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RESEARCH ARTICLE

Use of a breeding approach for improving biocontrol efficacy of *Phlebiopsis gigantea* strains against *Heterobasidion* infection of Norway spruce stumps

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Abstract

Sixty-four wild heterokaryotic isolates of *Phlebiopsis gigantea* were analysed for asexual spore production, growth rate and competitive ability against *Heterobasidion in vitro*, as well as growth rate in Norway spruce wood. These *P. gigantea* traits were considered important for controlling infection of Norway spruce stumps by spores of *Heterobasidion* spp. Ten most promising *P. gigantea* isolates were crossed with each other and 172 F_1 progeny heterokaryons were analysed for the above-mentioned traits. Thirteen most promising progeny heterokaryons were selected and their biocontrol ability against infection by *Heterobasidion* was compared with the parental isolates in stem pieces of Norway spruce. The results indicated that the progeny strains had generally better traits and control efficacy than the parental strains. The genetic effects accounted for a part of the variations between progeny and parental strains. This further suggests that there is a potential to improve the biocontrol properties of *P. gigantea* through breeding.

Introduction

Heterobasidion root rot, caused by the closely related fungal species belonging to the *Heterobasidion annosum* complex, is a common and economically important disease of conifers in the northern temperate forests (Woodward *et al*., 1998; Asiegbu *et al*., 2005). Strategies for the control of *Heterobasidion* root rot include silvicultural, chemical and biological methods. For both practical and environmental reasons, biocontrol has been proposed as a preferable treatment (Westlund & Nohrstedt, 2000; Vasiliauskas *et al*., 2005). *Phlebiopsis gigantea* (Fr.) Jül., a common saprotrophic wood-decay fungus and a highly competitive primary colonizer on conifer wood, is currently used as an effective biological control agent against *Heterobasidion*. In Europe, commercial *P. gigantea* products have been developed in the United Kingdom (PG suspension), Poland (PG IBL) and Finland (Rotstop) (Pratt *et al*., 2000).

The biocontrol mechanism of *P. gigantea* against the establishment of *H. annosum* on fresh stump surfaces is probably largely based on higher nutrient acquisition capability by *P. gigantea* (Asiegbu *et al*., 2005; Adomas *et al*., 2006), but possibly also on hyphal interference (Rishbeth, 1952; Ikediugwu *et al*., 1970; Holdenrieder & Greig, 1998; Tubby *et al*., 2008). Three other properties of *P. gigantea* that could be considered as important factors related to effective biocontrol include: (1) ability for rapid colonization of stump wood, (2) competitive ability against *H. annosum* and (3) ability to produce a sufficient number of asexual spores in culture (the latter property is important for the production of effective preparation). As there are clear advantages in having a number of isolates available for use in the products (Pratt *et al*., 1999), different selection processes have been applied to *P. gigantea* strains isolated from nature in the development of the three products (Pratt *et al*., 2000). In the case of Rotstop preparation, the same heterokaryotic isolate has been used in the product since 1991 in Finland. Despite generally good results obtained with this preparation, a poor controlling effect on Norway spruce stumps was recently reported by Berglund & Rönnberg (2004).

Phlebiopsis gigantea is a heterothallic fungus with bipolar (monofactorial) mating system. Pairing experiments (Korhonen & Kauppila, 1988; Korhonen *et al*., 1997; Grillo *et al*., 2005) and DNA fingerprinting analysis (Vainio *et al*., 1998; Vainio & Hantula, 2000) showed that *P. gigantea*

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strains in Europe are freely interbreeding, and the European population is interfertile also with the North American population. Moreover, the fungus showed high variability in biocontrol properties in nature (Sun *et al.*, 2008). Therefore, the breeding method, which has been commonly used in crops and domestic animals by crossing with desired traits, may offer a chance to improve the performance of *P. gigantea* in controlling *Heterobasidion* root rot and to obtain potential strains for use in practical control. A very useful property of *P. gigantea* for breeding is that it fruits easily in pure culture.

In an earlier study (Sun *et al.*, 2008), we identified *P. gigantea* isolates showing effective biocontrol properties. In the present study, these isolates were crossed and the properties of the progeny were tested. The aim of the study was to improve the biocontrol properties of *P. gigantea* through breeding.

