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IMPACT OF *TRICHODERMA* SP. ON THE DEVELOPMENT OF *HETEROBASIDION ANNOSUM* IN DECAYED UNDERSTORY *PICEA ABIES* STUMPS

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Heterobasidion annosum *(Fr.) Bref. s.l. causes significant economic losses in conifer forests. Therefore, to reduce the spread of the infection surfaces of freshly cut conifer, stumps are commonly treated with biological control agents.* Trichoderma *sp. shows very strong antagonistic effect against* H. annosum in vitro*, but relatively few field studies have been conducted. Moreover, most of previous studies examined the impact of* Trichoderma *sp. on* H. annosum *in healthy conifer stumps. The aim of our work was to evaluate the effect of* Trichoderma *sp. against* H. annosum *in already decayed understory spruce stumps. In total, 75 decayed spruce stumps were surveyed. Part of the spruce stumps were left as a control, and the others were treated with one of two* Trichoderma *isolates (T472 and T945) belonging to two different species:* T. viridescens *and* T. viride*. The occurrence of* H. annosum *was evaluated 3 and 12 months after treatment. The main results were that the area of previously healthy wood occupied by* H. annosum *was larger in control stumps in comparison with treated stumps, but the differences were not statistically significant.*

Key words: Picea abies*, decayed stumps,* Trichoderma*,* Heterobasidion*.*

INTRODUCTION

Heterobasidion annosum (Fr.) Bref. s.l. causes significant economic losses in conifer forests. In Latvia, the losses are estimated to be 800–4790 EUR ha^{-1}, depending on stand age and productivity (Gaitnieks *et al*., 2007). In stands already infected, *H. annosum* spreads from infected to healthy trees via root contacts. Spread of *H. annosum* is very rapid in forest sites previously used for agriculture (Korhonen and Stenlid, 1998). It is considered that one of the reasons why conifer plantations established on agricultural lands have a high risk of *H. annosum* infection is the lack of antagonistic microflora in soil, especially *Trichoderma* sp. (Johansson and Marklund, 1980; Sierota and Kwaśna, 1998; Gaitnieks *et al*., 2009).

To reduce the formation of new infection centres in healthy stands during logging, the surfaces of freshly cut conifer stumps are treated with chemical or biological control agents (Thor, 2005). The most frequently used biological control agent contains spores of fungus *Phlebiopsis gigantea* (Fr.) Jülich (Pratt *et al*., 2000). The Finnish preparation Rotstop® has been used for stump treatment in Latvia since 2007 (Kenigsvalde *et al*., 2011). Stump treatment with *P. gigantea* reduces the infection of stumps by spores of *H. annosum*, but if a stump is already infected by *H. annosum*, *P. gigantea* is not able to replace it, and *H. annosum* can live in the stump for more than 40 years (Piri, 1996). Instead, *P. gigantea* disappears from pine stumps after about three years and from spruce stumps after six years (Vainio *et al*., 2001).

Several fungal species (as *Fomitopsis pinicola*, *Resinicium bicolor*, *Sistotrema brinkmannii*, *Trichodema* sp., and others) have been studied as possible antagonists against *H. annosum*. However, the obtained results were variable, and only a few species have been tested under field conditions (Holdenrieder and Greig, 1998).

Biological preparations containing *Trichoderma* sp. spores are widely used for control of different plant pathogens in agriculture (Lielpetere, 2009) and their inhibiting effect on

the growth of *Ganoderma adspersum, Inonotus hispidus*, and *Polyporus squamosus* in pruning wounds of broad-leaved trees was demonstrated (Schubert *et al*., 2008). *Trichoderma* sp. shows very strong antagonistic effect against *H. annosum in vitro* (Hanso and Hanso, 1985). At present, however, only few data are available on the effect of *Trichoderma* sp. on development of *H. annosum* in conifer stumps, and the obtained results are contradictory. For example, Kallio and Hallaksela (1979) showed that *T. viride* had a negative impact on the development of *H. annosum* in spruce stumps, but Nicolotti *et al.* (1999) concluded that *T. harzianum* did not protect stumps against *H. annosum* infection. La Porta *et al.* (2001) analysed the effect of *Trichoderma* treatment on Norway spruce in Italy; better results were obtained on billets but no control effect was observed on stumps.

