

# Influence of *Trichoderma* isolates and *Phlebiopsis gigantea* on the growth of *Heterobasidion parviporum* and wood decay of Norway spruce in controlled conditions

Laura Alksne<sup>1</sup>, Vizma Nikolajeva<sup>1\*</sup>, Zaiga Petriņa<sup>1</sup>, Daina Eze<sup>2</sup>, Tālis Gaitnieks<sup>3</sup>

<sup>1</sup>Department of Microbiology and Biotechnology, Faculty of Biology, University of Latvia, Jelgavas 1, Riga LV–1004, Latvia

<sup>2</sup>Microbial Strain Collection of Latvia, Faculty of Biology, University of Latvia, Jelgavas 1, Riga LV–1004, Latvia

<sup>3</sup>Forest Sector Competence Center, LLC “MNKC”, Dzerbenes 27, Riga LV–1006, Latvia

\*Corresponding author, E-mail: vizma.nikolajeva@lu.lv

## Abstract

*Heterobasidion parviporum*-caused root rot is a destructive infection to conifers, especially Norway spruce *Picea abies*. The disease can be limited by the biocontrol agent fungus *Phlebiopsis gigantea*. The aim of this work was to study the impact of four previously selected psychrotrophic fast-growing strains of *Trichoderma*, *P. gigantea* and combinations of both agents on the growth of *H. parviporum* in spruce wood blocks and on the structure and chemical composition of wood in laboratory conditions. Stained wood cuts were examined under a microscope and the chemical composition of wood was determined. All investigated *Trichoderma* strains belonging to species *T. viride* and *T. viridescens* inhibited the growth of *H. parviporum* in the wood. Protective efficiency of *Trichoderma* spp. against spruce dry weight losses reached 77 to 79%. The study indicated a need for development of combined *Trichoderma* spp. and *P. gigantea* plant protection products for biocontrol of root rot. We propose that fast-growing *Trichoderma* could restrict the colonization of wood surface by rot fungi, while *P. gigantea* competes with them in deeper parts of wood.

**Key words:** biocontrol, *Heterobasidion*, Norway spruce, *Phlebiopsis*, *Trichoderma*, wood.

**Abbreviations:** CFU, colony-forming units; MSCL, Microbial Strain Collection of Latvia; *r*, Pearson correlation coefficient; W, moisture content.

## Introduction

Root rot caused by *Heterobasidion annosum* s.l. is a common and economically important disease of coniferous trees in the Northern Hemisphere (Woodward et al. 1998). *H. annosum* (Fr.) Bref. s.s. preferentially attacks pine, while *H. parviporum* Niemelä & Korhonen has a preference for spruce (Mason, Huber 2002). The main route of infection is by basidiospores germinating on stumps after tree felling (Tainter, Baker 1996). Biological methods of controlling the pathogen involve application of commercial preparations on fresh stumps. Among them, the PG Suspension was developed in the UK, PG IBL in Poland and Rotstop in Finland (Pratt et al. 2000). These products contain asexual spores (oidia) of *Phlebiopsis gigantea* (Thor 2001), a saprotrophic and antagonistic basidiomycete (Kües 2007). PG Suspension and PG IBL are applied to pine stumps, while Rotstop is as effective on both pine and Norway spruce stumps (Pratt et al. 2000).

Saprotrophic soil fungi from the genus *Trichoderma*/*Hypocrea* (Ahmad, Baker 1986; Kubicek, Harman 1998) are well-known fungal antagonists which also work on wood decay fungi (Highley, Ricard 1988). *Trichoderma* has a high rate of growth and sporulation, variable metabolism,

mycoparasitism and the ability to produce volatile and soluble antibiotics (Monte 2001; Shalini, Kotasthane 2007). *Trichoderma* can inhibit pathogens on plants and plant remnants, and in the soil (Shalini, Kotasthane 2007). *Trichoderma* strains are able to restrict *H. annosum* s.l. *in vitro* (Nicolotti, Varese 1996; Schubert et al. 2008; Nikolajeva et al. 2012). However, the effectiveness has been low in field conditions (Kallio 1971; Nicolotti et al. 1999; Berglund et al. 2005). It is known that *Trichoderma* species degrade cellulose (Song et al. 2010) while *P. gigantea* degrades both cellulose and lignin (Mgbeahuruike et al. 2011; Hori et al. 2014). To the best of our knowledge, the effect of combined suspensions of *Trichoderma* and *P. gigantea* on *H. annosum* has not been previously investigated.

Gymnospermous wood consists primarily of tracheids (90 to 95% of cells) and uniseriate xylem rays (5 to 10% of cells) (Sjöström 1993). The wood cell wall is organized in layers: a thin primary wall and much thicker secondary wall, consisting of three layers. The cell wall consists of cellulose, hemicellulose and the matrix material lignin. The walls of adjacent cells are bonded together by the middle lamella, consisting of lignin, calcium and pectic substances (Albersheim et al. 2010). The typical composition of Norway spruce *Picea abies* dry wood is 27.5% lignin,

39.5% cellulose, 30.6% hemicellulose and 2.1% extractives (Sjöström 1993).

Fungal decay types fall into three categories according to their mode of degradation of the woody cell walls: brown rot, white rot and soft rot (reviewed in Schwarze 2007). However, the latest analyses of basidiomycete genomes demonstrate inadequacy of the prevailing paradigm of white vs brown rot in the understanding of the diversity of fungal wood decay mechanisms (Riley et al. 2014). Lignin presents the most significant barrier to wood decay, but white rot fungi such as *Heterobasidion* spp. (Peek et al. 1972) and *P. gigantea* (Hori et al. 2014) are known to degrade it.

During early stages of colonization, most hyphae grow in the cell lumina (Schweingruber 2007). *Heterobasidion* shows typical delignification within the compound middle lamella between tracheids without any alterations in the primary and secondary walls. In Norway spruce, delignification occurs from the lumina of the latewood tracheids. Sheaths of the inner secondary wall detach and peel off towards the lumina (Peek et al. 1972). Individual hyphae grow transversely via minute bore holes to adjacent cells and produce chlamydospores in the cell lumen (Schwarze et al. 2012). Soft rot fungal hyphae penetrate the secondary wall and erode channels, which usually develop into cavities (reviewed in Blanchette 2010). Ascomycetes and deuteromycetes, including *Trichoderma* species, cause soft rot. Soft rots are chemically more similar to brown rots than white rots, since cellulose and hemicellulose is decomposed while lignin is modified slightly (Schwarze 2007; Schweingruber 2007). Water saturation of wood impedes the availability of oxygen necessary for fungal colonization and wood decay (Boddy 1992). Formation of chlamydospores may be crucial to survival under dry or very moist conditions, or other adverse conditions for a range of wood decay fungi (Powell 2002).

The aim of this work was to study the impact of four

previously selected psychrotrophic fast growing strains of *Trichoderma* spp. (Nikolajeva et al. 2012), *P. gigantea* and combinations of both genera, on the growth of *H. parviporum* in spruce wood blocks and on the structure and chemical composition of wood in laboratory conditions.