Materials and methods

Fungal isolates

Ten heterokaryotic isolates of *P. gigantea* for breeding were selected from the 64 wild isolates used in an earlier study (Table 1) (Sun *et al.*, 2008). They were originally collected from southern Finland. Selection was mainly based on growth rate in Norway spruce wood and competitive ability against *Heterobasidion* spp. on malt extract agar (MEA, containing 1.5% Bacto malt extract and 2% Bacto agar; Difco Laboratories, Becton, Dickinson & Co., Sparks, MD) in Petri dishes. The isolates were cultured to fruit by incubating at *c.* 22 °C for about 8 weeks. Twenty single-basidiospore cultures were isolated from each heterokaryotic isolate and cultured on MEA. Ten vigorously growing strains, supposedly homokaryons, were selected from each progeny and cultured for further use. Their properties were tested for spore production, growth and competitive ability against *Heterobasidion* spp. on MEA. Two most promising homokaryotic strains from each of the 10 parental isolates were selected for breeding.

Crossing the strains

The selected 20 homokaryons were paired in all combinations (except intrastock combinations) on MEA in Petri dishes, 180 pairing combinations in total. The dishes were incubated at *c.* 22 °C for 1 week, after which a piece of mycelial colony was taken from the confrontation (somatic compatibility) zone and transferred on to a new MEA Petri dish (Korhonen & Kauppila, 1988). After a 4–day incubation, an inoculum from the supposedly heterokaryotic hyphal tips was transferred into another Petri dish (Fig. 1). Original pairing cultures and the transferred cultures were incubated for 3 months at *c.* 22 °C in order to check their ability for fruiting.

Testing the progeny properties

The methods for determining the spore production ability, growth rate and competitive ability against *Heterobasidion* on MEA, growth rate in spruce wood and control efficacy against *Heterobasidion* in spruce billets have been described previously (Korhonen, 2001; Sun *et al.*, 2008). Briefly, for spore production ability, the 3-week-old culture of each

Table 1. The parental isolates of *Phlebiopsis gigantea* and their spore production, growth on agar medium and in spruce wood, and control efficacy against *Heterobasidion* in stem pieces of spruce

Isolate no.	Spore production (million per plate)	Growth on agar (mm day^{-1}) Alone	Growth on agar (mm day^{-1}) Against *Heterobasidion**	Growth in wood (mm day^{-1})†	Control efficacy (%)‡ *Heterobasidion* concentration§ 1	2	3
Parental							
04128	10.3	10.7	1.6	1.3	97.2	70.6	74.2
04154	10.3	10.2	2.4	2.2	88.3	55.1	57.5
04161	5.1	9.8	1.5	2.1	100.0	82.2	97.1
97084	15.5	10.8	1.8	2.1	95.5	80.8	94.8
03006	56.8	8.5	1.6	2.1	89.9	63.7	92.8
04314	5.2	8.0	1.8	1.8	95.3	79.5	67.6
04116	10.3	9.9	1.6	2.1	94.7	91.7	82.7
Rotstop	15.5	10.8	1.9	1.8	100.0	83.4	73.1
04160	12.9	9.3	1.7	1.6	85.1	67.9	60.8
97085	12.9	10.2	1.9	1.3	99.5	96.1	81.0

*Mean growth rate over three heterokaryotic isolates of *Heterobasidion parviporum* and two isolates of *Heterobasidion annosum* s.s.
†Mean growth rate in stem pieces cut from four spruce individuals.
‡Mean control efficacy against four isolates of *H. parviporum* and two *H. annosum* s.s. in stem pieces cut from four spruce individuals.
§*Heterobasidion* concentration: 1, 300 spores mL^{-1}; 2, 2000 spores mL^{-1}; 3, 10 000 spores mL^{-1}.

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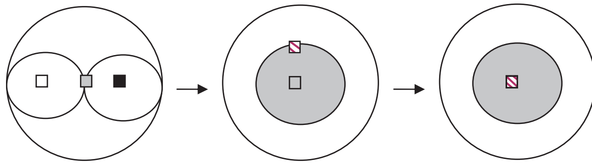

Fig. 1. Schematic illustration of the experimental design for crossing homokaryotic strains of *Phlebiopsis gigantea* to produce heterokaryotic strain: different homokaryons of *P. gigantea* (□, ■); transferring hyphae from confrontation zone (▣); transferring hyphae from hyphae tips (▨); □ with different colour indicates inocula of *P. gigantea* strains.

