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# MICROBIOLOGICAL AND GENETIC ANALYSIS OF FUNGI IN DISCOLOURED SILVER BIRCH (*BETULA PENDULA* ROTH) WOOD

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Discolouration of silver birch (*Betula pendula* Roth) wood results in lower added value for wood products, especially in plywood production. Microbial wood discolouration does not affect mechanical timber properties but is undesirable due to visual considerations. Pure fungal cultures were isolated from discoloured birch wood, aged 40-70 years, from different sites in Western, Eastern and central regions of Latvia. Identification of microorganisms inhabiting living birch wood was done by direct sequencing of fragments amplified with conserved fungal primers from total DNA. Additionally, quantification and diversity analysis of total fungal DNA was performed using qPCR and capillary electrophoresis. No single species of microorganism was found to be present in all discoloured wood samples, although preliminary results from DGGE shows that discolouration may be connected with such fungal species as *Pholiota squarrosa*, *Cadophora fastigiata, Phialophora* sp. Discoloured wood contained a much higher quantity and diversity of microorganisms as compared to the unaffected wood samples. Results of the present study showed that a wide range of fungi may be responsible for discolouration of both living and dead birch wood.

Key words: artificial inoculation, fungal microdiversity, hardwoods, ITS, timber, wood staining.

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## INTRODUCTION

The visual properties of wood, including colour, is an important factor influencing commercial value. The commercially most desirable birch wood is light, white to slightly yellow or orange. In plywood manufacturing this is particularly significant, with discoloured wood increasing the proportion of lower quality classes of plywood produced, resulting in lower added value. In trees with discoloured wood, the area around the heartwood is reddish brown or distinctly orange, but it has no significant effect on the mechanical properties of wood

(Giroud et al. 2008). There are few reports concerning the discolouration of birch wood in living trees (e.g., Siegle 1967, Hallaksela & Niemisto 1998, Przybyl 2001, Belleville et al. 2011, Havreljuk 2013, Vasaitis et al. 2012), with other studies investigating wood discolouration during drying (Luostarinen & Möttönen 2004, Fuentealba et al. 2008). It has been reported that the main cause of discolouration is mechanical injury and physiological damage (Giroud et al. 2008). In addition, it is known that colour of the wood is also affected by activity of microscopic fungi and bacteria. There are relatively more reports on wood discolouration in beech (Sorz & Hietz 2008). In beech, the discolouration is caused by tree defence reactions against fungi inhabiting the wood, even if hyphae in wood are not observed (Sorz & Hietz 2008). In discoloured wood, the number of bacteria may also be increased (Shortle et al. 1978). A number of fungal species were identified after culturing from discoloured birch wood samples (Hallaksela & Niemisto 1998), however it has been reported that healthy wood samples also can be inhabited by a range of fungi (Chapela 1989).

Root and trunk endophytes are considered as a part of the natural microbial complex in many plants. Endophytic fungi may be harmless to plants or may cause negative effects in the event of, for example, environmental changes (Kowalski & Gajosek 1998). Several studies, although based on culture methods, showed high fungal diversity in birch stems, branches and leaves (Przybyl 2001, Kowalski & Gajosek 1998).

