pinus sylvestris* L.). Besides pine, Norway spruce and other conifers are also prone to *H. annosum* and even deciduous trees such as birch can be infected when growing near an infected conifer (Korhonen et al., 1998). Despite a wide host range, *H. annosum* causes the greatest damage in pure pine forests, particularly if the fungus has an opportunity to spread from one pine generation to another. After regeneration, the first pine seedlings in the subsequent tree generation die about five years after planting when the pathogen from the stumps of the previous tree generation spreads to the seedling's root system. The mycelial spread of *H. annosum* below ground level can be intensive resulting in annually expanding disease centres (DCs). Moreover, infected trees produce fruiting bodies which increase the risk of spore infections in subsequent thinnings (Piri et al., 2021). Heterobasidion root rot may directly kill pine trees, or indirectly contribute to mortality due to windthrow or insects by weakening the root structure and function. In addition to mortality and decay, *Heterobasidion* infections lead to growth losses (Wang et al. 2014). Thus, new control methods to restrict the secondary,

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mycelial spread of *H. annosum* are necessary to maintain the productivity of pine forests on infested sites.

A possible method to control the secondary spread of *H. annosum* via root contacts from infected to healthy pines could be via blocking its pathway with the competing fungus *Phlebiopsis gigantea* (Fr.) Jül. (Sierota and Małecka, 2004; Sierota et al., 2007; Sierota, 2013). The saprophytic white rot fungus *P. gigantea* is a pioneer colonizer of freshly exposed conifer sapwood and is already used as a biological stump treatment agent against *Heterobasidion* spore infections in conifer stumps (Pratt et al., 2000). In Finland, the commercial product of *P. gigantea*, Rotstop®, was formulated by Verdera Inc. in 1991 and has been used thereafter in practical forestry to prevent *Heterobasidion* spore infections on both pine and spruce stumps (Korhonen et al., 1994). *P. gigantea* is a strong competitor of *Heterobasidion* sp. on stump surface due to its rapid colonization and hyphal interference with *Heterobasidion* sp., i.e., a mechanism that triggers the death of the cytoplasm when two hyphae from different species meet (Ikediugwu et al., 1970; Ikediugwu, 1976; Holdenrieder and Greig, 1998). Being better adapted to pine than spruce wood, *P. gigantea* colonises pine sapwood more effectively (Kallio, 1971; Korhonen, 2003; Webber and Thorpe, 2003) and can grow into the stump roots and, at least to some extent, replace *H. annosum* in pine stumps (Rishbeth, 1951, 1963; Meredith, 1960; Kallio, 1971; Tubby et al., 2008). Although numerous potential biocontrol agents have been tested against *Heterobasidion* sp., so far *P. gigantea* has been shown to be the most effective and is the only one widely used as biological stump treatment agent (Holdenrieder and Greig, 1998).

In addition to currently available control approaches, Heterobasidion partitivirus 13-an1 (HetPV13-an1), a disease-causing virus of *H. annosum* (Vainio et al., 2018), could reduce damage by an already established *Heterobasidion* infection at a forest site. This virus has been shown to considerably reduce the hyphal growth of its host on artificial media by widely affecting the expression of its genes, and when transmitted to *H. parviporum*, to reduce its growth within the trunk of living Norway spruce trees (Vainio et al., 2018). However, tolerance against the detrimental effects of HetPV13-an1 infection on artificial medium is common among *H. annosum* strains, and the presence of other fungi may enhance or reduce its transmission between mycelia and/or its phenotypic effects on the host (Kashif et al., 2019; Hantula et al., 2020). Moreover, even the infected mycelium may be able to partially recover (Kashif et al., 2021). Therefore, the usefulness of HetPV13-an1 against *H. annosum* in practical forestry is unclear.

The first aim of the present study was to test whether enlargement of *Heterobasidion* DCs can be restricted by inoculating healthy pine stumps around a DC with *P. gigantea* (Rotstop®) and thereby prevent the spread of *H. annosum* through the treated buffer zone to the healthy pines. Simultaneously, we tested whether the spread of *Heterobasidion* mycelium could be further retarded by the application of the HetPV13-an1 virus to diseased stumps, with virus treatments aiming to reduce the competitiveness of *H. annosum* against *P. gigantea*. Moreover, the growth rate and colonization capacity of *P. gigantea* in mechanically treated pine stumps was determined.

2. Material and methods

2.1. Study sites

This study was conducted in seven Scots pine stands at five different localities in southern and central Finland. The developmental stage of the study forests varied from sapling stands to commercial thinning stands. All stands showed symptoms associated with Heterobasidion root rot, i.e., single symptomatic trees or small groups of dead or dying trees. The experimental stands were quite poor dryish heaths, i.e., *Vaccinium* type according to Finnish forest type classification by Cajander (1949). The stand characteristics, the number of study plots and sectors (see Chapter 2.2.) in each stand and the length of the study period are given in Table 1.

2.2. Establishment of study plots

The study plots were established in summer 2016, except in Kauhava and Säkylä where the plots were established in 2014 and 2017, respectively (Table 1). Each study plot corresponded to one DC. The number of infected trees in a DC varied from one to 12 (mean 3.8 trees), and the area of the DCs ranged from a single tree centre, to a centre with a diameter of 12.5 m (mean 2.7 m).

First, all diseased trees on the plot (disease centre) were mapped, labelled, and cut down, the stump diameter measured, and a sample disc for isolation of *Heterobasidion* mycelium was then sawn from the stump. Following this, one to three rows of healthy-looking trees on the buffer zone were felled proceeding outwards from the edge of the DC (Fig. 1). At this stage of the study, it so happened that especially in the oldest experimental stands, the most asymptomatic pines, which were classified as healthy and considered to form a buffer zone, were actually infected as stump surfaces showed typical signs of *Heterobasidion* infection such as resin-soaked patches and/or decay (Laine, 1976; Kurkela, 2002). Those stumps were excluded from the buffer zone and included to the DC.

The stumps on the buffer zones were mapped. Although these stumps looked visually healthy, a sample disc was sawn immediately above ground level from all stumps to detect possible incipient *Heterobasidion* infection not yet visible on stump surface. All sample discs collected from the study plots were placed in separate paper bags and brought to

Table 1
Stand characteristics, number of sample plots and sectors, location, and length of the study period of each experimental stand.