P. gigantea strain was rinsed three times into 1 L water and 0.5 mL suspension evenly spread on MEA. After 36-h incubation at room temperature, the germinating spores were counted under a microscope. The total number of viable spores of each strain in one culture plate was calculated based on the microscope field of view and the area of the agar plate. For competitive ability, each *P. gigantea* strain was confronted with three heterokaryotic isolates of *Heterobasidion parviporum* (nos 03014, 04007, 05318) and two isolates of *H. annosum* (Fr.) Bref. (nos 03012, 05044). After the two mycelial colonies had met each other, the advancing front of *P. gigantea*, growing over the colony of *Heterobasidion*, was marked and recorded.

Growth rate in spruce wood

The experiment for determining the growth rate in spruce billets was carried out between June 28 and July 26, 2007. In total, 61 *P. gigantea* strains were tested: 50 progeny heterokaryons, 10 parental heterokaryons (including Rotstop) and Rotstop S. The latter is the strain used in Rotstop preparation in Sweden. Stem billets, 30 cm long and 15–20 cm in diameter, were cut from four spruce stems, originating from Ruotsinkylä Experimental Forest, *c.* 20 km from Helsinki. The fresh upper surface of the billet was divided into four sectors. One-third of each sector was sprayed with oidial suspension of different *P. gigantea* strains at a concentration of *c.* 5000 oidia mL^{-1} (*c.* 500 oidia cm^{-2} on the billet surface). During spraying, the other sectors were covered with a paper sheet. Each treatment was repeated in four different Norway spruce individuals. The billets were incubated outdoors on moist sand. After 4 weeks of incubation, five 3-cm-thick discs were cut from each billet. They were debarked, washed under running tap water and incubated in loosely closed plastic bags at room temperature. The area covered by *P. gigantea* (orange–brown colour) on the disc surfaces was recorded after 11 and 17 days incubation.

Biocontrol efficacy

The experiment for testing the control efficacy against *Heterobasidion* was carried out between August 23 and September 10, 2007. The spore suspension of *Heterobasidion* used was a mixture of conidia obtained from Petri plate of three heterokaryotic isolates of *H. parviporum* (nos 03014, 04007, 05318) and two isolates of *H. annosum* (Fr.) Bref. (nos 03012, 05044). The spore concentration used was higher than in natural conditions, *c.* 300, 2000 and 10 000 spores mL^{-1}. For inoculation, 22-cm-long Norway spruce billets were cut from four Norway spruce individuals. In total, 24 *P. gigantea* strains were tested (13 progeny heterokaryons, 10 parental strains and Rostop S). The upper surface of the billet was divided into four sectors. Three sectors were sprayed with oidial suspension of different *P. gigantea* strains in a concentration of *c.* 5000 oidia mL^{-1}. One sector was left empty as a control. An empty space *c.* 2 cm wide was left between the sectors to avoid cross contamination. After 1–3 h, the whole upper surface of the billet was sprayed with spore suspension (conidia) of *Heterobasidion*. Consequently, three sectors on the billet surface were treated both with *P. gigantea* and *Heterobasidion*, and one sector with *Heterobasidion* only. Each *P. gigantea* strain and *Heterobasidion* spore–concentration combination was repeated four times, and each repeat was on different Norway spruce individuals (Fig. 2).

After 6 weeks of incubation outdoors, four 3-cm-thick discs were cut from the billets and treated as above. After 1 week of incubation in plastic bag, a transparent grid consisting of 1 × 1 cm squares was pinned to the upper surface of the disc, and the area occupied by *Heterobasidion* (conidiophores) was recorded under dissection microscope. The efficacy control was calculated by comparing the relative area occupied by *Heterobasidion* in different sectors.