## Materials and methods

### Biological material

The studied microorganisms were obtained from the Microbial Strain Collection of Latvia (MSCL). One *H. parviporum* (MSCL 1023), one *P. gigantea* (MSCL 702) and four *Trichoderma* strains (MSCL 472, 585, 945 and 969) were used. For long-term storage, fungi were maintained frozen in liquid nitrogen in the MSCL, University of Latvia. Two types of fresh cut wood blocks of Norway spruce *Picea abies* (Olaine, Latvia) were used, i.e. 2 × 1 × 1 cm blocks, and blocks 8 to 9 cm long and 7 to 9 cm in diameter.

### Fungal growth studies

Fungal growth was observed on 8 to 9 cm long and 7 to 9 cm diameter wood blocks. Cell suspensions were made from fungi after 14-day long incubation on Difco™ Malt Extract Agar (Becton & Dickinson, USA) at 21 °C. Concentrations of inocula were as follows: *T. viridescens* 472 10<sup>5</sup> colony-forming units (CFU) mL<sup>-1</sup>; *T. viride* 585 10<sup>9</sup> CFU mL<sup>-1</sup>; *T. viride* 945 10<sup>7</sup> CFU mL<sup>-1</sup>; *T. viride* 969 10<sup>7</sup> CFU mL<sup>-1</sup>; *P. gigantea* 702 10<sup>5</sup> CFU mL<sup>-1</sup>, and *H. parviporum* 1023 10<sup>5</sup> CFU mL<sup>-1</sup>. The blocks were sterilized twice in an autoclave at 121 °C for 1 h and placed in sterile polypropylene plant tissue culture containers. At first, top surfaces of blocks were moistened with sterile water where appropriate and then inoculated with fungal suspensions according to the experimental design (Table 1). Each treatment had five replications. The inoculated blocks were incubated in darkness at 20 ± 2 °C.

**Table 1.** Experiment setup of treatment of wood blocks for observation of fungal growth

Treatment	Amount of suspension (mL)	Amount of water (mL)
<i>H. parviporum</i> 1023	0.3	0.6
<i>T. viridescens</i> 472	0.3	0.6
<i>T. viride</i> 585	0.3	0.6
<i>T. viride</i> 945	0.3	0.6
<i>T. viride</i> 969	0.3	0.6
<i>P. gigantea</i> 702	0.3	0.6
<i>H. parviporum</i> 1023 + <i>T. viridescens</i> 472	0.3 + 0.3	0.3
<i>H. parviporum</i> 1023 + <i>T. viride</i> 585	0.3 + 0.3	0.3
<i>H. parviporum</i> 1023 + <i>T. viride</i> 945	0.3 + 0.3	0.3
<i>H. parviporum</i> 1023 + <i>T. viride</i> 969	0.3 + 0.3	0.3
<i>H. parviporum</i> 1023 + <i>P. gigantea</i> 702	0.3 + 0.3	0.3
<i>H. parviporum</i> 1023 + <i>P. gigantea</i> 702 + <i>T. viridescens</i> 472	0.3 + 0.3 + 0.3	0.0
<i>H. parviporum</i> 1023 + <i>P. gigantea</i> 702 + <i>T. viride</i> 945	0.3 + 0.3 + 0.3	0.0
<i>H. parviporum</i> 1023 + <i>P. gigantea</i> 702 + <i>T. viride</i> 969	0.3 + 0.3 + 0.3	0.0
Control	0.0	0.9

Visual observations of blocks were performed every week. After nine months, one block from each treatment was rinsed with sterile water. The bark was damaged and dislodged during washing. Fungal spores and/or mycelium were carefully scraped from the surface where appropriate and one block from each variant was sawn in half. The bottom part was kept in a closed container and fungi developed on the top surface were examined under a microscope with 400× magnification and identified by micromorphological features after one week. After incubation for 6 and 15 months, one block from each treatment was used for histological studies of wood. After 16 and 26 months, one block from each treatment was used for chemical analyses.

#### Histological preparations

After 6 and 15 months of incubation with fungal isolates, wood blocks 8 to 9 cm long and 7 to 9 cm in diameter were washed under running water, 1-cm thick discs were cut from the upper surface and samples (1 × 1 × 1 cm) were made from the cut disc under this (Fig. 1). Samples were fixed with a solution of formaldehyde-acetic acid-ethanol-water (2:1:10:7), gradually dewatered according to the histovax/*terc*-butanol method and paraffinized (Ruzin 1999). Tangential and radial sections (35-µm thick) were cut using a Leica 23-TMB (Germany) rotary microtome, and 60-µm thick non-paraffinized sections were cut using a hand microtome.

Sections were stained with either a solution of astra blue and safranin (1:5) or toluidine blue (Braune et al. 1999). Stained sections were examined using a Leica DM 2000 microscope under 400× magnification and images were recorded digitally with a Leica DFC 420 camera (Germany) and processed with software Image-Pro Express v. 6.0. Observations were made on several sections for the staining experiments.

#### Wood degradation studies

Wood degradation studies were carried out using wood blocks, 2 × 1 × 1 cm each. The blocks were sterilized in an autoclave at 121 °C for 1 h. Cell suspensions were made from

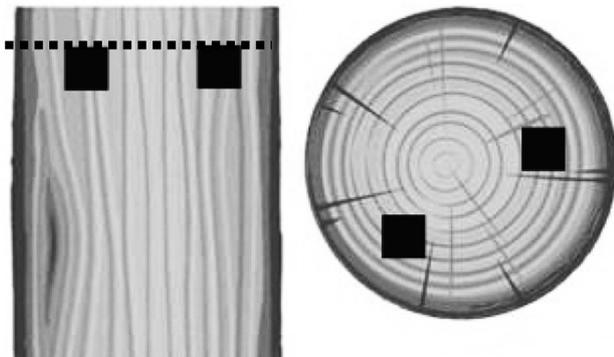


Fig. 1. Schematic illustration of the histological sample cut.

*Trichoderma* spp. and *P. gigantea* after 14-day incubation on Difco™ Malt Extract Agar (Becton & Dickinson, USA) at 21 °C. Concentrations of inocula were as follows: *T. viride* 585 10<sup>8</sup> CFU mL<sup>-1</sup>; *T. viride* 945 10<sup>8</sup> CFU mL<sup>-1</sup>; *T. viride* 969 10<sup>4</sup> CFU mL<sup>-1</sup>; and *P. gigantea* 702 10<sup>7</sup> CFU mL<sup>-1</sup>. Each wood block was weighed and numbered. Then each block was immersed in one of the fungal suspensions, or in sterile water (control), for 2 s and placed to flow off on napkins in aseptic conditions. The treated blocks were placed in Petri dishes on top of the mycelium of 18-day old *H. parviporum* 1023, but sterile control blocks were placed on water agar (agar 2%, Bio-Rad Laboratories, USA). Five blocks were placed in each Petri dish (Schubert et al. 2008). Three replications were used. Petri dishes were sealed with parafilm and incubated in the dark at 20 ± 2 °C. Samples were removed after 6, 12, 18, 24 and 30 weeks of incubation. Fungal spores and/ or mycelium were scraped from the surface where appropriate. The blocks were weighed, dried at 105 °C for 48 h and repeatedly weighed.