Experimental stand	Regeneration method	Avg. stand age, yrs.	Avg. no. of trees/ 100 m^2	No. of sample plots[1]	No. of sectors	Location	Study period
Hausjärvi	natural, seeding	10	173	18	29	N60° 46.892′ E24° 53.390′	2016–2020
Säkylä	natural	9	60	7	10	N61° 10.189′ E22° 17.207′	2017–2020
Uusikaupunki	seeding	20	28	4	6	N60° 41.853′ E21° 27.932′	2016–2020
Kauhava 1	natural	30	11	3	5	N63° 13.630′ E23° 11.232′	2014–2020
Kauhava 2	planting	22	14	5	8	N63° 13.663′ E23° 11.314′	2014–2020
Salo 1	natural	40	8	3	5	N60° 25.095′ E23° 37.312′	2016–2020
Salo 2	natural	23	22	2	3	N60° 25.255′ E23° 37.850′	2016–2020

[1] Includes 17 undivided plots and 25 plots divided to sectors. See Chapter 2.2.

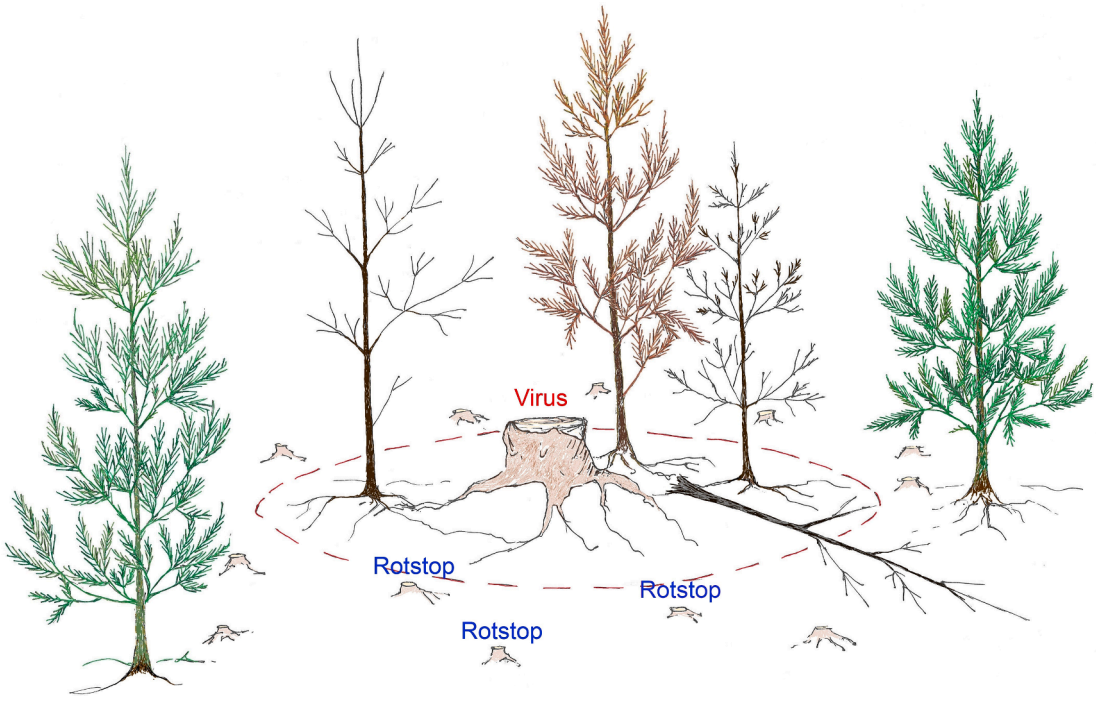

Fig. 1. Schematic drawing showing a *H. annosum* disease centre with dead and declining trees (circled) and our treatment plan: In a DC, all trees showing symptoms of Heterobasidion root rot (e.g., declining crowns) will be cut down and the stumps as well as infected stumps of the previous tree generation will be treated with HetPV13-an1 (virus). In the buffer zone surrounding the DC, one to three rows of healthy-looking pines will be cut down and their stumps will be treated with *P. gigantea* (Rotstop®).

the laboratory for further processing. If there were old pine stumps of the previous tree generation in the plot, they were also sampled by taking a disc from both stump body and main roots. In the laboratory, the discs were washed, incubated, and sampled for *Heterobasidion* mycelium as described earlier (Piri et al., 2021). The *Heterobasidion* pure cultures were stored at 4 °C on MEA for later pairing tests.

Immediately after sampling, the stumps on the buffer zone were sprayed manually with Rotstop®-solution, with the exception being the Kauhava experiment (Stands 1 and 2), where the Rotstop® treatment was applied mechanically by a harvester in connection with the thinning. Both in the manual and mechanical treatments, the same production batch of Rotstop® was used. After thinning in the Kauhava experiment, those treated stumps in the buffer zone showing symptoms of *Heterobasidion* infection were sampled by means of an increment borer. After incubation, all 10 symptomatic stumps proved to be infected by *H. annosum*. In addition, immediately after the sample disc was taken, a virus treatment was carried in a subset of DCs as described in Vainio et al. (2013) in all other experimental stands except for those in Säkylä. In the virus treatment, all pine stumps considered visually infected by *H. annosum*, i.e., stumps of both of symptomatic trees initially assigned to DCs, and stumps of non-symptomatic trees that initially were assigned to the buffer zones with decay and/or resin patches on the stump surface, were treated with HetPV13-an1 by spreading a liquid solution of the virus hosting *H. annosum* strain 04120/1b* over the stump surface. The treatments were carried out between May and August in dry weather with day temperatures ranging from approximately 15 °C to 25 °C.

In the eight control plots, without a buffer zone, the trees showing external symptoms of *Heterobasidion* infection were mapped, felled, and sampled in the same way as in the treated plots. With the same method, in four DCs in the Säkylä experiment, a sector (a triangle with its vertex in the centre of the DC) was left untreated as a control sector while the other parts of the plot were provided with a buffer zone on which the stumps were treated with Rotstop®. These control sectors were established to supplement the control plots. The control plot in the Salo experiment (Stand 1) could not be used because it had been destroyed in a thinning operation. The number of plots/sectors for each study stand are given in Table 2.