Calculation and statistics

The methods used for calculating the growth depth and control efficacy of *P. gigantea* were described by Sun *et al.* (2008). Briefly, the growth rate was calculated for each billet on each repetition, simply by dividing the growth depth by

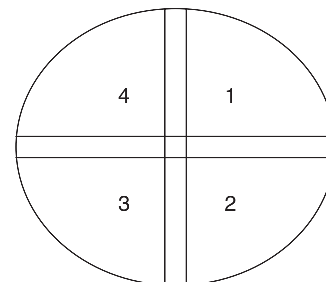

Fig. 2. Schematic illustration of the experimental design for biocontrol efficacy testing of *Phlebiopsis gigantea* strains against *Heterobasidion* infection on Norway spruce stem: each billet surface was inoculated by three different *P. gigantea* strains, *P. gigantea* + *Heterobasidion* sectors (1, 2, 3), *Heterobasidion* only sector (control sector, 4) and 2 cm empty space between each sector.

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incubation days outdoors after inoculation. The growth rate of each isolate was calculated as mean growth rate from four billet repetitions on the basis of a 17-day incubation after disc cutting. The control efficacy was a measure of the reduction of infection in treated sector with both *Heterobasidion* and *P. gigantea* compared with the sector treated only with *Heterobasidion*. For each billet, the control efficacy was calculated as mean of control efficacy of all four discs using the data from the upper surface of disc (for the first disc, data from the lower surface was used). The control efficacy of each *P. gigantea* isolate was calculated as mean efficacy from four billet repetitions.

The statistical analyses for genetic heritability (h^2) were performed according to Marmeisse (1989) using SPSS software 15.01 for Windows. The model describes the total phenotypic variance (V_p) as being the sum of environmental (V_E) and genetic (V_G) effect: $V_p = V_E + V_G$. Tukey's test was used to test significance level at 5% or 1%.

Results

Fruiting of progeny isolates

In total, 96% of the *P. gigantea* progeny isolates (172 out of 180) obtained through pairing fruited after 2–3 months incubation and were distinctly heterokaryotic. No fruit bodies were observed in single-basidiospore cultures originating from 10 parental heterokaryotic isolates used for the breeding.

Traits of parental and progeny isolates

The highest values in all the tested traits were observed in the progeny heterokaryons and the differences, compared with parental isolates, were particularly substantial in spore production ability and growth rate in spruce wood (Table 1; Fig. 3). One hundred and seventy-two progeny isolates were tested for spore production ability, growth rate and antagonism against *Heterobasidion* isolates on MEA. The limits of variation among the progeny isolates were as follows (variation of 10 parental isolates in brackets): spore production ability 1.5–250 (5.1–56.8) million spores per plate, growth rate 6.7–12.3 (7.3–10.8) mm day^{-1}, mean growth rate over *Heterobasidion* colonies 0.4–2.0 (1.5–1.8) mm day^{-1}. Fifty progeny isolates were tested for growth rate in spruce wood; the variation among them was 0.9–3.6 (1.3–1.8) and the average 2.1 (1.8) mm day^{-1}. Almost half of the progeny strains (21 out of 50) had faster growth rates than the best parental isolate (Table 1, Fig. 3). The growth rate of the