Five blocks were weighed before and after immersion in water to estimate the average amount of absorbed water. Moisture content of blocks W was calculated as:

$$W = (A - B - C) / (A - B) \times 100\%,$$

where A is weight before treatment with fungi, g; B is average amount of absorbed water, g; and C is weight after drying, g. Five blocks were left untreated and weighed without immersion in water or suspension, and average weight loss was estimated after drying. Percentage of dry weight loss (M) of wood blocks was calculated as follows:

$$M = (M_b - M_{av} - M_d) / (M_b - M_{av}) \times 100\%,$$

where  $M_b$  is weight before treatment with fungi, g;  $M_{av}$  is average amount of water, g;  $M_d$  is weight after drying, g. Fungal biocontrol effectiveness (E) was calculated as:

$$E = (M_{inf} - M_T) / M_{inf} \times 100\%,$$

where  $M_{inf}$  is dry weight loss of control blocks immersed in water and treated with *H. parviporum*;  $M_T$  is dry weight loss of blocks immersed in suspension of *Trichoderma* strain and treated with *H. parviporum*.

#### Chemical analyses

After 16 and 26 months of incubation with fungal isolates, wood blocks, 8 to 9 cm long and 7 to 9 cm diameter, were quickly washed under running water, 1-cm thick discs were cut from the upper surfaces and chips (<0.4 mm for determination of lignin and >0.4 mm for determination of cellulose) were made from the next discs. The chips were oven dried at 105 °C for 18 h and the percentage moisture content was calculated.

Extractives were isolated from wood chips with acetone in a Soxhlet extractor for 8 h. Concentration of cellulose was estimated according to the Kirshner-Hofer method (Abelson et al. 1988). Concentration of lignin was estimated according to the Klason method (Wood, Kellogg 1988). Hemicellulose was determined by subtracting the values of cellulose and lignin (%) from that of dry matter without extractives (100).

### Statistical analysis

An *F*-test was used for estimation of homogeneity of samples and for comparison of variance. The significance of the difference in values was determined at a significance level of 0.05. Program R.2.12.1. was used for Pearson correlation analysis.

## Results

### Fungal growth on spruce wood blocks

*Trichoderma* spp. mycelium was observed on inoculated wood blocks as of one week after the beginning of incubation when these fungi had colonized about half of the block surface. At that time, colonies of *P. gigantea* were scarcely visible. Growth of *H. parviporum* was noticed at two weeks (Fig. 2 a to d). *P. gigantea* later developed branched mycelium in all of the *P. gigantea*-containing variants and *Trichoderma* strains were overgrown by *P. gigantea* after two months (Fig. 2 e to h). *P. gigantea* overgrew the surface and sides of blocks after three months and one week, and retained dense mycelium and arthrospores throughout the experiment.

We observed mycelium of *H. parviporum* only in the treatment with single *H. parviporum* (Fig. 2h) but not in coculture with *Trichoderma* and/or *P. gigantea* during the entire experiment period of nine months. *Trichoderma* strains, in single cultures as well as in cocultures with *H. parviporum*, developed a dense cover of mycelium and conidia which was visible during all nine months (Fig. 2).

*Trichoderma* spp. formed conidia first on the sapwood (Fig. 2b) and later on heartwood. *P. gigantea*-caused brown coloration was observed mainly in the centre of the heartwood (Fig. 2).

After incubation for nine months, the inoculated fungi

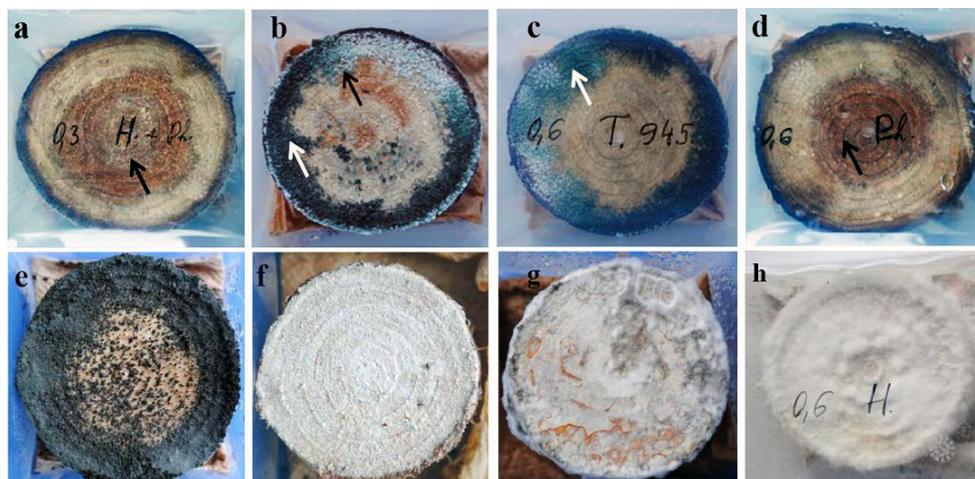
were identified in 4 to 4.5 cm deep layers of spruce blocks with the exception of three treatments, i.e. *T. viride* 969 + *H. parviporum* 1023, and *P. gigantea* 702 + *H. parviporum* 1023 where *Heterobasidion* was not observed, and *T. viridescens* 472 + *P. gigantea* 702 + *H. parviporum* 1023 where *Trichoderma* was not observed (Table 2).

### Histological studies

Wood blocks (8 to 9 cm long and 7 to 9 cm diameter) used for fungal growth studies were also used for histological studies. Structural changes of tracheids and presence of fungal cells were investigated. The structure of blocks incubated with *P. gigantea* was too much degraded, deformed and soft that we did not achieve good quality of cuts.

Observations under a light microscope showed that spruce blocks after 6 and 15 months of incubation were colonized by *Trichoderma* hyphae located in the tracheid lumen (Fig. 3a), the hyphae grew through bordered pits (Fig. 3c) and formed chlamydospores (Fig. 3b, Fig. 4a), and occasionally conidiospores (Fig. 3d) also. Similarly, hyphae in the lumen and penetration through bordered pits were observed in wood after incubation with *H. parviporum*; also dipyrnidal probably oxalate crystals were observed (Fig. 3e). *H. parviporum* also grew perpendicularly to the tracheids through lateral pores of tracheids and showed degraded bordered pits with hyphae in the middle (Fig. 4 b to d).

Stained cross-sections demonstrated visual differences between wood inoculation treatments (Fig. 5). Colour varied from red (safranin) to blue (astra blue), which are indicative of lignin and cellulose in the absence of lignin, respectively. Only the control showed bright rot borders in the latewood zone. *Trichoderma* and particularly *H. parviporum* decreased rot colouration.



**Fig. 2.** Fungal growth on spruce wood blocks after two weeks (a to d) and after nine months (e to h) of incubation. a, *H. parviporum* 1023 + *P. gigantea* 702; b, *H. parviporum* 1023 + *P. gigantea* 702 + *T. viride* 945; c, *T. viride* 945; d, *P. gigantea* 702; e, *H. parviporum* 1023 + *T. viride* 945; f, *H. parviporum* 1023 + *P. gigantea* 702; g, *H. parviporum* 1023 + *P. gigantea* 702 + *T. viride* 945; h, *H. parviporum* 1023; black arrows show *P. gigantea* coloration; white arrows show *T. viride* conidia.