Due to the infected stumps on the buffer zone and uneven distribution of seedlings around DCs (especially in naturally established and seeded stands), the number of rows of healthy, Rotstop®-treated stumps varied around individual DCs. Therefore, 25 of the 42 study plots were divided into sectors according to the number of rows with healthy stumps on the buffer zone. When the number of rows is 1, it means that on the buffer zone one row of healthy stumps are treated with Rotstop®. Correspondingly, if the number of rows is two or three, there are two and three rows of healthy stumps treated with Rotstop®, respectively. “0 row” means that although the trees on the buffer zone looked externally healthy, the wood samples taken from the stumps showed that they had an incipient *Heterobasidion* infection before Rotstop® treatment (Table 2). On average, there were 5, 7 and 11 healthy Rotstop®-treated stumps per sector in the “1 row”, “2 rows” and “3 rows” sectors, respectively. The total number of sectors was 66 ranging from one to three per plot (Table 1). The number of trees infected through the buffer zone as well as the proportion of infected trees of the total number of trees bordering the buffer zone, i.e., the nearest trees bordering the outer edge of the zone, were recorded for each sector separately. A total of 17 plots/13 sectors were rejected because of pre-existing infections adjacent to the outer edge of the buffer zone. In all, 382 pine stumps on the buffer zone surrounding 42 DCs (study plots) were treated with Rotstop®. Of these stumps visually classified as healthy and treated with Rotstop®, a total of 43 were later – after incubation and checking the disc sample – found to be infected by *H. annosum*.

At the end of the experiment, the health status of a total of 506 trees bordering the buffer zone and DCs (control plots/sectors) were analysed based on their external condition. Those trees showing any visible signs of *Heterobasidion* infection were sampled. In addition, 30 healthy-looking trees bordering the buffer zone in the experimental stands in

Table 2
Number of plots/sectors, total number of edging trees, and proportion of edging trees infected by *H. annosum* through the buffer zone with 0, 1, 2, or 3 rows of healthy stumps treated with Rotstop® by treatments in seven experimental stands. "0 row" means that the stumps were infected by *H. annosum* before Rotstop® treatment.

Experimental stand	Number of rows consisting of healthy stumps treated by Rotstop® by treatments												
	Rotstop® treatment alone				*Rotstop® and virus treatment*				*Both treatments combined*				*Controls*[2]
	0[1]	1	2	3	0[1]	1	2	3	0[1]	1	2	3	
Hausjärvi													
No. of plots/sectors	1/5	2/3	0/5	0/1	4/3	1/2	0/2	0/0	5/8	3/5	0/7	0/1	2/0
Total no. of edging trees	38	32	30	8	55	19	6	0	93	51	36	8	23
Infected trees, %	18.4	15.6	6.7	0	36.0	0	0	–	28.4	9.8	5.6	0	52.2
Säkylä													
No. of plots/sectors	0/0	2/5	0/1	0/2	0/0	0/0	0/0	0/0	0/0	2/5	0/1	0/2	1/4
Total no. of edging trees	0	39	4	6					0	39	4	6	39
Infected trees, %	–	5.1	0	0	–	–	–	–	–	5.1	0	0	30.8
Uusikaupunki													
No. of plots/sectors	2/1	0/1	0/0	0/0	0/2	0/0	0/0	0/0	2/3	0/1	0/0	0/0	1/0
Total no. of edging trees	28	8	0	0	10	0	0	0	38	8	0	0	15
Infected trees, %	32.1	0	–	–	0	–	–	–	23.7	0	–	–	33.3
Kauhava 1													
No. of plots/sectors	0/0	1/1	0/1	0/0	0/0	0/1	0/1	0/0	0/0	1/2	0/2	0/0	1/0
Total no. of edging trees	0	15	7	0	0	11	7	0	0	26	14	0	19
Infected trees, %	–	6.7	0	–	–	0	0	–	–	3.8	0	–	31.6
Kauhava 2													
No. of plots/sectors	0/0	1/0	1/0	0/0	0/1	0/1	0/2	0/2	0/1	1/1	1/2	0/2	2/0
Total no. of edging trees	0	7	8	0	10	5	11	20	10	12	19	20	24
Infected trees, %	–	0	0	–	40.0	0	9.1	0	40.0	0	5.3	0	50.0
Salo 1													
No. of plots/sectors	1/0	0/0	0/0	0/0	0/3	0/1	0/0	0/0	1/3	0/1	0/0	0/0	0/0
Total no. of edging trees	14	0	0	0	22	5	0	0	36	5	0	0	0
Infected trees, %	35.7	–	–	–	22.7	20.0	–	–	27.8	20.0	–	–	–
Salo 2													
No. of plots/sectors	0/0	1/0	0/0	0/0	0/1	0/0	0/1	0/0	0/1	1/0	0/1	0/0	1/0
Total no. of edging trees	0	4	0	0	6	0	10	0	6	4	10	0	13
Infected trees, %	–	25.0	–	–	0	–	0	–	0	25.0	0	–	60.0
All stands													
No. of plots/sectors	4/6	7/10	1/7	0/3	4/10	1/5	0/6	0/2	8/16	8/15	1/13	0/5	8/4
Total no. of edging trees	80	105	49	14	103	40	34	20	183	145	83	34	133
Infected trees, %	26.3	8.6	4.1	0	28.2	2.5	2.9	0	27.3	13.8	3.6	0	41.4

[1] includes stumps infected by *H. annosum* before Rotstop® treatment.
[2] without buffer zone.

Uusikaupunki, Kauhava and Salo were sampled by taking two to three cores from the butt of the tree with the aid of an increment borer. All those samples were healthy.

2.3. Preparation of treatments solutions

Rotstop®-solution was prepared according to the manufacturer's instructions in the morning of the same day as it was used. The virus solution for stump treatments was prepared as described previously (Vainio et al., 2013) by growing *H. annosum* strain 04120/1b* harboring the alphapartitivirus HetPV13-an1 in a liquid culture with 2% malt extract and Sipernat 22S silica powder (Algol Chemicals, Evonik Degussa GmbH, Germany) dissolved in 200 ml of sterile water for 2 weeks. Following this, the cultures were homogenized in the morning of the treatments and diluted 1:3 with tap water and thereafter kept cool in a cold box containing ice bricks. The *H. annosum* strain 04120/1b* used for stump treatments had been prepared by transmitting HetPV13-an1 from its original host *H. annosum* 94233 (Kashif et al., 2015) by inoculating it on the same agar plate as the recipient strain *H. annosum* 04120/1b and allowing their hyphae to grow into contact. The growth rate of *H. annosum* strain 04120/1b* was very slow after virus introduction (Vainio et al., 2018). This was considered beneficial because a slow-growing *Heterobasidion* isolate would not easily establish itself in the treated stumps and would likely be outcompeted by the indigenous *Heterobasidion* strains pre-existing in the stumps, still being able to engage in cellular contact and transmit the virus as demonstrated in our earlier investigation using the *Heterobasidion* alphapartitivirus HetPV4 (Vainio et al., 2013).