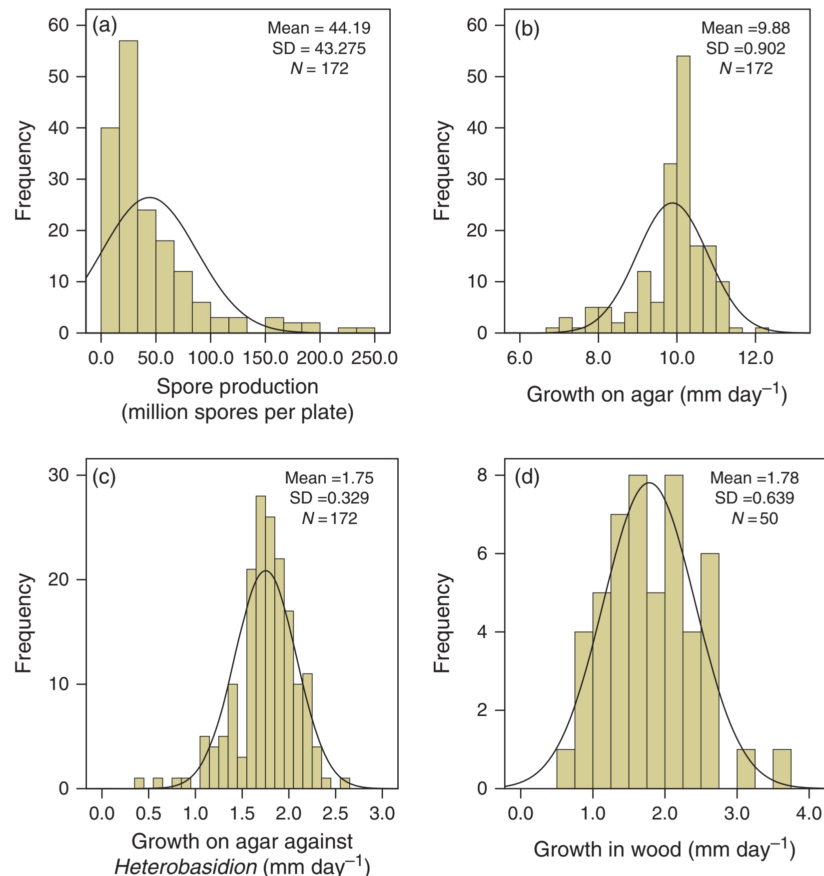

Fig. 3. The frequency distribution of spore production, growth on agar medium alone and against *Heterobasdion* and growth in spruce wood (50 selected progeny isolates) of progeny isolates of *Phlebiopsis gigantea*. (a) Spore production (million spores per plate), (b) growth on agar (mm day^{-1}), (c) growth on agar against *Heterobasidion* spp. (mm day^{-1}) and (d) growth in wood (mm day^{-1}).

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fastest progeny (3.6 mm day^{-1}) was twice as fast as that of isolate Rotstop (1.8 mm day^{-1}) and 64% faster than the fastest of the parental strains (2.2 mm day^{-1}). Among the 10 parental isolates, Rotstop showed moderate performance, except for the growth rate on agar, which was the fastest among the isolates (10.8 mm day^{-1}). Rotstop S showed poor growth rate (1.1 mm day^{-1}) in spruce wood but high spore production ability.

The genetic heritability analysis for tested traits showed that the genetic variance appeared to represent the major part of the phenotypic variation for growth on agar medium alone and against *Heterobasidion*, and spore production ability. Because of high variability of spruce wood tissues, however, low heritability of growth rate in wood was observed and only about 20% variance was contributed to the growth in wood (Table 2).

The growth rate in spruce wood and control efficacy of *P. gigantea* showed positive correlation ($P < 0.01$) at higher spore concentrations of *Heterobasidion* (*c.* 2000 and 10 000 spores mL^{-1}) in the treatment suspension (Fig. 4). For instance, the strain with faster growth rate (3.3 mm day^{-1}) also has better control efficacy (97.8%), whereas the Rotstop S with low growth rate (1.1 mm day^{-1}) has low control efficacy as well.

Table 2. Estimate of component of variation in traits of progeny strains obtained from parental strains

Trait	V_P (%)	V_E (%)	V_G (%)
Spore production	100	55	45
Growth on agar	100	39	61
Growth against *Heterobasidion*	100	31	69
Growth in wood	100	79	19

V_P, phenotypic variance; V_E, environmental variance; V_G, genetic variance.

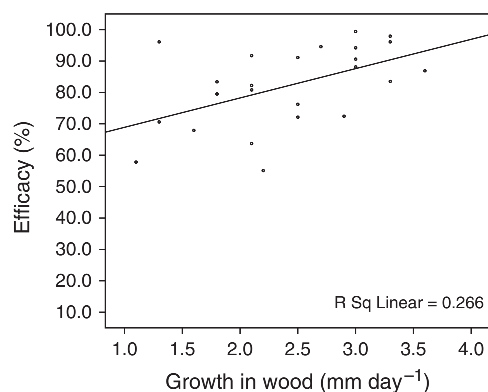

Fig. 4. Correlation between the growth rate of *Phlebiopsis gigantea* strains and their control efficacy against *Heterobasidion* in spruce billets ($P < 0.01$). The concentration of *Heterobasidion* spores applied on the cutting surface of the billet was *c.* 2000 spores mL^{-1} (treatment 2).