Most of the previous experiments were performed using *Trichoderma* for the protection of healthy spruce stumps against *H. annosum* infection. To our knowledge there are no investigations on the impact of *Trichoderma* in spruce stumps with *H. annosum* decay. Stumps of spruce trees with *H. annosum* butt rot contain also sound wood, and biological stump treatment may restrict the colonisation of this wood by the pathogen. Rishbeth (1951) observed that *Trichoderma* sp. can replace *H. annosum* in decayed pine roots under controlled conditions. Also Capretti and Mugnai (1989) observed that *Trichoderma* sp. can colonise decayed roots. Kuhlman and Hendrix (1964) noted that *T. viride* could grow in *Pinus echinata* wood that is fully colonised by *H. annosum* and even replace it in decayed stumps. Meredith (1960) concluded that *Trichoderma* sp. is not able to compete with *P. gigantea* or *H. annosum* on freshly cut wood, but its frequency of occurrence and activity increase at later stages of decay. Also Vasiliauskas *et al.* (2005) and Varese *et al.* (2003) observed the increase of *Trichoderma* sp. with time in Norway spruce stumps.

Therefore, it seems meaningful to investigate the impact of other fungi on *H. annosum* in already infected substrate. The aim of this work was to study the impact of *Trichoderma* sp. on the development of *H. annosum* mycelium in decayed stumps of understory spruce.

MATERIALS AND METHODS

The experiment was conducted in the Kalsnava Forest Research Station management area in eastern Latvia, in compartment 13, subcompartment 13. The size of the experimental plot was approximately 60 × 100 m. The stand was composed of 113-year-old Scots pine with 21–46-year- old Norway spruce in the understory.

In 4 August 2011, a total of 258 randomly selected Norway spruce trees were manually cut leaving 0.5 m high stumps. A wood disc with 3 cm thickness was cut from all stumps showing signs of root and butt rot (138 stumps) and transported to the laboratory.

The discs were debarked, washed with a stiff brush under running tap water, and incubated for 5–7 days in loosely closed plastic bags at room temperature. The lower side of each disc was investigated. Decay caused by *H. annosum* was found in 76 stumps. A grid consisting of 0.49 cm^2 squares was fixed on to the disc with pins, and the area colonised by *H. annosum* conidiophores was marked on the disc using a dissection microscope (Sun *et al*., 2009). Then a sheet of transparent paper was placed on the surface of the disc and the area of *H. annosum* was redrawn and measured using a planimeter (PLANIX 10S "Marble", Tamaya).

The stumps containing *H. annosum* decay were divided in three equal groups, each with approximately the same distribution of different diameter classes and different degrees of decay. Twenty-four stumps were treated with *Trichoderma* T472, 26 stumps with *Trichoderma* T945, and 25 stumps with tap water only (below these stumps are called "untreated"). The stump treatment was carried out one week after cutting the trees. Before treatment, a disc with 2 cm thickness was cut from the stump and discarded.