2.4. Analysing the spread of Heterobasidion genotypes

The sample plots were monitored annually for the next three to six years. If any new trees showing signs of *Heterobasidion* infection (i.e., dead or dying trees or trees with thin or chlorotic crowns, reduced height growth or distress cones) were found outside the buffer zone, they were felled and sampled. At the end of the experiment, all *Heterobasidion* isolates collected from the same plot and outside, behind the buffer zone, were paired with each other to identify their genotypes (Stenlid, 1985). If a *Heterobasidion* genotype present in the DC was isolated from an infected tree behind the buffer zone, the fungus was considered to have spread vegetatively through the blockade to a healthy tree. Finally, a representative from each genet was identified at the species level by pairing with homokaryotic tester strains of *H. annosum* s.s. and *H. parviporum* (Korhonen, 1978). All genets proved to be *H. annosum*.

The transmission of HetPV13-an1 into native strains of *H. annosum* s. s. pre-existing in the treated stumps was tested two years after the inoculation by taking wood samples from 28 virus-treated stumps in the Hausjärvi, Uusikaupunki, Kauhava and Salo experiments. The *Heterobasidion* mycelium was cultured, and the presence of HetPV13-an1 was tested by isolating RNA and conducting RT-PCR as described in Vainio et al. (2015) using virus specific primers 95122midFor2 and 95122midR (Kashif et al., 2015).

2.5. Determining the growth rate of P. gigantea in pine stumps

The growth rate of *P. gigantea* was measured in two Kauhava experimental stands. A total of 72 pine stumps, treated mechanically by a harvester during the thinning carried out in August 2014, were randomly selected for the analysis. The advance of the mycelium of *P. gigantea* downwards from the stump surface was determined one, two and three years after stump treatment (thinning). One year after the Rotstop-treatment, the first 30 stumps (20 in Stand 1 and 10 in Stand 2) were analysed by sawing two sample discs 10 and 15 cm below the stump surface and one cross-sectional disc from the base of three main roots. Two years after the treatment, a below-ground sampling was performed by manually excavating three horizontal main roots of 30 previously untouched stumps (10 in Stand 1 and 20 in Stand 2) and excising five transversal discs 30, 40, 50, 60 and 70 cm from the root collar. The final 12 stumps (in Stand 1) were sampled three years after the treatment, when root samples were taken in 10 cm intervals (starting at 30 cm) towards the root tip, i.e., until the root diameter was<2 cm (Fig. 2). In the laboratory, the sample discs were processed in the same way as the discs to identify *Heterobasidion* infection. After incubation, isolations were made by transferring hyphae of *P. gigantea* from the wood surface on malt extract agar (MEA) media. To verify that the infection in stumps originated from the inoculated Rotstop®-strain, somatic compatibility tests were performed between isolated and inoculated strains (Korhonen and Kauppila, 1987).

2.6. Calculations

A nonparametric Kruskal-Wallis H test (one-way ANOVA on ranks) was used to determine whether there was a statistically significant difference in the proportion of trees infected by *H. annosum* among treatments (treatment both with *P. gigantea* and virus, treatment only with *P. gigantea*, both treatments combined, control without *P. gigantea* and virus). Following a significant Kruskal-Wallis test ($p < 0.001$), *post hoc* Mann-Whitney tests were conducted for pairwise comparisons. Within the treatments, the infection rates were compared between different numbers of treated stump rows. A Spearman rank correlation was used to test the correlation between the proportion of trees infected through the buffer zone and the minimum distance from the outer edge of the buffer zone to the nearest tree, the diameter of treated stumps, and the age of the trees. The significance level in all tests was $p \leq 0.05$. The analyses were performed using IBM SPSS Statistics, version 28.0.1.0.

3. Results

3.1. Effect of the buffer zone on the mycelial spread of Heterobasidion genotypes

When the infected trees in DCs were separated from the surrounding stand by one, two or three rows of healthy, Rotstop®-treated stumps, the proportion of trees infected vegetatively through the buffer zone was 10.8, 3.1 and 0%, respectively. The corresponding figures for sectors, where the Rotstop® treatment was combined with a virus treatment, were 3.3, 4.2 and 0%, respectively, and for the treated sectors together (including both Rotstop treated and Rotstop and virus treated sectors) 8.8, 3.6 and 0%, respectively (Table 3). Only the nearest trees bordering the outer edge of the buffer zone are included in the calculations.

In the control plots (eight plots) and control sectors without a buffer zone (four sectors), a total of 50 trees, i.e., 42.1% of the nearest trees bordering DCs, were infected vegetatively by expanding *Heterobasidion* genets.

Two years after the inoculation of the virus donor, the transfer of HetPV13 into the local *Heterobasidion* strains was tested in four experimental areas. The virus was detected in 16 out of the 28 *Heterobasidion* isolates successfully cultured from these sites (Table 4). Therefore, the average transmission rate of HetPV13-an1 from the donor to the recipient was 57.1%, and the lowest efficacy was observed in Salo, where spread of *Heterobasidion* was observed more often than in other Rotstop and virus treated plots.

A significant difference ($p < 0.05$) was observed between the *Heterobasidion* infection rates of trees in control and treated sectors (Rotstop® with and without HetPV13-an1), mostly due to the low rate of infection passing through the row 1. If the stumps were not healthy before Rotstop treatment (0 row in Table 3), the infection rate did not differ significantly from the control although the infection rate was lower in treated sectors than in controls (Table 3).

There were no significant differences in the infection rate between the sectors with one, two or three rows treated stumps between the treatments (Rotstop, Rotstop and virus, and both combined) nor within the treatments. It should be noted, however, that the total number of sectors with two and three stump rows was small. However, no trees were found to be infected through buffer zone with three rows of stumps (Table 3).