**Table 2.** Fungi developed in spruce wood blocks in 4 to 4.5 cm deep layer nine months after surface treatment with fungal inocula

Inoculated fungi	Fungi identified in deep layer
<i>H. parviporum</i> 1023	<i>Heterobasidion</i>
<i>P. gigantea</i> 702	<i>Phlebiopsis</i>
<i>T. viridescens</i> 472	<i>Trichoderma</i>
<i>T. viride</i> 585	<i>Trichoderma</i>
<i>T. viride</i> 945	<i>Trichoderma</i>
<i>T. viride</i> 969	<i>Trichoderma</i>
<i>T. viridescens</i> 472 + <i>H. parviporum</i> 1023	<i>Trichoderma</i> , <i>Heterobasidion</i>
<i>T. viride</i> 585 + <i>H. parviporum</i> 1023	<i>Trichoderma</i> , <i>Heterobasidion</i>
<i>T. viride</i> 945 + <i>H. parviporum</i> 1023	<i>Trichoderma</i> , <i>Heterobasidion</i>
<i>T. viride</i> 969 + <i>H. parviporum</i> 1023	<i>Trichoderma</i>
<i>P. gigantea</i> 702 + <i>H. parviporum</i> 1023	<i>Phlebiopsis</i>
<i>T. viridescens</i> 472 + <i>P. gigantea</i> 702 + <i>H. parviporum</i> 1023	<i>Phlebiopsis</i> , <i>Heterobasidion</i>
<i>T. viride</i> 945 + <i>P. gigantea</i> 702 + <i>H. parviporum</i> 1023	<i>Trichoderma</i> , <i>Phlebiopsis</i> , <i>Heterobasidion</i>
<i>T. viride</i> 969 + <i>P. gigantea</i> 702 + <i>H. parviporum</i> 1023	<i>Trichoderma</i> , <i>Phlebiopsis</i> , <i>Heterobasidion</i>
Control	No

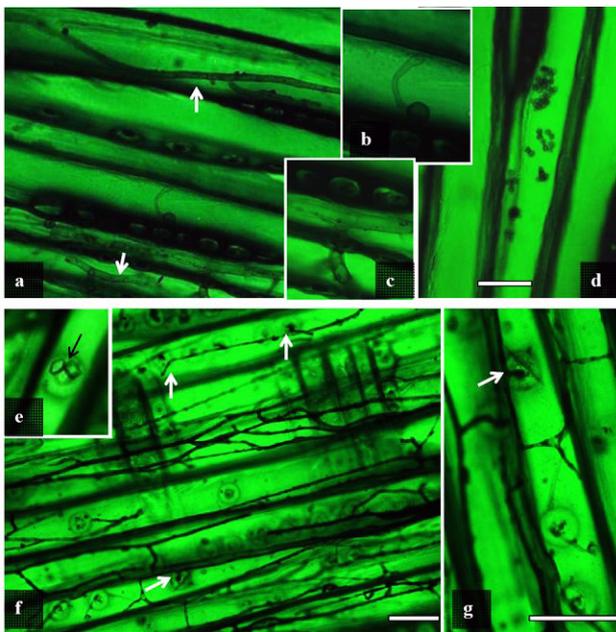
Secondary wall detachment from the middle lamella was observed in wood inoculated with *H. parviporum* and *Trichoderma* (Fig. 6 a to c). Axially oriented hyphae of *Trichoderma* and *H. parviporum* were detected in the middle lamella, in the secondary cell wall of tracheids and lamella (Fig. 6 d, f). *H. parviporum* caused tracheid ray cell splitting (Fig. 6e).

Blue colouration of detached layers indicated intense

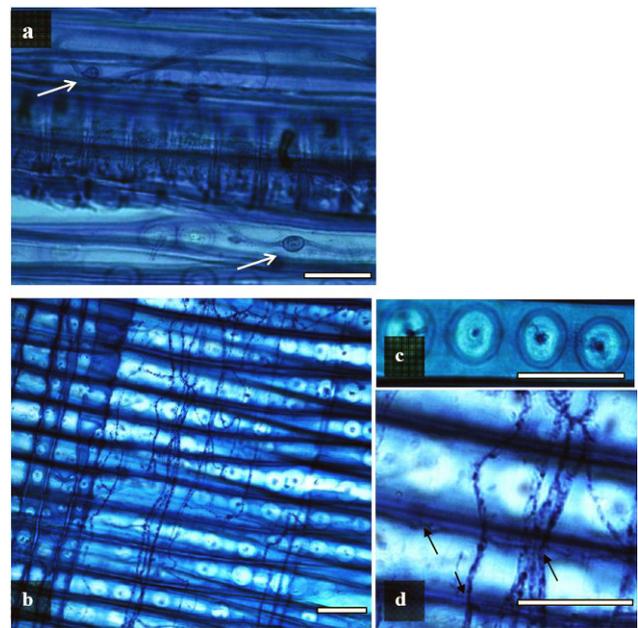
delignification, which was more pronounced in earlywood tracheids than in latewood tracheids. Detachment was mostly located at the edges of tracheids in comparison with corners.

#### Influence of fungal growth on the chemical composition of wood

The proportions of lignin, cellulose and hemicellulose in



**Fig. 3.** Micrographs of 35-µm thick tangential (a to d) and radial (e to g) cuts of spruce tracheids after six months of incubation with *T. viride* 945 (a to d) and *H. parviporum* 1023 (e to g) and toluidine blue staining taken with the green filter in 400× magnification. a, hyphae in tracheid lumen (arrows); b, chlamydospore; c, hypha growing through bordered pit; d, conidiospores; e, oxalate crystals (black arrow); f, hyphae in tracheid lumen; g, tracheid bordered pits and penetrating hyphae (arrows). Scale bars = 50 µm.



**Fig. 4.** Micrographs of 35-µm thick radial cuts of spruce tracheids after 15 months incubation with *T. viridescens* 472 (a) and *H. parviporum* 1023 (b to d) and toluidine blue staining. White arrows show hyphae and chlamydospores. b, hyphae growing perpendicularly to the tracheids; c, degraded bordered pits with hyphae in the middle; d, hyphae growth through tracheid lateral pores (arrows). Scale bar = 50 µm.

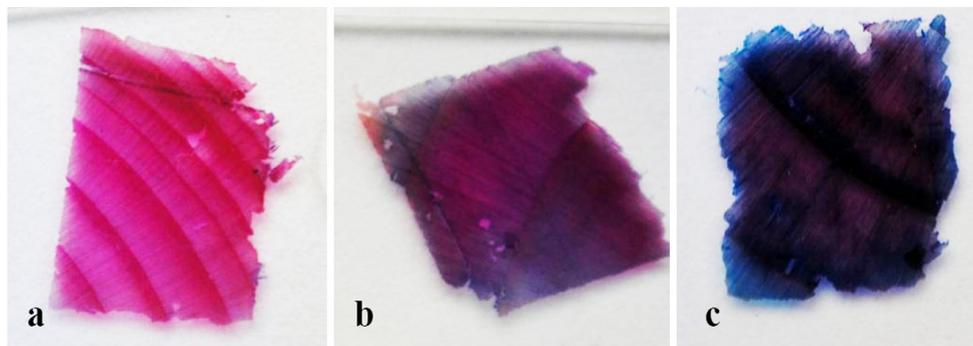


Fig. 5. Photographs of 60-µm thick cross-sections of spruce wood after 15 months of incubation and astra blue-safranin staining. a, control, incubated without fungi; b, incubated with *T. viridescens* 472; c, incubated with *H. parviporum* 1023.

spruce wood incubated with and without fungi during 16 and 26 months were determined (Table 3). We did not observe any differences between these periods of time with the exception of decreasing moisture content during long-term incubation. This might be because the experimental conditions did not provide enough moisture to support fungal growth.