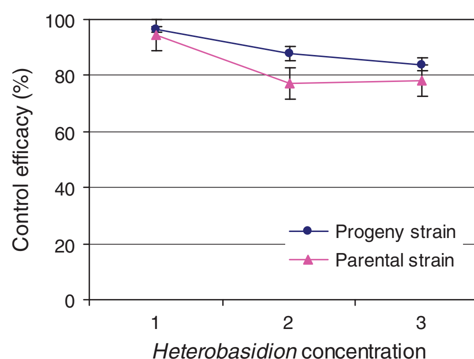

Fig. 5. The mean control efficacy of 10 parental and 13 progeny isolates of *Phlebiopsis gigantea* against *Heterobasidion* in stem pieces of spruce. *Heterobasidion* concentration: 1, 300 spores mL^{-1}; 2, 2000 spores mL^{-1}, 3. 10 000 spores mL^{-1}.

Biocontrol efficacy of progeny and parental strains

All the tested progeny and parental strains reduced *Heterobasidion* infection in spruce billets under three different loads of *Heterobasidion* spores. The control efficacy decreased with increasing *Heterobasidion* spore load. On average, the progeny heterokaryons showed better control efficacy than parental isolates, especially at higher *Heterobasidion* spore concentrations (Fig. 5). However, the difference was significant ($P < 0.028$) only at *Heterobasidion* spore concentration of 2000 spores mL^{-1} (which corresponds to *c.* 200 spores cm^{-2} applied on the cutting surface). Rotstop showed good control efficacy (100%) at low *Heterobasidion* spore concentration but was less effective (below average) at higher concentrations. Rotstop S showed lowest control efficacy among all tested strains (Supporting Information, Table S1).

Discussion

The aim of this study was to investigate breeding as a possible approach to improve the performance of *P. gigantea* in controlling *Heterobasidion* spore infection. The overall result showed that the investigated traits, considered important for biocontrol efficacy, were generally better in the progeny strains than in the parental strains and the same also for the control efficacy.

It is crucial that the progeny strains produced in pairings between single-basidiospore isolates were heterokaryons. The control efficacy of homokaryons is not known, and a practical problem with homokaryons is that they degenerate easily. In other fungi, Garbelotto *et al.* (1996) have also reported that homokaryons of *H. annosum* are short lived in nature. Distinction between homo- and heterokaryons of *P. gigantea* is somewhat problematic as there is no clear morphological difference between them (Korhonen &

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Kauppila, 1988; Grillo *et al*., 2005). The most useful indicator of heterokaryotic cultures is their ready fruiting. Conical cystidia emerge as the first signs of fruiting after 4–5 weeks at room temperature, and the ripe hymenium develops as a white covering generally after 6 weeks. In our study, the progeny strains were identified as heterokaryons mainly on the basis of ready and abundant fruiting.

Positive correlation was observed between the growth rate in spruce wood and the control efficacy of *P. gigantea*. This is consistent with the result obtained in the former study: growth rate in spruce wood was the most important characteristic indicating control efficacy against *Heterobasidion* spp. (Sun *et al*., 2008). The progeny strains showed faster growth in spruce wood compared with parental strains. As regards the other traits investigated (spore production, growth rate on agar medium and growth rate over *Heterobasidion* colonies on agar medium), similarly, the progeny heterokaryons showed better performance than the parental isolates. However, these traits did not directly correlate to control efficacy against spore infection by *Heterobasidion*, and they were to a lesser degree used as a basis for strain selection.

There is a certain level of positive relationship between the concentration of *P. gigantea* spores applied to the cutting surface of spruce billets and the control efficiency (Korhonen *et al*., 1993). However, besides a sufficient number of *P. gigantea* spores, an important factor for the control effect is also the number of *Heterobasidion* spores on the cutting surface. In our efficiency test, the number of *P. gigantea* spores in different treatment suspensions was between 3.9 and 9.3 million spores L^{-1}. It was in all cases well above the minimum amount that should be applied to spruce stumps [*c*. 2 million spores L^{-1}, corresponding to *c*. 200 spores cm^{-2} applied on cutting surface (Korhonen & Kauppila, 1988)]. As a control, in which we recounted the number of spores in the suspension of *Heterobasidion* after the experiment, the concentration of *Heterobasidion* spores in three treatments was relatively higher, with 300, 2000 and 10 000 spores mL^{-1} (corresponding to *c*. 30, 200 and 1000 spores cm^{-2}). Under such high infection pressure, the control efficacy of *P. gigantea* was relatively low. This is consistent with the assumption presented in the study of Berglund & Rönnberg (2004) that the poor controlling effect of Rotstop in their experiments was due to the exceptionally high spore load of *Heterobasidion*.