No correlation was observed between the minimum distance from the outer edge of the buffer zone to the nearest tree and the proportion of trees infected through the buffer zone. Neither was the stand age, nor the diameter of the treated stumps significantly correlated with the proportion of trees infected through the buffer zone.

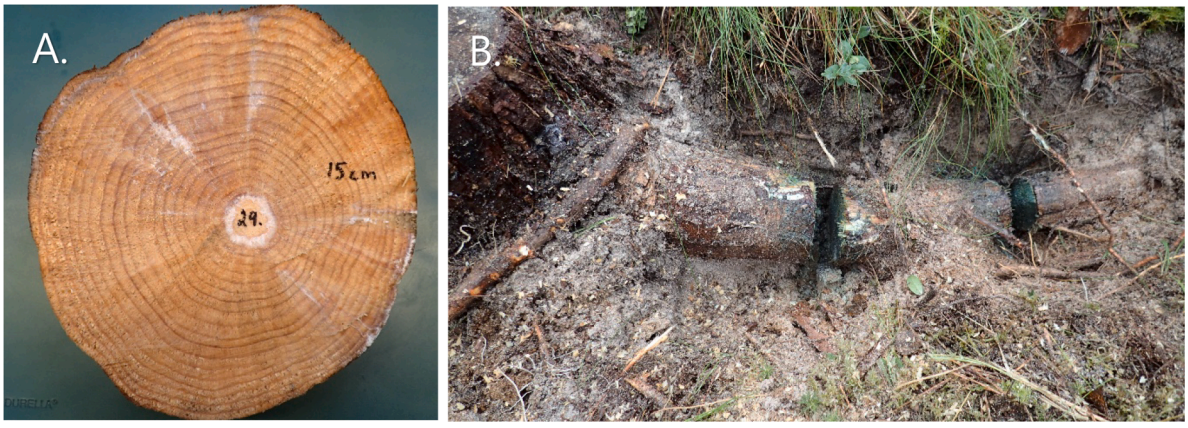

Fig. 2. A. A cross section 15 cm below the surface of a Rotstop®-treated pine stump (no. 29) one year after treatment. The area colonized by *P. gigantea* is characterized by a brownish-orange discoloration. **B**. Three years after Rotstop® treatment, the first two cross-sectional discs were taken at a distance of 30 and 40 cm from the root collar. Root sampling proceeds every 10 cm towards the root tip. After sampling, the cut ends in the roots have been treated with Rotstop® (revealed as a blue color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Number and proportion of trees infected by *H. annosum* through the buffer zone given as an average of all sectors/plots by treatments and by number of rows consisting of healthy stumps inoculated with Rotstop® on the buffer zone (rows 1–3). "0 row" means that the stumps on the buffer zone were infected before Rotstop® treatment, or the stumps were left untreated (controls). The differences in the frequency of *Heterobasidion* infections between control and other treatments with 1–3 stump rows were statistically significant ($p < 0.05$).

Treatment	No. of healthy stump rows treated with Rotstop®							
	0[1,2]		1		2		3	
	Infected trees							
	no.	%	no.	%	no.	%	no.	%
Rotstop® treated sectors/plots	21[1]	30.7[3] (0–100)	9	10.8 (0–40.0)	2	3.1 (0–25.0)	0	0
Rotstop® and virus-treated sectors/plots	18[1]	24.9 (0–75.0)	1	3.3 (0–20.0)	1	4.2 (0–25.0)	0	0
Both treatments combined	43[1]	26.9 (0–100)	10	8.8 (0–40.0)	3	3.6 (0–25.0)	0	0
Controls	50[2]	42.1 (16.7–88.9)						

[1] Stumps infected by *H. annosum* before Rotstop® treatment.
[2] Stumps not treated.
[3] Mean (min.-max.) of all sectors/plots combined by treatments.

Table 4
Virus transmissions from the donor to the natural *H. annosum* in the stumps.

Experimental stand	No. of *H. annosum* isolates analyzed	Transmission		
		Yes	No	Success rate, %
Hausjärvi	10	9	1	90.0
Uusikaupunki	5	3	2	60.0
Kauhava 1 & 2	2	1	1	50.0
Salo 1 & 2	11	3	8	27.3
Total	28	16	12	57.1

3.2. Growth rate of *P. gigantea* in pine stumps

One year after the Rotstop® treatment, *P. gigantea* had infected all 30 analysed pine stumps (8.5–20.5 cm in diameter; mean 13.9 cm) and colonized on average 81 and 74% of the stump surface area at the depth of 10 and 15 cm below the stump top, respectively. In eight roots (9.1% of all analysed roots) of seven stumps, the fungus had reached the root collar, advancing on average 24.3 cm (to a maximum of 36 cm) from the top of the stump.

Two years after the treatment, a random sample of 102 roots of 30 stumps (8–19.5 cm in diameter; mean 13.4 cm) was analysed. *P. gigantea* was found in 73 roots of 11 stumps. Most frequently (43% of all infected roots) the fungus had spread 40–50 cm, corresponding to an annual growth rate of 20–25 cm. At its longest, the fungus had spread 70 cm in two years.

Three years after the treatment, the fungus was present in eight out of the 12 sampled stumps (10.5–20 cm in diameter; mean 14.8 cm). In those stumps, 17 roots (68% of all analysed roots) were colonized by *P. gigantea*. In most infected roots (82.3%), *P. gigantea* had spread 50–100 cm from the stump surface indicating an annual growth rate of about 17–30 cm. *P. gigantea* had spread more than one metre in 11.8% and less than half a metre in 5.9% of roots (Table 5).

4. Discussion

The experimental stands were heterogeneous in terms of both stand age and incidence of Heterobasidion root rot. However, more than half of the study plots were covered by about ten-year-old trees making young stands the main focus of the investigation. The youngest stand in Säkylä, where the incidence of Heterobasidion root rot was low and DCs were scattered and small in size, was best suited for the blocking method in terms of practical implementation. The control efficiency (one row stumps treated with Rotstop®) was also better than in our study material on average. In the other young sapling stand in Hausjärvi, the previous pine generation was severely infected which was reflected in numerous infections in the regeneration. In addition, the stand was exposed to *Heterobasidion* spore infections already at an early stage due to a summer tending carried out without stump treatment. Though the high disease frequency made the blocking of individual DCs challenging in this site, nonetheless the positive control effect was clear. Despite the young age of the trees, high disease frequency was also observed in the experimental stand in Uusikaupunki, where a high diversity of *Heterobasidion* genotypes was most likely the result of numerous spore infections due to an early precommercial summer thinning. In such stands where DCs are closely spaced and concentrated in limited parts of the stand, it could be more reasonable to block the complex of several nearby DCs instead of blocking each individual DC separately.