*H. parviporum* significantly ( $P < 0.05$ ) decreased the proportion of lignin in the wood. *Trichoderma* treatment

significantly prevented lignin losses caused by *H. parviporum* (Table 3). A strong negative correlation ( $r = -0.9561$ ,  $P = 0.011$ ) was observed between the proportion of lignin and cellulose. The greatest degradation of cellulose was observed in the case of *P. gigantea*. The proportion of hemicellulose significantly decreased only in the treatment with *Trichoderma*.

#### Effectiveness of *T. viride* strains and *P. gigantea* against wood dry weight loss caused by *H. parviporum*

Wood degradation experiments were carried out using wood blocks with dimensions of  $2 \times 1 \times 1$  cm. *H. parviporum* developed on the agar surface, while the top and edges of the blocks were colonized by *T. viride* or *P. gigantea* (Fig. 7).

Wood dry weight loss and moisture content did not change ( $P > 0.05$ ) during the experiment in the control (Table 4), i.e. sterile blocks placed on water agar. Blocks incubated with *T. viride* strains did not cause weight losses in comparison with the control. The white rot fungi, *H. parviporum* singly and together with *P. gigantea* caused significant ( $P < 0.05$ ) weight losses which gradually increased to 20.42 and 36.43% respectively after 24 weeks of incubation.

*H. parviporum* and all of the investigated cocultures caused a significant ( $P < 0.05$ ) increase of moisture content (Table 4). *H. parviporum* together with *P. gigantea* increased the moisture content more rapidly than in other treatments. At the end of the experiment, the moisture content reached 31.72 to 43.18, 58.23 and 62.22% in the case of *H. parviporum* together with *T. viride* strains, single *H. parviporum*, and *H. parviporum* together with *P. gigantea*, respectively.

A strong correlation ( $r = 0.9073$  and  $r = 0.9238$ ) was found between wood moisture content and weight losses for variants with single *H. parviporum*, and *H. parviporum* together with *P. gigantea*, respectively. Treatments with *T. viride* showed a weak and insignificant correlation ( $r = 0.1105$ ,  $P > 0.05$ ).

Fungal biocontrol effectiveness was calculated. We were unable to evaluate the effectiveness of *P. gigantea* because it in itself degraded wood samples and caused weight losses. Effectiveness of 9 to 14% for all three *T. viride* strains

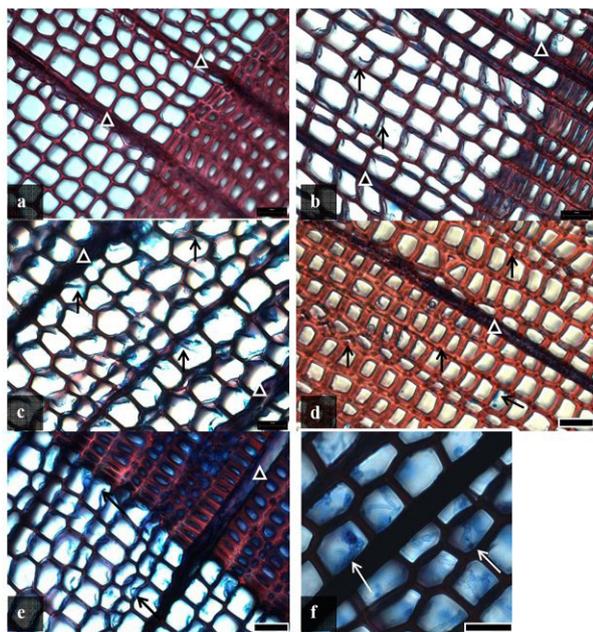


Fig. 6. Micrographs of 60-µm thick cross-sections of spruce tracheids after 15 month's incubation and astra blue-safranin staining. a, tracheid structure of control, incubated without fungi; b, incubated with *T. viridescens* 472, tracheid secondary wall detachment from the middle lamella (black arrows); c, incubated with *H. parviporum* 1023, tracheid secondary wall detachment from the middle lamella (black arrows); d, incubated with *T. viridescens* 472, axially oriented hyphae in the middle lamella, in the secondary cell wall of tracheid and lamella (black arrows); e, incubated with *H. parviporum* 1023, secondary wall detachment (black arrows); f, incubated with *H. parviporum*, hyphae clusters in lumens (white arrows); Δ, ray tracheids. Scale bars = 50 µm.

**Table 3.** Proportion (%) of lignin, cellulose, hemicellulose, and moisture content in spruce wood after 16- and 26-month incubation with fungi. <sup>a</sup>, single measurement; <sup>b</sup>,  $P < 0.05$  in comparison to control incubated without fungi; <sup>c</sup>,  $P < 0.05$  in comparison with *H. parviporum* 1023

Treatment	Lignin	Cellulose	Hemicellulose	Moisture <sup>a</sup>
16 months <sup>a</sup>				
Control	28.22	50.71	21.07	15.43
<i>H. parviporum</i> 1023	23.65	54.30	22.05	55.22
<i>T. viride</i> 969	27.48	53.86	18.66	55.14
<i>H. parviporum</i> 1023 + <i>T. viride</i> 969	27.56	53.16	19.28	59.36
26 months				
Control	28.80 ± 0.07 <sup>c</sup>	49.70 ± 0.37 <sup>c</sup>	21.50 ± 0.24 <sup>c</sup>	17.74
<i>H. parviporum</i> 1023	23.60 ± 0.26 <sup>b</sup>	53.40 ± 0.24 <sup>b</sup>	23.00 ± 0.25 <sup>b</sup>	40.50
<i>T. viride</i> 945	27.90 ± 0.03 <sup>bc</sup>	51.90 ± 0.74 <sup>c</sup>	20.20 ± 0.55 <sup>bc</sup>	35.85
<i>H. parviporum</i> 1023 + <i>T. viride</i> 945	27.50 ± 0.08 <sup>bc</sup>	52.00 ± 0.88 <sup>c</sup>	20.50 ± 0.76 <sup>c</sup>	27.97
<i>H. parviporum</i> 1023 + <i>T. viride</i> 945 + <i>P. gigantea</i> 702	34.3 ± 0.49 <sup>bc</sup>	43.70 ± 0.40 <sup>bc</sup>	22.00 ± 0.42 <sup>c</sup>	39.20

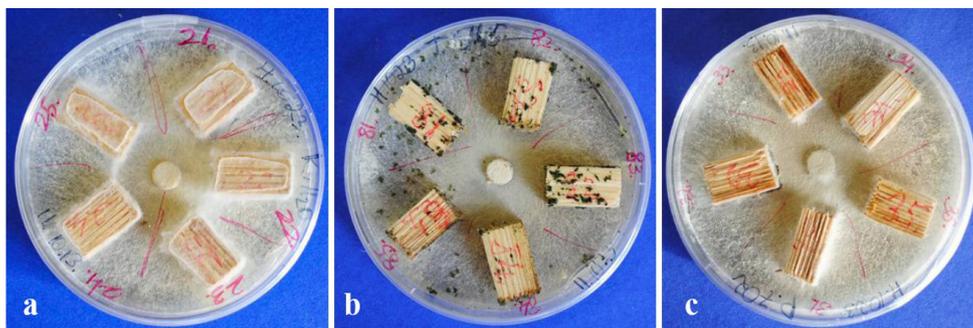
was found after 6 weeks of incubation (Fig. 8) and which increased during subsequent weeks to 77 to 79%, without significant difference ( $P > 0.05$ ) between strains after 24 and 30 weeks.