A significant proportion of microorganisms are not culturable in standard laboratory conditions; therefore traditional microbiological methods cannot reveal the full extent of microbial biodiversity (O'Brien et al. 2005). With the development of molecular genetic techniques, the analysis of environmental or total DNA samples, which contain DNA from many different species, has become feasible. Sequencing of PCR products amplified from total DNA samples enables direct assessment of the range of diversity (Weber et al. 2009). Conserved primers can be used to amplify a wide range of species; however, they may also decrease the estimates of biodiversity, due to the mismatches and PCR artefacts (Anderson et al. 2003, Lindahl et al. 2013). In addition, species identification based on sequence data is dependent on the presence and accuracy of sequence information in public databases (Anderson et al. 2003, Nilsson 2012). In this study, amplification of fungal DNA from the total DNA extracted from wood samples was done with two fungi specific sets of primers: ITS1f/ITS4 and ITS2 (White et al. 1990, Gardes & Bruns 1993) and nu-SSU-0817-5/nu-SSU-1196-3 (Borneman & Hartin 2000). The ITS1f/ITS4 primer pair amplifies the non-coding ITS1 and ITS2 and the 5.8S coding sequences, which are located between the Small Subunit-coding (18S gene) and Large Subunit-coding (ribosomal 28S gene) sequences (White et al. 1990). Primers nu-SSU-0817-5/nu-SSU-1196-3 amplifies most of Small Subunit-coding region (ribosomal 18S gene). The ITS1f/ITS4 primers can also be used to characterise fungal DNA abundance and diversity from mixed-template samples using qPCR (Manter & Vivanco 2007). This technique was used to compare the quantity and diversity of total fungal DNA extracted from discoloured and non-discoloured birch wood samples in present study.

This study utilised a range of techniques to investigate the microorganisms found in discoloured and non-discoloured birch wood, as well as using inoculations to determine the effect of fungal isolates on birch wood colour. The aim of this study was to investigate fungi in discoloured silver birch wood using standard microbiological and molecular methods.

## MATERIAL AND METHODS

### Plant material

Birch wood discolouration in the central part of the stem was assessed during felling of mature stands (approximately 40-70 years old)

and thinning of a younger stand consisting of open-pollinated half-sib families (14 years old). The three mature stands were located near Liepāja (Western Latvia, 56.619°N, 21.607°E), Salaspils (Central Latvia, 56.907°N 24.276°E) and Rēzekne (Eastern Latvia, 56.620°N, 27.614°E). The 14 year old stand was located near Rembate (Central Latvia, 56.879°N, 24.590°E). The Rembate plantation consisted of half-sib families, and so it was possible to assess the discolouration in families. The number of individuals assessed per family ranged from 7 to 17 (average 12.7). In total 45 stumps in Liepāja, 57 stumps in Salaspils, 88 stumps in Rēzekne were visually examined for discolouration with increment borer and 419 stumps in Rembate were visually examined for discolouration after felling. Different and smaller sets of samples from these localizations were used for further analysis, collected with sterilized increment borer (d=5 mm, length – from inner bark to pith) and stored at -80°C until processing. Samples for the microbiological experiments were not collected from individuals from Rembate plantation.

**Isolation of fungi and bacteria**

In the laboratory, 10 mm long parts from 13 discoloured and three non-discoloured part were cut out of collected wood cores from sample site "Liepāja", were washed with sterile water, surface-sterilized in 70% ethanol for 10 seconds and flamed for 1-2 seconds and placed on medium for incubation on 3.5% (w/v) malt extract agar (Roth, Germany) and R2A (Difco, USA) agar for approximately four to eight weeks at room temperature in the dark. Pure cultures were used for DNA extraction.

**DNA extraction and analysis**

Total DNA extraction was done using a multistep procedure described by Doyle and Doyle (1987) with modifications. Approx. 200 mg of wood or 100 mg of fungal mycelia were used for DNA extraction. Sample material were sterilized as mentioned previously, submerged in liquid nitrogen for 5 min, then homogenized separately by mill Retsch MM 400 (Retsch, Germany) by bead beating for 40 seconds at frequency 30 Hz. Homogenized wood material were suspended in extraction buffer (2% CTAB, 0.1M Tris-HCl, 20mM EDTA, 1.4M NaCl, 1% beta-mercaptoethanol (v/v), 4% polyvinylpyrrolidone (w/v)). The cell lysate was extracted for two times with chloroform/isoamyl alcohol (24:1). The aqueous phase was treated again with extraction buffer and then with chloroform/isoamyl alcohol. The DNA was precipitated with 0.7 vol. isopropanol, washed twice with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)).