Although the obtained results were promising and the buffer zone clearly inhibited enlargement of DCs regardless of the age of the experimental stand, it must be noted that the follow-up might have been too short especially in older stands. In young and dense pine regenerations, where Heterobasidion root rot progresses rapidly from tree to tree and where trees die quickly after being infected (Piri et al., 2021), the study period (three and four years) seemed to be long enough to prove the effectiveness of the blocking method. This view was also supported by the fact that on the control plots in Hausjärvi and Säkylä new perimeter trees were infected at a maximum distance of 3.95 m and 2.75 m from the outer edge of the DC, exceeding the width of the buffer zones, that were 0.8 and 1.2 m on average, respectively. Compared to young regeneration stands, the distance between trees was greater in the older commercial thinning stands (e.g., mean distance 2.9 m in Salo 1), increasing the time of the movement of *H. annosum* from one tree to another. So far, there is little data available about the growth rate of *H. annosum* s.s. in pine roots in Nordic conditions, but according to an inoculation experiment performed in a 40-year-old Scots pine stand in south-eastern Finland, the average growth rate of *H. annosum* in living pine roots averaged 10 cm/year (Piri, 2000). Though *Heterobasidion* mycelium spreads in dead stump roots two to three times faster than in roots of a living tree (Bendz-Hellgren et al., 1999; Pettersson et al., 2003), it takes many years for the fungus to advance through the buffer zone and cause visible symptoms in surrounding living trees. Even though the study lasted six years in Kauhava and five years in Salo, it can be expected that new trees may become infected through the buffer zone in the coming years. It would have been helpful, especially in older stands, to fell all the nearest edge trees to identify possible incipient infections at the base of the trees, but unfortunately that was not possible during this study conducted on private land.

Table 5
P. gigantea infections in pine roots two and three years after Rotstop® treatment.

Root infections	Distance from the top of the stump, cm							
	31–40	41–50	51–60	61–70	71–80	81–90	91–100	>100
2 years after treatment[1]								
Infected roots, no. (%)	14 (13.7)	44 (43.1)	13 (12.8)	2 (2.0)	0	0	0	0
Root diameter at the furthest point, min.-max. (mean), cm	2.4–8.1 (5.5)	1.4–6.6 (4.0)	3.4–5.8 (4.3)	2.7–4.3 (3.5)				
3 years after treatment[2]								
Infected roots, no. (%)	1 (4.0)	0	3 (12.0)	3 (12.0)	4 (16.0)	2 (8.0)	2 (8.0)	2 (8.0)
Root diameter at the furthest point, min.-max. (mean), cm	(2.6)		3.7–5.7 (5.0)	1.6–5.0 (2.9)	0.7–3.0 (2.2)	0.6–3.0 (1.8)	1.9–2.3 (2.1)	0.6–1.8 (1.2)

[1] 102 roots of 30 stumps analyzed; stump diameter 8–19.5 cm (mean 13.4 cm).
[2] 25 roots of 12 stumps analyzed; stump diameter 10.5–20 cm (mean 14.8 cm).

The identification of diseased pines is challenging as *Heterobasidion* infections may occur latently in the root system without any symptoms in the canopy (Kurkela, 2002; Wang et al., 2014), which was also observed in this study when study plots were established in older stands. Particularly in the Salo experimental stand, the boundaries of infection centres were difficult to determine accurately. In most cases, small resin and/or decay patches indicative for *Heterobasidion* infection were observed on fresh stump surface cuts just above ground level when externally healthy-looking trees surrounding DCs were felled. In other words, the area of DCs was clearly larger (even 18 m in diameter) than what was supposed from the external condition of the standing trees. Such big openings in pole-sized or small sawtimber-sized stands are not advisable unless the opening is replanted with broad leaf trees more resistant to *H. annosum* (Korhonen, 1978). On the other hand, in the Kauhava experiment, all sampled stumps showing resin and/or decay patches on the cut surface proved to be infected by *H. annosum*, indicating that *H. annosum* was the predominant causal agent of root rot.

A more attractive alternative to blocking individual DCs (without replanting) in older pine stands might be a conventional thinning carried out during the thermal growing season (i.e., when daily mean temperature is permanently above + 5 °C) including stump treatment with Rotstop®. Our results indicate that the Rotstop® treatment might have a preventive impact on the progress of Heterobasidion root rot even when the stumps were infected by *H. annosum* before treatment (Table 3; 0 row). This is also supported by earlier studies showing that *P. gigantea* is able to spread into the root system of Scots pine stumps and thereby limit the mycelial spread of *H. annosum* (Rishbeth, 1951; Meredith, 1960; Holdenrieder, 1984; Zaļuma et al., 2019). The high growth rate of *P. gigantea* in stump roots (on average 20–30 cm/yr. with a maximum of 36 cm/yr.) observed in the present study, and its ability to colonize most main roots of pine stumps also supports the positive effect of the Rotstop® treatment in controlling the mycelial spread of *H. annosum* via root contacs in infested stands. Consequently, Rotstop® treatment likely also limits the production of *Heterobasidion* basidiocarps. However, more detailed information is required on the effectiveness of stump treatment in preventing mycelial spread of *H. annosum* in infested pine forests before practical recommendations can be made.

As shown in this study, the growth rate of *P. gigantea* can vary widely not only between individual stumps but also between stump roots. Moreover, the obtained results revealed that although *P. gigantea* is an effective colonizer of pine stumps, it does not spread to all stump roots, even if the stump surface is completely occupied by the fungus. Thus, it is understandable that a single row of treated stumps cannot stop disease spreading; on average 8.8% of the nearest trees bordering the buffer zone were infected through the zone. Compared to the proportion of infected trees on controls without any buffer zone (42.1%) the control effect was, however, substantial.

This study did not prove that the control efficiency of the blocking method would increase significantly with an increasing number of stump rows inoculated with Rotstop® alone or in combination with HetPV13-an1. Nevertheless, the proportion of through-buffer-zone-infected trees decreased with an increasing number of stump rows, and therefore such an effect is probable, though the differences were not significant due to the small data for sectors with two and three stump rows.