Biological control by fungal organisms is commonly used in agriculture and horticulture. Most of the biocontrol agents are selected from natural microbial populations and followed by numerous commercial products and extensive use. Despite long history and successful applications, the quest for more effective isolates selection is still ongoing. For example, Nobre *et al*. (2005) selected new effective strains of *Clonostachys rosea* in controlling *Botrytis cinerea* from Brazilian ecosystems; meanwhile, he also found out the variation between the colonization and suppression performance among strains. Moreover, biocontrol agent of *C. rosea* and *Trichoderma virens* strains has been enhanced by genetic engineering (Baek & Kenerley, 1998; Rey *et al*., 2001; Lübeck *et al*., 2002). The manipulation of genomes is, however, in many cases still limited by the lack of detailed information on the potential impact on other genes and their products. So far, there is no report on fungal biocontrol agent selection using conventional breeding methods. In our study, the progenies of *P. gigantea* obtained through breeding showed better mean control efficacy than parental strains at all the three different *Heterobasidion* spore loads applied. As several progeny strains showed relatively high control efficacy even under high spore concentration of *Heterobasidion*, they could be used as promising strains for replacing Rotstop strain for practical control of the pathogen on stump surface in the future. Moreover, analysis of component of variation within progeny showed that genetic component accounts for the major part of the phenotypic variation in most of the tested traits of *P. gigantea*. The breeding method used in this study seems to be an alternative approach for biocontrol agent selection.

It is known that moisture content of the stumps and their nutrient status play important roles in the decay of stumps (Smiley *et al*., 1972; Sierota, 1997; Redfern & Stenlid, 1998). In billet experiments, the moisture content of the wood may cause great variation in the results because it is difficult to keep the moisture content uniform in all billets. To minimize potential variations due to moisture content, higher numbers of inoculated billets are needed, but this is very laborious when many isolates have to be tested. In efficacy tests, the moisture problem can largely be eliminated by dividing the billet into two or more sectors. The protective treatment is made on one sector, while another sector is left as untreated control. The effect of treatment is compared with the untreated control in the same billet, and the mean efficacy is counted from several inoculated billets. However, for practical reasons, only relatively few strains can be included in such a test, particularly when considering the great variation between different spruce individuals (Sun *et al*., 2008). Therefore only 24 isolates were analysed during this investigation.

Phlebiopsis gigantea has for a long time been known as a strong competitor against *H. annosum* (Rishbeth, 1952); however, almost nothing is known about the physiological and molecular basis of the interspecific interaction between these fungi. There is no evidence of either antibiotics or toxins being secreted by *P. gigantea* (Holdenrieder & Greig, 1998). Recently, a macroarray differential gene analysis by Adomas *et al*. (2006) reported that a range of genes encoding proteins important for nutrient acquisition were shown to be preferentially expressed during the interaction. It would

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be of interest to investigate transcriptomic profile of *P. gigantea* strains with different biocontrol efficacy from the wild and hybrid strains, to identify candidate genes contributing to the control efficacy as well as genes that are affected by the breeding process. The availability of whole-genome sequences of *P. gigantea* could also help to facilitate a large-scale transcriptomic study.

In conclusion, the results obtained in this study suggest that the traits of *P. gigantea* related to biocontrol against *Heterobasidion* can be improved through breeding, and the breeding approach seems to be a useful way to obtain more effective strains for the control of *Heterobasidion* root rot. It is likely that the traits obtained through breeding are stable, but this should be monitored in long-term experiments. We consider that the breeding approach should be considered as an optional tool for the study and development of biological control agents against *H. annosum* root rot.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The parental and progeny isolates of *Phlebiopsis gigantea* and their spore production, growth on agar medium and in spruce wood (50 selected progeny isolates), and control efficacy against *Heterobasidion* in stem pieces of spruce (10 selected progeny isolates).

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