## Discussion

Biocontrol mechanisms include competition, antibiosis, parasitism and induction of host systemic resistance (reviewed in Lo 1998; Pal, McSpadden Gardener 2006). These mechanisms have all been used in the biological control of wood decay fungi although the relative importance of these potential mechanisms *in vivo* remains obscure (Schoeman et al. 1999). The Basidiomycete *P. gigantea* is the only fungus that has routinely been used for the biocontrol of *H. annosum* s.l., but the effectiveness of *P. gigantea* in biocontrol has been shown empirically (Sun et al. 2011). The results obtained by Mgbeahuruike et al. (2011) suggest that the antagonistic and competitive advantages of *P. gigantea* are based on the ability to degrade the different structural components of wood. Sun et al. (2011) revealed that *P. gigantea* provokes a very strong necrotic reaction on the host, as caused by the pathogenic *H. annosum* in the initial stages of infection, and that induced localized resistance could be part of the

mechanism for the biocontrol action. Concerning fungi from genus *Trichoderma*, biocontrol mechanisms are much clearer. *Trichoderma* species successfully manages all of the classic interaction mechanisms (reviewed in Benitez et al. 2004; Mukherjee et al. 2013) such as competition, antibiosis, parasitism and induction of host defence. Recently, there has been increasing interest among researchers in using combinations of biological control agents with multiple biocontrol mechanisms to exploit potential synergistic effects among them (Xu et al. 2011).

Our experiments demonstrated the combined action of selected *Trichoderma* strains and *P. gigantea* on the growth of *H. parviporum* in spruce wood blocks and on the structure and chemical composition of wood in laboratory conditions. All four studied *Trichoderma* strains (*T. viride* MSCL 585, MSCL 945 and MSCL 969, and *T. viridescens* MSCL 472) colonized the surface of fresh spruce blocks faster than *P. gigantea* and *H. parviporum* (Fig. 1). *Trichoderma* spp. were able to grow in undamaged softwood without addition of nutrients and formed spores. Hyphae of *Trichoderma* were found in tracheid lumens where they penetrated through bordered pits (Fig. 3c) like *Heterobasidion* and other white rot fungi (reviewed in Schwarze 2007). Visual observations of wood colonization and decay are in agreement with changes in structural



**Fig. 7.** Blocks of spruce wood after six weeks of incubation with fungi. Sterile wood blocks were immersed in sterile water (a) or in suspension of *T. viride* 945 (b), or *P. gigantea* 702 (c), and placed in Petri dishes on the top of mycelium of 18-day old *H. parviporum* 1023.

**Table 4.** Percentage of spruce wood dry weight loss (M), and wood moisture content (W) after 6, 12, 18 and 24 weeks of incubation with fungi. Sterile wood blocks (2 × 1 × 1 cm) were immersed in suspension of *T. viride* strains or *P. gigantea* 702 and placed in Petri dishes on the top of mycelium of 18-day old *H. parviporum* 1023. Control blocks were placed on water agar. <sup>a</sup>, wood was immersed in water instead of suspension of *T. viride* or *P. gigantea*; <sup>b</sup>, significant difference ( $P < 0.05$ ) from control in the corresponding column; <sup>c</sup>, significant difference ( $P < 0.05$ ) from water in the corresponding column

Treatment	6 weeks		12 weeks		18 weeks		24 weeks	
	M	W	M	W	M	W	M	W
Control	3.77 ± 0.64	29.86 ± 0.88	4.16 ± 1.50 <sup>c</sup>	30.47 ± 0.03 <sup>c</sup>	3.73 ± 0.25 <sup>c</sup>	32.93 ± 2.16 <sup>c</sup>	4.75 ± 0.47 <sup>c</sup>	26.63 ± 2.73 <sup>c</sup>
Water <sup>a</sup>	4.67 ± 0.34	31.95 ± 0.73	12.88 ± 3.24 <sup>b</sup>	39.90 ± 3.47 <sup>b</sup>	14.29 ± 1.23 <sup>b</sup>	43.38 ± 2.57 <sup>b</sup>	20.42 ± 2.82 <sup>b</sup>	58.23 ± 0.32 <sup>b</sup>
<i>T. viride</i> 585	4.00 ± 1.02	29.50 ± 1.65	4.00 ± 0.27 <sup>c</sup>	42.35 ± 2.88 <sup>bc</sup>	3.69 ± 0.75 <sup>c</sup>	49.14 ± 1.96 <sup>bc</sup>	4.67 ± 0.37 <sup>c</sup>	42.19 ± 3.61 <sup>bc</sup>
<i>T. viride</i> 945	4.08 ± 0.80	33.23 ± 4.26	4.91 ± 0.06 <sup>c</sup>	36.35 ± 3.35 <sup>b</sup>	5.20 ± 1.29 <sup>c</sup>	47.18 ± 2.98 <sup>bc</sup>	4.45 ± 0.27 <sup>c</sup>	43.18 ± 8.64 <sup>bc</sup>
<i>T. viride</i> 969	4.27 ± 1.21	35.34 ± 3.30 <sup>b</sup>	4.10 ± 0.19 <sup>c</sup>	34.27 ± 5.78	4.21 ± 0.13 <sup>c</sup>	39.43 ± 9.78	4.47 ± 0.09 <sup>c</sup>	31.72 ± 0.13 <sup>bc</sup>
<i>P. gigantea</i> 702	5.54 ± 0.69 <sup>b</sup>	34.69 ± 3.02 <sup>b</sup>	26.72 ± 2.32 <sup>bc</sup>	61.21 ± 3.88 <sup>bc</sup>	30.20 ± 2.66 <sup>bc</sup>	58.63 ± 0.04 <sup>bc</sup>	36.43 ± 3.03 <sup>bc</sup>	62.22 ± 1.96 <sup>bc</sup>