The two step PCRs were performed with ITS primers for denaturing gradient gel electrophoresis (DGGE). First amplifications was performed using DNA extracted from discoloured birch wood ("Salaspils", n=12) and a universal primer pair ITS1f/ITS4 (White et al. 1990, Gardes & Bruns 1993), second – using 2 mL of previous amplification as a template DNA and primer pair ITS1f-GC/ITS2 with adition 40 nucleotides long GC-tail (specific for DGGE analysis) at 5' end for primers ITS1f (Rajala et al. 2010). Also fungi-specific primers nu-SSU-0817-5/nu-SSU-1196-3 (Borneman & Hartin 2000) were used to amplify partial sequence from ribosomal 18S gene from the total DNA extracted from discoloured and non-discoloured birch wood ("Rēzekne", n=88?).

The PCR mix for all primer sets contained 2mL DNA, 1x *Taq* PCR buffer, 2mM $MgCl_2$, 0.2mM each dNTP, 0.2mM each primer, 5µg BSA, 0.6U *Taq* polymerase (Thermo Fisher Scientific, Lithuania) in a total volume of 20mL. Thermocycling conditions were: initial denaturation at 94°C for 5min, 13 cycles (95°C 0:35 min, 55°C 0:55 min, 72°C 0:45 min), 13 cycles (95°C 0:35 min, 55°C 0:55 min, 72°C 2 min), 9 cycles (95°C 0:35 min, 55°C 0:55 min, 72°C 3 min) and final extension at 72°C for 10 min for primer pair ITS1f/ITS4, 94°C for 5 min, 35 cycles at 94°C for 0:30 min, 55°C for 0:30 min, 72°C for 0:30 min, with final extension at 72°C for 10 min for primer pair ITS1f-GC/ITS2 and 94°C for 2 min, 35 cycles at 94°C

for 0:30 min, 56°C for 0:30 min, 72°C for 0:30 min, with final extension at 72°C for 30 min for primer pair nu-SSU-0817-5/nu-SSU-1196-3. After the amplification nu-SSU-0817-5/nu-SSU-1196-3 PCR products were purified using Sephadex50 (Sigma, Germany), and quantified using a Qubit fluorometer (Life Technologies). The fragments were then ligated into plasmids using the InsTAclone PCR Cloning Kit (Thermo Scientific) according to the manufacturer's protocol. Ligated plasmids were transformed into E. coli DH5α cells; blue/white selected, and sequenced using M13 primers after PCR amplification of inserts.

DGGE was performed with a CBS DGGE-2001 (C.B.S. Scientific, ASV) using a denaturing gradient of 18% to 58% for ITS primer set in 0.75 mm thick 7.5% polyacrylamide gel (acrylamide/bisacrilamide – 37.5:1). Electrophoresis was performed in 1x TAE running buffer for 17 h at 60°C and 110V. For initial comparison of fungal communities only 12 discoloured wood samples were used for DGGE. Gel was stained with ethidium bromide and bands from DGGE gel were excised with sterile scalpel, incubated in 30 µl TE buffer overnight at +4°C, vortexed and then centrifuged. 2mL of supernatant was used for PCR.

After the amplification DNA extracted from pure cultures or DGGE bands, PCR products were purified using Sephadex50, treated with SAP and *Exo*I (Thermo Fisher Scientific, Lithuania) and sequenced with 3130 xl Genetic analyzer (Applied biosystems, Singapore) using Big Dye Terminator kit 3.1 (Applied Biosystems, USA). Sequences were edited with Chromas Lite (Technelysium, Australia) compared with NCBI nucleotide database using BLAST-N option (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990).

Characterisation of total fungal abundance and diversity analysis for eight samples from sample site "Salaspils", was assessed using the ITS1f/ITS4 primers and total DNA extracted from discoloured and non-discoloured part separately. qPCR and subsequent length heterogeneity of amplicons was done by capillary electrophoresis following a protocol by Manter and Vivanco (2007), except that the fungal quantitation was not done using an external standard curve as described, but a relative quantitation method (Pfaffl 2001*)* was utilised using the birch pal2 (phenylalanine ammonia-lyase 2) gene as a reference (NCBI accession AJ289609) to normalise the data. Therefore PCR primers (Bpal2F: ggcatgattcatagggttgg; Bpal2R: ggtattccatgcaccacaca) were designed using the program Primer3 *(*Rozen & Skaletsky 2000). The extent or intensity of the discolouration in the trees was not assessed.