A similar study on blocking trees infected by *H. annosum* by treating healthy surrounding stumps with a *P. gigantea* preparate has been carried out in approximately 30-year-old Scots pine stands on former agricultural land in Poland. Six years after stump treatment, no infected trees outside the treated area with a radius of five metres (the number of rows with treated stumps surrounding DCs was not given) were observed (Sierota et al., 2007). The result shows that *P. gigantea* is a strong competitor of *H. annosum* even on the old agricultural land where soil properties are favourable for an accelerating spread of *H. annosum* (Bruna et al., 2019).

The isolation of *Heterobasidion* mycelia from two-year-old, decayed

stumps to confirm virus transmission resulted in being difficult. Small stumps in sapling stands were too decayed to obtain wood samples for fungal isolation, and therefore no significant differences in the infection rate were found between sectors with Rotstop treatment only, and with combined Rotstop and virus treatment. However, based on the 28 successful mycelial isolations, 57.1% had received the virus suggesting for an efficient transmission of HetPV13-an1. As a result, the spread of the infesting *Heterobasidion* mycelium through one row of Rotstop®-treated stumps (Table 3, row 1) was significantly reduced by the virus treatment. Therefore, the use of HetPV13-an1 to control *H. annosum* in combination with *P. gigantea* as in this investigation, or alone, deserves further testing despite the high rate of tolerance among this species observed previously (Vainio et al., 2018).

5. Conclusions

Our study has shown that it is possible to prevent the spread of *H. annosum* via root contacts and reduce the expansion of DCs in Scots pine stands by treating the stumps of healthy pines surrounding a DC with Rotstop® (*P. gigantea*). The obtained results indicate that the control efficiency can be further improved if, in addition to the Rotstop® treatment, the infected stumps in a DC are inoculated with a Heterobasidion virus (HetPV13-an1). One row of treated stumps surrounding a DC gave a significant control effect. Because *P. gigantea* is, however, not able to colonize all stump roots, more than one row of Rotstop® treated stumps seems to be necessary to stop mycelial spread of *H. annosum*. The greatest benefit of the method can be achieved in young pine stands on slightly infested sites where the disease centres are scattered and small in size.

Declaration of Competing Interest

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

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References

Bendz-Hellgren, M., Brandtberg, P.-O., Johansson, M., Swedjemark, G., Stenlid, J., 1999. Growth rate of *Heterobasidion annosum* in *Picea abies* established on forest land and arable land. Scand. J. For. Res. 14, 402–407. https://doi.org/10.1080/02827589950154104.

Bruna, L., Klavina, D., Korhonen, K., Zaļuma, A., Burņeviča, N., Gaitnieks, T., 2019. Effect of soil properties on the spread of Heterobasidion root rot. In: Proceedings of the Latvian Academy of Sciences. Section B. 73(6): 466-471.

Cajander, A.K., 1949. Forest types and their significance. Acta For. Fenn. 56, 1–71.

Hantula, J., Mäkelä, S., Xu, P., Brusila, V., Nuorteva, H., Hyder, R., Vainio, E.J., 2020. Multiple virus infections of *Heterobasidion parviporum*. Fungal Biol. 124, 102–109. https://doi.org/10.1016/j.funbio.2019.12.004.

Holdenrieder, O., 1984. Untersuchungen zur biologischen Bekämpfung von *Heterobasidion annosum* an Fichte (*Picea abies*) mit antagonistischen Pilzen II. Interaktionstests auf Holz. Eur. J. For. Pahol. 14, 137–153. https://doi.org/10.1111/j.1439-0329.1984.tb00938.x.

Holdenrieder, O., Greig, B.J.W., 1998. Biological methods of control. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*: biology, ecology, impact, and control. CAB International, pp. 235–258.

Ikediugwu, F.E.O., 1976. The interface in hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum*. Trans. Br. Mycol. Soc. 66, 291–296. https://doi.org/10.1016/S0007-1536(76)80055-1.

Ikediugwu, F.E.O., Dennis, C., Webster, J., 1970. Hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum*. Trans. Br. Mycol. Soc. 54, 307–309. https://doi.org/10.1016/S0007-1536(70)80044-4.

Kallio, T., 1970. Aerial distribution of the root-rot fungus *Fomes annosus* (Fr.) Cooke in Finland. Acta For. Fenn. 107, 1–55.

Kallio, T., 1971. Protection of spruce stumps against *Fomes annosus* (Fr.) Cooke by some wood-inhabiting fungi. Acta For. Fenn. 117 (20).

Kashif, M., Hyder, R., De Vega Perez, D., Hantula, J., Vainio, E.J., 2015. *Heterobasidion* wood decay fungi host a diverse and globally distributed viruses related to *Helicobasidion mompa* partitivirus V70. Virus. Res. 195, 119–123. https://doi.org/10.1016/j.virusres.2014.09.002.

Kashif, M., Jurvansuu, J., Vainio, E.J., Hantula, J., 2019. Alphapartitiviruses of *Heterobasidion* wood decay fungi affect each other's transmission and host growth. Front. Cell. Inf. Microbiol. 9, 64. https://doi.org/10.3389/fcimb.2019.00064.

Kashif, M., Jurvansuu, J., Hyder, R., Vainio, E., Hantula, J., 2021. Phenotypic recovery of a *Heterobasidion* isolate infected by a debilitation-associated virus is related to altered host gene expression and reduced virus titer. Front. Microbiol. 12, 661554 https://doi.org/10.3389/fmicb.2021.661554.

Korhonen, K., 1978. Intersterility groups of *Heterobasidion annosum*. Commun. Inst. For. Fenn. 94 (6), 25.

Korhonen, K., Kauppila, P., 1987. The sexuality of *Phlebiopsis gigantea*. Karstenia 27: 23-30.

Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., Seiskari, P., Niemi, M., 1994. Control of *Heterobasidion annosum* by stump treatment with 'Rotstop', a new commercial formulation of *Phlebiopsis gigantea*. In: Johansson, M., Stenlid, J. (Eds.), Proc. of the 8th Int. Conf on Root and Butt Rots, Sweden and Finland, Aug. 9-16, 1993. Swedish University of Agricultural Sciences, Uppsala, Sweden, Uppsala. pp. 675-685. ISBN 91-576-4803-4.

Korhonen, K., Delatour, C., Greig, B.J.W., Schönhar, S., 1998. Silvicultural control. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.). *Heterobasidion annosum*: biology, ecology, impact, and control. CAB International, pp. 283-313.