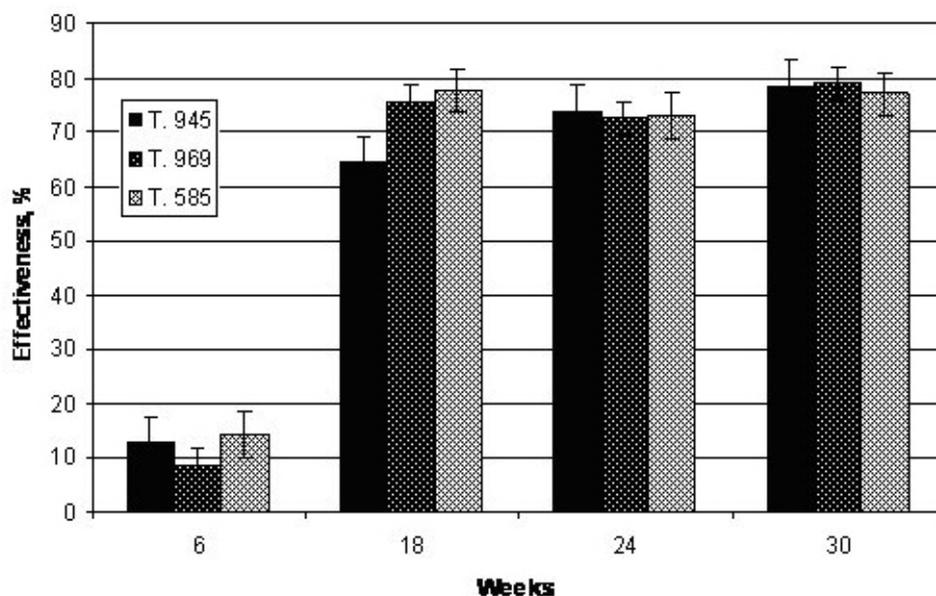
components (Table 3) and dry weight losses (Table 4). *P. gigantea* and *H. parviporum* caused significant dry weight losses of wood. *Trichoderma* did not reduce dry weight nor did it change the proportions of lignin and cellulose, but decreased the proportion of hemicellulose (Table 4).

Cocultivation of *H. parviporum*, *P. gigantea* and *Trichoderma* resulted in degradation of cellulose by *P. gigantea* and protection of lignin perhaps by both potential biocontrol agents. In the presence of *H. parviporum*, spruce wood was much more protected from weight loss by *Trichoderma* strains than by *P. gigantea* (Table 4). *Trichoderma* also showed 77–79 % biocontrol effectiveness after 24–30 weeks of incubation (Fig. 8). This is consistent with results obtained by Schubert et al. (2008) where the efficacy of several *Trichoderma* isolates against weight losses caused by wood decay fungi exceeded 80%.

It is expected that biocontrol efficacy is variable over time (Xu et al. 2011). We observed a succession of fungal

growth, i.e. rapid growth of *Trichoderma* on the wood with appearance of colonies in the first week of incubation (Fig. 1), whereas growth of *P. gigantea* and especially *H. parviporum* were slower. Later after two months *Trichoderma* strains were overgrown by *P. gigantea*. This means that *Trichoderma* does not destroy or inhibit *P. gigantea*, and does not affect growth of the slowest colonizer. At the end of the experiment, both *T. viride* and *P. gigantea* were found in 4–4.5 cm deep layers of wood both in single culture and in cocultures (Table 2).

Based on these results, we consider that it is appropriate to continue research on *Trichoderma* and *P. gigantea* interaction for wood protection against *Heterobasidion* and perhaps other wood decay fungi. Rapid growth and antagonism with *Heterobasidion*, mainly on the wood surface is an advantage of using *Trichoderma* at least in short-term protection. However, degradation of cellulose and growth and antagonism with *Heterobasidion* in the



**Fig. 8.** Biocontrol effectiveness (E) of *T. viride* strains (T. 945, T. 969 and T. 585) against *H. parviporum* caused spruce wood dry weight loss at different times of simultaneous incubation, in percents. The means for each strain with different letters are significantly ( $P < 0.05$ ) different at any given time.

deeper layers of wood are advantages of using *P. gigantea*. Such combined preparations would correspond to biological control with different protection mechanisms and opportunities (Xu et al. 2011). Furthermore, *P. gigantea* may have limitations when applied at a lower temperature (Zhao 2013). Our selected psychrotrophic fast growing *Trichoderma* strains have the ability to completely overgrow *Heterobasidion* at temperatures in the range of 4 to 21 °C (Nikolajeva et al. 2012). We must accept that attempts at biologically controlling basidiomycetes in forests on a commercial scale have met with limited success and the environmental factors influencing efficacy must be taken into account (Schoeman et al. 1999). We propose to examine combinations of selected *Trichoderma* strains and *P. gigantea* in further experiments in field conditions.

### Acknowledgements

We thank Kristine Dokane, Dace Megre and Iluta Dauskane for assistance with histological preparations, Edgars Kuks and Eduards Bakis for chemical analyses, and Ilze Kreismane for language editing. This study was partly financed by European Regional Development Fund project “Methods and technologies for increasing forest capital value” (No. L-KC-11-0004).

