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Screening of *Phlebiopsis gigantea* isolates for traits associated with biocontrol of the conifer pathogen *Heterobasidion annosum*

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ABSTRACT

Stump treatment with *Phlebiopsis gigantea* has been widely used as the preferred method for the control of the conifer pathogen, *Heterobasidion annosum* sensu lato. However, the traits underlying this biocontrol process are not fully known. Sixty-four heterokaryotic isolates of *P. gigantea* from diverse geographical sources were screened for growth rate, laccase production, wood decay capability and antagonistic ability under *in vitro* conditions. In parallel, spore production, antagonism and growth rates were tested on a subset of 17 homokaryotic isolates and were compared with the heterokaryon strains of the same isolates. The data were statistically analysed using multiple regression and principal component analysis. The result showed that 90% of the *P. gigantea* isolates were able to replace *H. annosum* on wood medium compared to 4% on glucose-rich medium. The growth rate on modified nutrient medium supplemented with sawdust showed positive interrelationship with growth rate on the medium with xylan ($P = 0.0001$), % weight loss in pine ($P = 0.02$) and interaction in xylan ($P = 0.08$); but was negatively interrelated with growth rate on nutrient medium containing ferulic acid ($P = 0.03$). Antagonism on sawdust was positively interrelated with laccase production ($P = 0.03$) and % weight loss in pine ($P = 0.05$) but negatively dependent on growth rate in xylan ($P = 0.03$). There was a significant variation in spore production between the homokaryons and the heterokaryons of the same strains. The results show that the antagonistic interaction is partly dependent on the ability of the two fungi to degrade the different structural components of wood.

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1. Introduction

Root and butt rot disease of conifers caused by *Heterobasidion annosum* (Fr.) *Bref.sensu lato* (s.l)) is an economically important disease of the coniferous forest in the northern hemisphere (Asiegbu et al., 2005). The disease constitutes a serious threat to the economic growth of the wood industry both in Europe and globally. In Europe, the fungus attacks particularly trees of the genera *Pinus* and *Juniperus* (P), *Picea* (S), *Abies* (F) and in North America, *Pinus* (P), *Picea*, *Tsuga* and *Abies* (S/F). To a lesser extent, it causes root rot on some deciduous trees (*Betula* and *Quercus*) (Asiegbu et al., 2005).

The estimated annual losses attributable to this disease range from 100 to 200 million Euros in the Nordic countries, and at the global level the disease is of high economic importance (Woodward et al., 1998). In Europe, *H. annosum* sensu lato (s.l.) exists as a complex of three different species – *H. annosum* sensu stricto (s.s.) which has special preference for pine, *H. parviporum* (Niemelä and Korhonen) which preferentially attacks spruce and *H. abietinum* (Niemelä and Korhonen) which attacks silver fir. The fungus spreads through aerial basidiospores to stump surfaces and wounds; and by mycelia via root contacts from tree to tree (Stenlid and Redefern, 1998; Piri and Korhonen, 2007, 2008).

Control of the infection has mostly been achieved through prophylactic stump treatment immediately after tree felling, using chemicals (urea and borax) or biological control agents. The use of chemicals (urea) have been shown to cause severe damage to common ground vegetation species (Westlund and Nohrsted, 2000) as well as have a major influence on fungal community structure in freshly cut spruce stumps (Vasiliauskas et al., 2004). Biological control is therefore the preferred alternative measure of controlling and managing the spread of the infection (Vasiliuaskas et al., 2005). The first use of *Phlebiopsis gigantea* (Fr.) for biological stump treatment in forest plantations was in 1960 by Rishbeth (1952, 1963). The fungal preparation is commercially marketed as Rotstop® in Scandinavia, PG suspension® in UK and PG IBL® in Poland.

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Several mechanisms of action of biological control agents including competition for nutrients, secretion of inhibitory compounds and mycoparasitism have been reported (Mohammed and Caunter, 1995; Leifert et al., 1995; Benhamou and Chet, 1997; Poppe et al., 2003). However, little is known about the mode of action of *P. gigantea* against *H. annosum* on the stump surface. There has not been any evidence of antibiosis or toxin secretion by the biological control agent (Holdenrieder and Greig, 1998). Hyphal interference, characterised by granulation and loss of opacity of the hyphae of *H. annosum* in contact with *P. gigantea* has been reported (Ikediugwu, 1976). However, this phenomenon is common to most interspecific fungal interactions. The biological control of *H. annosum* by *P. gigantea* has been reported to be partly dependent on the ability to rapidly colonise the stump surface as well as high growth rate (Korhonen et al., 1994; Asiegbu et al., 2005; Adomas et al., 2006; Turby et al., 2008). Secretion of higher levels of wood degrading enzymes such as laccases by the biocontrol fungus has also been reported to confer competitive advantage in terms of ability to obtain nutrient from the available niche (Asiegbu et al., 2005). Furthermore, Adomas et al. (2006) reported up-regulation of a number of genes encoding proteins involved in carbohydrate and nitrogen metabolism at the zone of interaction between the biocontrol fungus and the pathogen. It was concluded that it could partly explain the competitive advantage of *P. gigantea* over the pathogen (Asiegbu et al., 2005).