## RESULTS

### Frequency of birch wood discolouration

The presence of discoloured wood was assessed during tree felling in mature and 14-year-old birch stands. Signs of wood discolouration in stumps in mature stands Liepāja, Salaspils, Rēzekne varied and were with a frequency of 58%, 89% and 63%, respectively. In the 14-year-old stand, 131 trees of 419 trees examined showed signs of wood discolouration (31%). The frequency of individuals with discoloured wood varied largely among the families, ranging from 0 to 92% (average 32%). Discolouration was completely absent in stumps of three families.

### Bacteria and fungi isolated from wood

After culturing on media for four to eight weeks, 46 bacterial and 18 fungal colony morphotypes were obtained from both 13 discoloured and three non-discoloured wood samples, however, the quantity and diversity of microorganisms was higher in discoloured wood samples. Bacterial isolates were not further analysed. Microscopic examination of fungal pure cultures helped to identify three fungal genera: *Mucor*, *Alternaria* and *Cladosporium.* Sequencing of DNA extracted from pure fungal cultures allowed identification of *Cladosporium* sp. (teleomorph *Davidiella tassiana* (De Not.) Crous & U. Braun), *Trametes versicolor* (L.) Lloyd, *Tubaria*

sp., *Hyaloscypha* sp. and *Umbelopsis* sp.

**Microbial diversity and quantity in total DNA extracted from birch wood**

40 unique sequences (or operational taxonomic units (OTUs)) were obtained using the nu-SSU-0817-5/nu-SSU-1196-3 primer pair, of which 15 were obtained only from discoloured wood samples, 17 – only from non-discoloured wood, and eight were obtained from both. The majority of OTUs were similar to uncultured fungal sequences, even from completely different habitats, e.g. aquatic samples. However, there were some OTUs that identified at the genus level and might be connected to decay processes, e.g., *Cadophora* sp., *Candida* sp., *Pholiota* sp., *Fusarium* sp., *Cladosporium* sp., *Penicillium* sp., *Umbelopsis* sp.

DGGE was done for 12 discoloured wood samples from sample site "Salaspils" with PCR products from ITS1f-GC/ITS2 (Fig. 1). Average band number was 2.75 per sample, ranging from 0 to 5. Sequencing of the most intense DGGE fragments and comparison to the NCBI nucleotide database showed that *Cadophora* sp. and *Cadophora fastigiata* Lagerb. & Melin was