### References

- Abelson J.N., Simon N.I., Wood W.A., Kellogg S.T. (eds.) 1988. *Methods in Enzymology*. Vol. 161. Biomass. Part B: Lignin, Pectin, and Chitin. Academic Press, San Diego. 574 p.
- Ahmad J.S., Baker R. 1986. Rhizosphere competence of *Trichoderma harzianum*. *Phytopathology* 77: 182–189.
- Albersheim P., Darvill A., Roberts K., Sederoff R., Staehelin A. 2010. *Plant Cell Walls: from Chemistry to Biology*. Garland Science, Taylor and Francis Group, New York. 430 p.
- Benitez T., Rincon A.M., Limon M.C., Codon A.C. 2004. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* 7: 249–260.
- Berglund M., Rönnerberg J., Holmer L., Stenlid J. 2005. Comparison of five strains of *Phlebiopsis gigantea* and two *Trichoderma* formulations for treatment against natural *Heterobasidion* spore infections on Norway spruce stumps. *Scand. J. For. Res.* 20: 12–17.
- Blanchette R.A. 2010. Microbial degradation of wood from aquatic and terrestrial environments. In: Mitchell R., McNamara C.J. (eds) *Cultural Heritage Microbiology: Fundamental Studies in Conservation Science*. ASM Press, Washington, DC, pp. 179–206.
- Boddy L. 1992. Microenvironmental aspects of xylem defences to wood decay fungi. In: Blanchette R.A., Biggs A.R. (eds) *Defence Mechanisms of Woody Plants Against Fungi*. Springer Verlag, Berlin, New York, pp. 96–132.
- Braune W., Leman A., Taubert H. 1999. *Pflanzenanatomisches Praktikum*. I. Spektrum. Akademischer Verlag, Berlin, Heidelberg. 368 S.
- Brown H.L., Bruce A. 1999. Assessment of the biocontrol potential of a *Trichoderma viride* isolate: Part I: Establishment of field and fungal cellar trials. *Int. Biodeter. Biodegr.* 44: 219–223.
- Highley T.L., Ricard J. 1988. Antagonism of *Trichoderma* spp. and *Gliocladium virens* against wood decay fungi. *Mater. Organismen* 23: 157–169.
- Hori C., Ishida T., Igarashi K., Samejima M., Suzuki H., Master E., Ferreira P., Ruiz-Dueñas F.J., Held B., Canessa P., Larrondo L.F., Schmoll M., Druzhinina I.S., Kubicek C.P., Gaskell J.A., Kersten P., John F.St., Glasner J., Sabat G., BonDurant S.S., Syed K., Yadav J., Mgbeahuruike A.C., Kovalchuk A., Asiegbo F.O., Hoffmeister D., Rencoret J., Gutiérrez A., Sun H., Lindquist E., Barry K., Riley R., Grigoriev I.V., Henrissat B., Kües U., Berka R.M., Martínez A.T., Covert S.F., Blanchette R.A., Cullen D. 2014. Analysis of the *Phlebiopsis gigantea* genome, transcriptome and secretome provides insight into its pioneer colonization strategies of wood. *PLoS Genetics* 10: e1004759.
- Kallio T. 1971. Protection of spruce stumps against *Fomes annosus* (Fr.) Cooke by some wood inhabiting fungi. *Acta For. Fenn.* 117: 1–20.
- Kubicek C.P., Harman G.E. 1998. *Trichoderma & Gliocladium*. Vol. 1. Basic Biology, Taxonomy and Genetics. CRC Press, London. 293 p.
- Kües U. 2007. *Wood Production, Wood Technology, and Biotechnological Impacts*. Universitätsverlag Göttingen, Göttingen. 635 p.
- Lo C.-T. 1998. General mechanisms of action of microbial biocontrol agents. *Plant Pathol. Bull.* 7: 155–166.
- Mason P.G., Huber J.T. 2002. *Biological Control Programmes in Canada, 1981–2000*. CABI Publishing, Wallingford. 583 p.
- Mgbeahuruike A.C., Sun H., Fransson P., Kasanen R., Daniel G., Karlsson M., Asiegbo F.O. 2011. Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum*. *Biol. Control* 5: 118–129.
- Monte E. 2001. Understanding *Trichoderma*: between biotechnology and microbial ecology. *Int. Microbiol.* 4: 1–4.
- Mukherjee P.K., Horwitz B.A., Singh U.S., Mukherjee M., Schmoll M. 2013. *Trichoderma: Biology and Applications*. CABI, Wallingford. 344 p.
- Nicolotti G., Gonthier P., Varese G.C. 1999. Effectiveness of some biocontrol and chemical treatments against *Heterobasidion annosum* on Norway spruce stumps. *For. Pathol.* 29: 339–346.
- Nicolotti G., Varese G.C. 1996. Screening of antagonistic fungi against air-borne infection by *Heterobasidion annosum* on Norway spruce. *For. Ecol. Manage.* 88: 249–257.
- Nikolajeva V., Petrina Z., Vulfa L., Alksne L., Eze D., Grantina L., Gaitnieks T., Lielpetere A. 2012. Growth and antagonism of *Trichoderma* spp. and conifer pathogen *Heterobasidion annosum* s.l. *in vitro* at different temperatures. *Adv. Microbiol.* 2: 295–302.
- Pal K.K., McSpadden Gardener B. 2006. Biological control of plant pathogens. *The Plant Health Instructor* doi:10.1094/PHI-A-2006-1117-02.
- Peek V.R.-D., Liese W., Parmeswaran N. 1972. Infektion und Abbau der Wurzelrinde von Fichte durch *Fomes annosus*. *Eur. J. For. Pathol.* 2: 104–115.
- Powell M.R. 2002. A model for probabilistic assessment of phytosanitary risk reduction measures. *Plant Dis.* 86: 552–557.
- Pratt J.E., Niemi M., Sierota Z.H. 2000. Comparison of tree products based on *Phlebiopsis gigantea* for the control of *Heterobasidion annosum* in Europe. *Biocontrol Sci. Technol.* 10: 467–477.
- Riley R., Salamov A.A., Brown D.W., Nagy L.G., Floudas D., Held B.W., Lévassour A., Lombard V., Morin E., Otillar R., Lindquist E.A., Sun H., LaButti K.M., Schmutz J., Jabbour D., Luo H., Baker S.E., Pisabarro A.G., Walton J.D., Blanchette R.A.,

- Henrissat B., Martin F., Cullen D., Hibbett D.S., Grigoriev I.V. 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. *Proc. Natl. Acad. Sci., USA* 111: 9923–9928.
- Ruzin S.E. 1999. *Plant Microtechnique and Microscopy*. Oxford University Press, New York. 322 p.
- Schoeman M.W., Webber J.F., Dickinson D.J. 1999. The development of ideas in biological control applied to forest products. *Int. Biodeter. Biodegr.* 43: 109–123.
- Schubert M., Fink S., Schwarze F.W.M.R. 2008. *In vitro* screening of an antagonistic *Trichoderma* strain against wood decay fungi. *Arboric. J.* 31: 227–248.
- Schwarze F.W.M.R. 2007. Wood decay under the microscope. *Fungal Biol. Rev.* 21: 133–170.
- Schwarze F.W.M.R., Mattheck C., Engels J. 2012. *Fungal Strategies of Wood Decay in Trees*. Springer, Heidelberg. 185 p.
- Schweingruber F.H. 2007. *Wood Structure and Environment*. Springer, Berlin. 279 p.
- Shalini S., Kotasthane A.S. 2007. Parasitism of *Rhizoctonia solani* by strains of *Trichoderma* spp. *Electron. J. Environ. Agric. Food Chem.* 6: 2272–2281.
- Sjöström E. 1993. *Wood Chemistry: Fundamentals and Applications*. 2<sup>nd</sup> ed. Academic Press, San Diego. 292 p.
- Song F., Tian X., Fan X., He X. 2010. Decomposing ability of filamentous fungi on litter is involved in a subtropical mixed forest. *Mycologia* 102: 20–26.
- Sun H., Paulin L., Alatalo E., Asiegbu F.O., Peter G. 2011. Response of living tissues of *Pinus sylvestris* to the saprotrophic biocontrol fungus *Phlebiopsis gigantea*. *Tree Physiol.* 31: 438–451.
- Tainter F.H., Baker F.A. 1996. *Principles of Forest Pathology*. John Wiley and Sons, New York. 805 p.
- Thor M. 2001. Operational stump treatment against *Heterobasidion annosum* in European forestry – current situation. In: Laflamme G., Bérubé J., Bussièrès G. (eds) *Root and Butt Rot of Forest Trees*. Proceedings of the IUFRO Working Party 7.02.01 Quebec City, Canada, September 16–22, 2001. Canadian Forest Service, Information Report LAU-X-126, pp. 170–175.
- Wood W.A., Kellogg S.T. (eds.) 1988. *Methods in Enzymology*. Vol. 160. Biomass, Part A. Cellulose and Hemicellulose. Academic Press, San Diego. 774 p.
- Woodward S., Stenlid J., Karjalainen R., Hutterman A. (eds.). 1998. *Heterobasidion annosum: Biology, Ecology, Impact, and Control*. CAB International, Cambridge. 589 p.
- Xu X.-M., Jeffries P., Pautasso M., Jeger M.J. 2011. Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology* 101: 1024–1031.
- Zhao A. 2013. *Effects of Temperature and Heterobasidion Species on the Biological Control Efficacy of Phlebiopsis gigantea*. Master Thesis. Swedish University of Agricultural Sciences, Uppsala. 27 p.