Korhonen, K., 2003. Simulated stump treatment experiments for monitoring the efficacy of *Phlebiopsis gigantea* against *Heterobasidion*. In: Proceedings of the IUFRO International Conference on Root and Butt Rot 2001. Eds. G. Laflamme, J.A. Bérubé, G. Bussières. Quebec City, Canada, September 16-22, 2001. Pp. 206-210.

Kurkela, T., 2002. Crown condition as an indicator of the incidence of root rot caused by *Heterobasidion annosum* in Scots pine stands. Silva Fennica 36 (2), 451–457. https://doi.org/10.14214/sf.537.

Laine, L., 1976. The occurrence of *Heterobasidion annosum* (Fr.) Bref. in woody plants in Finland. Communicationes Instituti Forestalis Fenniae 93 (3), 53.

Meredith, D.S., 1960. Further observations on fungi inhabiting pine stumps. Ann. Bot. 24 (1), 63–78. https://doi.org/10.1093/oxfordjournals.aob.a083689.

Pettersson, M., Rönnberg, J., Vollbrecht, G., Gemmel, P., 2003. Effect of thinning and *Phlebiopsis gigantea* stump treatment on the growth of *Heterobasidion parviporum* inoculated in *Picea abies*. Scand. J. For. Res. 18, 362–367. https://doi.org/10.1080/02827580310007845.

Piri, T., 2000. Response of compensatory-fertilized *Pinus sylvestris* to infection by *Heterobasidion annosum*. Scand. J. For. Res. 15, 218–224. https://doi.org/10.1080/028275800750015037.

Piri, T., Hamberg, L., 2015. Persistence and infectivity of *Heterobasidion parviporum* in Norway spruce root residuals following stump harvesting. For. Ecol. Manage. 353, 49–58. https://doi.org/10.1016/j.foreco.2015.05.012.

Piri, T., Vainio, E.J., Nuorteva, H., Hantula, J., 2021. High seedling mortality of Scots pine caused by *Heterobasidion annosum* s.s. Forests 12 (9), 1289. https://doi.org/10.3390/f12091289.

Pratt, J., Niemi, M., Sierota, Z.H., 2000. Comparison of three products based on *Phlebiopsis gigantea* for the control of *Heterobasidion annosum*. Eur. Bio. Sci. Technol. 10, 467–477. https://doi.org/10.1080/09583150050115052.

Rishbeth, J., 1951. Observations on the biology of *Fomes annosus*, with particular reference to East Anglian pine plantations II. Spore production, stump infection, and saprophytic activity in stumps. Ann. Botany, N.S. 57, 1–21. https://www.jstor.org/stable/42907135.

Rishbeth, J., 1963. Stump protection against *Fomes annosus* III. Inoculation with *Peniophora gigantea*. Ann. Appl. Biol. 52, 63–67. https://doi.org/10.1111/j.1744-7348.1963.tb03728.x.

Sierota, Z., 2013. Heterobasidion root rot in forests on former agricultural lands in Poland: Scale of threat and prevention. Academic Journals. Sci. Res. Essays 8 (47), 2298–2305. https://doi.org/10.5897/SRE2013.5724.

Sierota, Z., Małecka, M., 2004. Formowanie „sztucznych luk" w celu ograniczania huby korzeni i inicjowania przebudowy monokultur sosnowych na gruntach porolnych (Abstract: Creation of „ artificial gaps" to reduce root rot effects and initiate reconstruction of pine monocultures established on post-agricultural land). Sylwan 148 (1), 6–11.

Sierota, Z.H., Malecka, M., Pratt, J., 2007. Eindämmung des Wurzelpilzes *Heterobasidion annosum* in Beständen mit Gemeiner Kiefer in Ackeraufforstungen. Archiv f. Forstwesen u. Landsch.ökol. 41, 29–35.

Stenlid, J., 1985. Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. Can. J. Bot. 63 (12), 2268–2273. https://doi.org/10.1139/b85-322.

Tubby, K.V., Scott, D., Webber, J.F., 2008. Relationship between stump treatment coverage using the biological control product PG Suspension, and control of *Heterobasidion annosum* on Corsican pine. *Pinus nigra* ssp. *laricio*. For. Pathol. 38, 37–46. https://doi.org/10.1111/j.1439-0329.2007.00519.x.

Vainio, E.J., Piri, T., Hantula, J., 2013. Virus community dynamics in the conifer pathogenic fungus *Heterobasidion parviporum* following an artificial introduction of a partitivirus. Micr. Ecol. 65, 28–38. https://doi.org/10.1007/s00248-012-0118-7.

Vainio, E.J., Müller, M.M., Korhonen, K., Piri, T., Hantula, J., 2015. Viruses accumulate in aging infection centers of a fungal forest pathogen. ISME J. 9, 497–507. https://doi.org/10.1038/ismej.2014.145.

Vainio, E.J., Jurvansuu, J., Hyder, R., Kashif, M., Piri, T., Tuomivirta, T., Poimala, A., Xu, P., Mäkelä, S., Nitisa, D., Hantula, J., 2018. Heterobasidion Partitivirus 13 mediates severe growth debilitation and major alterations in the gene expression of a fungal forest pathogen. J. Virol. 92, e01744–e01817. https://doi.org/10.1128/JVI.01744-17.

Wang, L.-Y., Zhang, J., Drobyshev, I., Cleary, M., Rönnberg, J., 2014. Incidence and impact of root infection by *Heterobasidion* spp., and the justification for preventative silvicultural measures on Scots pine trees: A case study in southern Sweden. For. Ecol. Manage. 315, 153–159. https://doi.org/10.1016/j.foreco.2013.12.023.

Webber, J.F., Thorpe, K.V., 2003. Potential for biological control of *Heterobasidion annosum* in the UK using Rotstop®. In: Proceedings of the IUFRO International Conference on Root and Butt Rot 2001. Eds. G. Laflamme, J.A. Bérubé, G. Bussières. Quebec City, Canada, September 16-22, 2001. Pp. 221-225.

Zaļuma, A., Bruna, L., Klavina, D., Burņeviča, N., Kenigsvalde, K., Lazdins, A., Gaitnieks, T., 2019. Growth of *Phlebiopsis gigantea* in wood of seven conifer species. For. Pathol. 2019 (49), e1255.