Understanding the physiological, biochemical and molecular mechanisms involved in the biological control processes is a prerequisite for enhancing control efficacy, reducing variability and inconsistencies as well as helping in isolate selection. Recently, we reported variation either in spore production, competitive ability against *H. annosum* or growth rate in several isolates of *P. gigantea* from diverse geographical sources (Sun et al., 2009a). It was also shown that growth in wood was one of the most important characteristics to be considered in assessing *P. gigantea* isolates for biocontrol. In this study, conducted under *in vitro* laboratory conditions, several variables that can impact on biological control were considered using the same group of 64 isolates as above and the results were compared against field data as reported in Sun et al. (2009a). Such knowledge could provide researchers with tools for enhancing or transferring a specific trait into ecologically superior microbial agents or combining important traits from one biocontrol agent to another through breeding. Equally important is the basic research derived from the fact that the interactions between *Heterobasidion* s.l. and *P. gigantea* offer a good model system to understand interspecific fungal–fungal interactions. The approach of studying nutritional preferences using such model system would increase the knowledge on competitive fungal interactions in boreal forests. The primary aim of the present investigation is to identify biological traits that are important for the biocontrol ability of *P. gigantea* against *H. annosum*. These traits could be used as criteria for assessing *P. gigantea* isolates for use in biological control.

2. Materials and methods

2.1. Growth performance of P. gigantea isolates

A total of 64 heterokaryotic isolates of *P. gigantea* used in the current study, including 2 Rotstop® isolates (Rotstop S® and Rotstop F®), from different geographical locations in Finland, Sweden, Lithuania and Latvia were obtained courtesy of Kari Korhonen (Finnish Forestry Research Institute, Finland). All isolates were maintained on Hagem agar media (Stenlid, 1985) at 20 °C.

The effect of temperature on the growth rates of *P. gigantea* was tested in Hagem agar at 10 °C, 20 °C or 30 °C. A 3 mm diameter agar plug was used to inoculate 90 mm diameter Petri plates containing solid Hagem agar. The plates were sealed with parafilm and incubated at either 10 °C, 20 °C or 30 °C. The growth rate was determined by measuring the diameter of the mycelial growth at two different time points (5 and 10 days). The growth rate of each isolate was also measured on either glucose rich Hagem medium or carbon limited modified nutrient medium (NM) (g L^{-1}: NH_4PO_4 – 1.5, $MgSO_4{\cdot}7H_2O$ – 1.5, $CaCl_2{\cdot}2H_2O$ – 0.5, NaCl – 0.25, KH_2PO_4 – 5, $FeCl_3{\cdot}6H_2O$ – 0.1 and Thiamine – 0.002), supplemented with 0.1% wv^{-1} cellulose, or 0.01% wv^{-1} ferulic acid, or 0.05% wv^{-1} xylan or 1% wv^{-1} sawdust as the sole source of carbon, in 2.5% wv^{-1} agar. The cultures were incubated at 20 °C and monitored for growth at two different time points as above. The growth rate was determined by measuring the diameter of the mycelial growth. In parallel to the above study, single spores (homokaryons) were generated from 17 heterokaryotic isolates following the procedures described in Sun et al. (2009a,b). The homokaryons were grown on malt extract agar (MEA) containing 1.5% Bacto malt extract and 2% Bacto agar (Difco Laboratories, Becton, Dickinson & Co., Sparks, MD) to determine their growth rates. The growth rates were compared with the heterokaryotic isolates of the same strains grown on MEA (Sun et al., 2009a). The ability to produce viable spores on MEA was also tested on the homokaryons following the method described in Sun et al. (2009a,b) and this was compared with the spore production ability of the heterokaryons.

2.2. Wood decay capability of P. gigantea

Wood degradation studies were carried out using wood blocks obtained from Scots pine (*Pinus sylvestris* L.). Three wood blocks (3 cm × 1 cm each) were dried at 65 °C to constant weight. The wood blocks were weighed and put into 100 ml Erlenmeyer flasks containing vermiculite of fraction size 1 mm and nutrient solution (g L^{-1}: NH_4NO_3 – 0.6, K_2HPO_4 – 0.4, KH_2PO_4 – 0.5, $MgSO_4{\cdot}7H_2O$ – 0.4 and glucose – 1.0) in the ratio of 1:6 (1 g vermiculite to 6 ml nutrient solution). The flasks were plugged with cotton wool and covered with aluminium foil, and autoclaved for 30 min. Three pieces of agar plugs of each *P. gigantea* isolate were inoculated into each flask after sterilization. The inoculated flasks were placed in an improvised incubation chamber overlaid with wet paper towels to prevent excessive water loss through evaporation. Each chamber was covered with a lid and incubated at 20 °C. The inoculated samples were maintained at a relative humidity of 60–80% by wetting the paper towels with water at intervals of 2 weeks. The cultures were harvested after 4 months of incubation. At harvest, wood blocks were removed from the incubation flask and the adhering mycelia were scraped off with a scalpel. The wood blocks were oven dried for 18 h at 105 °C and the percentage dry matter loss calculated with respect to the original dry weight of the wood blocks. The experiment was repeated using Norway spruce (*Picea abies*(L.) Karst.) wood blocks.

2.3. Light microscopic observations of wood decay

After four month's incubation with the fungal isolates, wood blocks of both pine and spruce colonised by one of the isolates (isolate 4118) were removed for observations of patterns of colonisation and decay. Sections (radial, tangential, and transverse) (ca 20 μm thick) for light microscopy were cut using a Leitz Wetzlar sliding microtome (Type 1300) and stained with either 1% wv^{-1} lactophenol blue (longitudinal sections) or 1% wv^{-1} safranin (transverse sections). Stained sections were covered with cover slips and examined using a Leica DMLB light microscope and images recorded digitally with a Leica DC 300 CCD camera and stored using a Leica IM50 Image Manager. Results reflect observations on several sections from different staining experiments.

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