Trichoderma T945 (*Trichiderma viride*, isolated from soil) and *Trichoderma* T472 (*Trichoderma viridescens*, isolated from rhododendron) were selected according to their known high antagonistic effect against *Heterobasidion* spp. (three strains of *H. annosum* s.s. and four strains of *H. parviporum)* in laboratory tests on malt extract agar media at low temperatures (Nikolajeva *el al.*, 2012). For stump treatment, the *Trichoderma* strains were cultivated in Petri dishes on malt extract agar (MEA) medium for 4 weeks at ca. 20 ^{o}C in the dark. Spore suspensions were prepared 2–4 hours before the experiment by rinsing the spores from one Petri dish to 250 mL of tap water. The colony was agitated with a glass triangle during rinsing. The spore suspension was shaken vigorously and 0.5 mL of it was spread evenly on a Petri dish with 2–3-week-old MEA medium. Using a microscope, spores within 30 sight fields per dish were counted, and the number of spores per dish was calculated taking into account the area of the sight field and the area of the agar plate. Spore concentration in the treatment suspension was adjusted to ca. 10^7 cfu mL^{-1}. Stumps were treated manually using a hand sprayer.

Three months after start of the experiment, in October 2011, the stumps of each of the three treatment variants (25, 26, and 24 stumps, respectively) were divided in two approximately equal parts, each consisting of 12 or 13 stumps (Table 1). One part of the stumps was sampled, the other part was left intact to be sampled later. First, a 2 cm thick disc was cut from the stump surface and discarded. The second disc (2 cm thick) and third disc (4 cm thick) were placed in plastic bags and transported to the laboratory. The area occupied by *H. annosum* on the lower side of each disc was measured. Hence the 50 cm high stumps were investigated at two heights, 46 and 42 cm.

The other part of the stumps was sampled as described above on August 2012, one year after start of the experiment.

Table 1

CHARACTERISTICS OF THE STUMPS AT THE BEGINNING OF THE EXPERIMENT IN AUGUST 2011

Treatment	Number of stumps	Average stump diameter, cm	Total area of sample discs, cm^2	Total area of *H. annosum*, cm^2	Percentage* of area occupied by *H. annosum*
Untreated	25	8.03 ± 0.46	1209	331	27.37 %
T945	26	7.51 ± 0.30	1006	346	34.39 %
T472	24	8.14 ± 0.36	1148	392	34.15 %

* The percentages are based on summed areas from all sample discs within each treatment variant

The effect of *Trichoderma* treatment on *H. annosum* was calculated on the basis of wood occupied by *H. annosum* on sample discs cut from treated and untreated stumps at the beginning of the experiment, after three months, and after one year. The area colonised by *H. annosum* on a sample disc was related to the total disc area including sapwood and heartwood. Statistical significance of differences between treatments was analysed using a Student's t-test.

RESULTS

Visible decay was observed on 138 of 256 analysed stumps (53.9%). Decay caused by *H. annosum* was identified from 76 stumps (29.7%). In the further experiment 75 stumps were analysed. Their diameter varied from 4 to 14 cm. Area occupied by *H. annosum* on sample discs taken from these stumps varied from 0.25 to 50.4 cm^2. Main characteristics of the stumps with *H. annosum* decay are shown in Table 1. There were no significant differences of any analysed parameters among treated and untreated stump groups at the beginning of the experiment ($p > 0.05$).

Three months after stump treatment, in October 2011, a part of the stumps were investigated at two heights, 46 cm and 42 cm. Statistical analysis revealed no significant differences between untreated stumps and stumps treated with *Trichoderma* (Fig. 1). *Trichoderma* was found only in two analysed stumps.

One year after stump treatment, in August 2012, the other part of the stumps were investigated at heights 46 cm and 42 cm (Table 2). There was one statistically significant change compared to October 2011: area occupied by *H. annosum* was significantly larger in stumps treated with *Trichoderma* T945 (at height 46 cm). There were two statistically significant changes compared to the beginning of the experiment in August 2011: the area occupied by *H. annosum* (at height 46 cm) was larger in untreated stumps ($p < 0.05$), but it was significantly larger also in stumps treated with *Trichoderma* T945 at height 46 cm ($p < 0.05$). When the area of *H. annosum* in stumps at height 42 cm was compared to the situation at the beginning of the experiment one year earlier, no statistically significant differences were found between any treatment variants. A general trend was that the proportion of *H. annosum* decay increased with time and towards the base of the stump (Fig. 1). *Trichoderma* was present only in two analysed stumps.

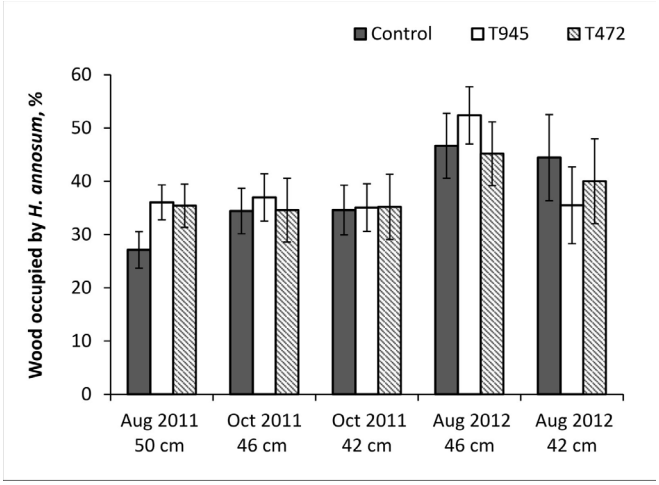

Fig. 1. Percentage of stump wood occupied by *H. annosum* at different sampling times and at different heights in stumps.

The effect of *Trichoderma* was analysed using two calculation methods — occupied area of *H. annosum* calculated from total area of all analysed discs (areas on sample discs summed) and average occupations of *H. annosum* calculated from individual stumps: for untreated control stumps at the beginning of experiment in August 2011 it was 27.37% and 27.10%, for stumps treated with *Trichoderma* T945 it was 34.39% and 36.00% and for *Trichoderma* T472 it was 34.15% and 35.40%, respectively. There were no statistically significant differences also comparing different treatments at different levels after one year using these calculation methods (data are not shown).

DISCUSSION

Our study did not reveal any clear effect of stump treatment with *Trichoderma* on the spread of *H. annosum* in stumps with *H. annosum* butt rot. A general trend was that the proportion of wood occupied by *H. annosum* in the stump increased with time and decreased with sampling height (Fig. 1), which can be explained by the conical form of the decay column in spruce stem stems. In Latvian forests, the mean length of stem decay caused by *H. annosum* in mature spruce (~100 years old) is 6.7 m (Arhipova *et al*., 2011). The spruces in our experimental plot were younger, 21–46 years old, and the mean length of the decay column was 2.7 m (Gruduls *et al*., 2012).

The increase of *H. annosum*-infected wood towards the stump base was greater in untreated stumps compared to the stumps treated with *Trichoderma* (Fig. 1) but the difference was not statistically significant. The slightly greater increase of *H. annosum*-infected wood in untreated stumps may be due to the inhibiting effect of *Trichoderma* in treated stumps, but it may also be an indication of spore infections by *H. annosum* to the sound wood of stumps. This kind of spore contamination is likely because fruit bodies of *H. annosum* were found in the experimental site. On the other hand, rapid drying of the relatively small-sized stumps in this experiment may restrict the germination of *H. annosum* spores on stump surfaces and subsequent mycelial growth (Benz-Hellgren and Stenlid, 1998). If the decreased expansion of *H. annosum* in lower parts of the stump in treated stumps is due to limiting effect of *Trichoderma*, this effect is very small.

In the present work, the poor growth of *Trichoderma* in stump wood, in particular in deeper parts of the stumps, seems to contradict the possible limiting effect of this fungus on spread of *H. annosum* in stump wood. Three months after stump treatment, typical *Trichoderma* infection was seen on sample discs of two stumps only (at levels 4 cm and 8 cm from stump surface). Also, one year after treatment, *Trichoderma* infection was found only in two other stumps. This was somewhat unexpected, because *Trichoderma* is a relatively common fungus in spruce stumps, particularly in older ones. Vasiliauskas *et al*. (2005) observed an increase of the proportion of *Trichoderma* with time in Norway spruce stumps: four years after stump treatment with Rotstop, *Trichoderma* sp. were isolated from 16.7–17.1% control stumps, as well as from treated stumps, but after six years *Trichoderma* was found in 37.1–40% of stumps. Also, Varese *et al*. (2003) reported an increase of the proportion of *Trichoderma* sp. with time in Norway spruce stumps.

There are limited data about presence *Trichoderma* on living wood. Living trees normally reject *Trichoderma* infection. Lygis *et al.* (2004) isolated *Trichoderma* sp. from *Pinus sylvestris* stem base. Schubert *et al.* (2008) treated pruning wounds of several broadleaved trees with *Trichoderma* and tried to reisolate the fungus at different time intervals (2–30 months). Maximum isolation frequency was recorded at depth 1 cm, but at depth 5 cm no isolates were obtained. In the present study, a number of tested stumps had root contacts with living spruce trees and may have remained alive for some time. This may have restricted the growth of *Trichoderma*. In another experiment, we treated coniferous billets with *Trichoderma* suspension; six weeks later pronounced growth of *Trichoderma* mycelium in treated sectors was observed (unpublished data).

Temperature has significant impact on *Trichoderma* sp. activity. Kallio and Hallaksela (1979) noted that *T. viride* cannot protect freshly cut stumps of Norway spruce from *H. annosum* in the cold season. Rishbeth (1951) analysing *T. viride* effect on *H. annosum* on *Pinus* roots in laboratory conditions at temperatures 5, 10, and 15 ^{o}C concluded that the impact of *Trichoderma* decreases at 5 ^{o}C. However, one of our *Trichoderma* isolations T945 *in vitro* showed pronounced antagonism against *H. annosum* also at +4 ^{o}C (Nikolajeva *el al*., 2012).

In our experiment, *Trichoderma,* in spite of its antagonistic properties against several plant pathogens, did not prove to be effective against *Heterobasidion* infection present in spruce stumps. Other control methods, such as change of tree species or removal of stumps may be necessary for controlling the disease in heavily infected sites (Vasaitis *et al*., 2008). However, it might be useful to continue experimentation with *Trichoderma* against *H. annosum* spore infection using different strains, and also in combination with other stump-colonising fungi, including *P. gigantea*.

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TRICHODERMA SP. IETEKME UZ *HETEROBASIDION ANNOSUM* ATTĪSTĪBU TRUPĒJUŠOS PAAUGAS EGĻU CELMOS

Heterobasidion annosum (Fr.) Bref. s.l izraisa ievērojamus ekonomiskos zaudējumus skujkoku mežos, tāpēc, lai samazinātu sakņu piepes izplatību mežistrādes laikā, svaigi celmi tiek apstrādāti ar bioloģiskajiem preparātiem. Laboratorijas apstākļos ļoti izteiktu antagonismu pret *H. annosum* uzrāda *Trichoderma* sp., tomēr ir samērā maz pētījumu lauka apstākļos. Turklāt līdzšinējos pētījumos analizēta *Trichoderma* sp. ietekme uz *H. annosum* veselos skujkoku celmos. Mūsu pētījuma mērķis bija novērtēt *Trichoderma* sp. ietekmi uz *H. annosum* attīstību trupējušos paaugas egļu celmos. Pavisam tika analizēti 75 egļu celmi. Daļa celmu atstāti kontrolei, bet daļa apstrādāti ar *Trichoderma* izolātiem. Darbā izmantoti *T. viridescens* un *T. viride* izolāti. *H. annosum* sastopamība analizēta 3 un 12 mēnešus pēc celmu apstrādes. Secināts, ka kontroles celmos *H. annosum* aizņemtais veselās koksnes laukums ir lielāks, salīdzinot ar apstrādātajiem celmiem, tomēr atšķirības nav būtiskas.