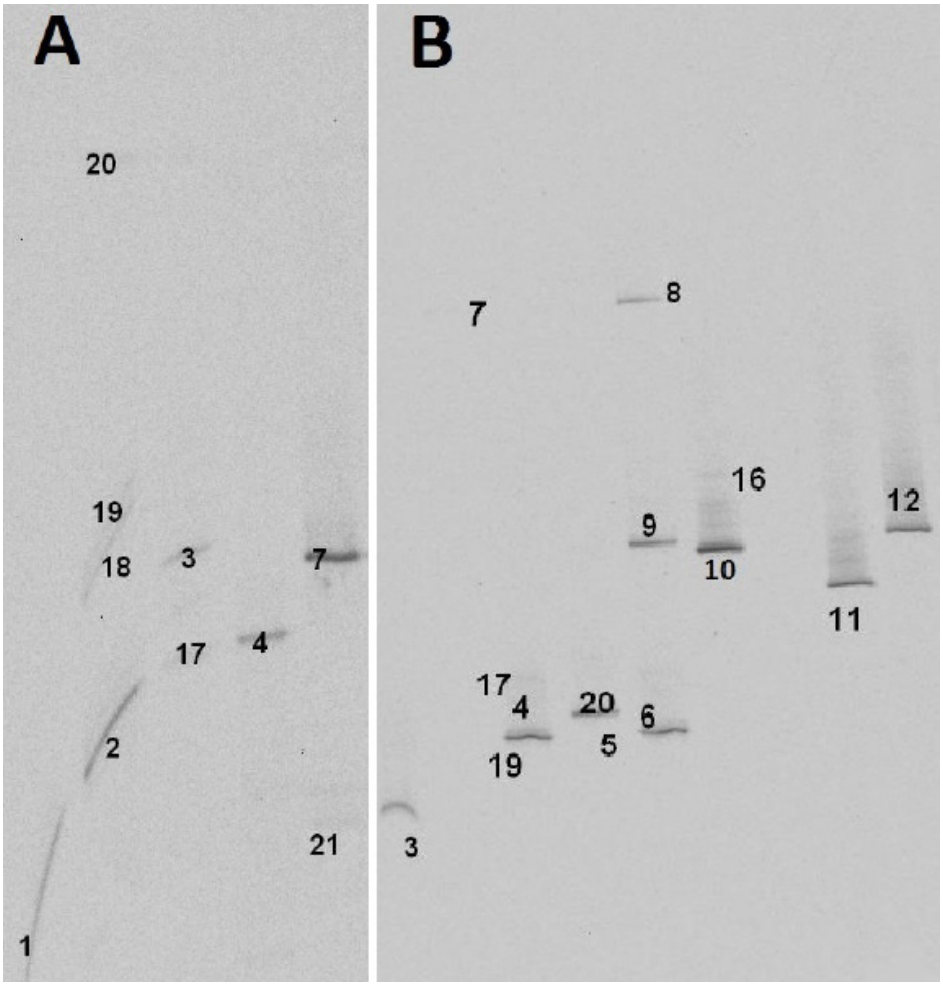


Fig. 1. DGGE profiles of amplicons from PCR with ITS1f-GC/ITS2 primers and DNA from 12 discoloured wood sample. Image of the DGGE gel consist of two separated runs without reference ladder.

identifiable in four samples of discoloured wood, *Helotiales* sp. in five samples, *Pholiota* sp. in two samples, *Phialophora* sp. In two samples, *Trametes versicolor* along with one unidentified basidiomycete in one sample. There was an attempt to adjust the DGGE protocol as described by Vainio and Hantula (2000) who showed that DGGE with PCR amplicons from ribosomal 18S gene larger than 500 bp are possible to analyse, but this protocol was rejected because of difficulties with reproducibility of results. However, analysis of ten samples of discoloured wood from sample site "Liepāja" allowed identification of *Pholiota squarrosa* (Oeder) Kumm. in six samples.

Characterisation of total fungal abundance and diversity by qPCR and length heterogeneity analysis showed that in six individuals the relative fungal quantity in the discoloured wood was significantly higher, ranging from approximately 20-1680 times more in the discoloured wood, relative to non-discoloured wood from the same tree. In two individuals, the relative fungal amounts in the discoloured and non-discoloured wood was slightly lower compared to other six tree individuals (trees 3 and 4, Fig. 2). The number of ITS1F/ITS4 PCR fragments, which indicates the total fungal diversity (Manter & Vivanco 2007), was also higher in discoloured wood in the majority of analysed trees, with the exception of trees no. 2 and no. 7 (Fig. 2).

## DISCUSSION

The survey performed in mature and juvenile birch stands showed that wood discolouration in living birch stems is very common. Differences in discolouration frequency were observed among the half-sib birch families, indicating that there may be a certain heritability of birch resistance against the fungi or other factors related to formation of discolouration.

Culturing of fungi and subsequent microscopic identification is the most established and utilised technique. However it is time-consuming, many species may not be cultured in standard laboratory conditions (O'Brien et al. 2005), and species resolution by microscopy may be low. The use of sequencing overcomes the requirement for culturing, but may still

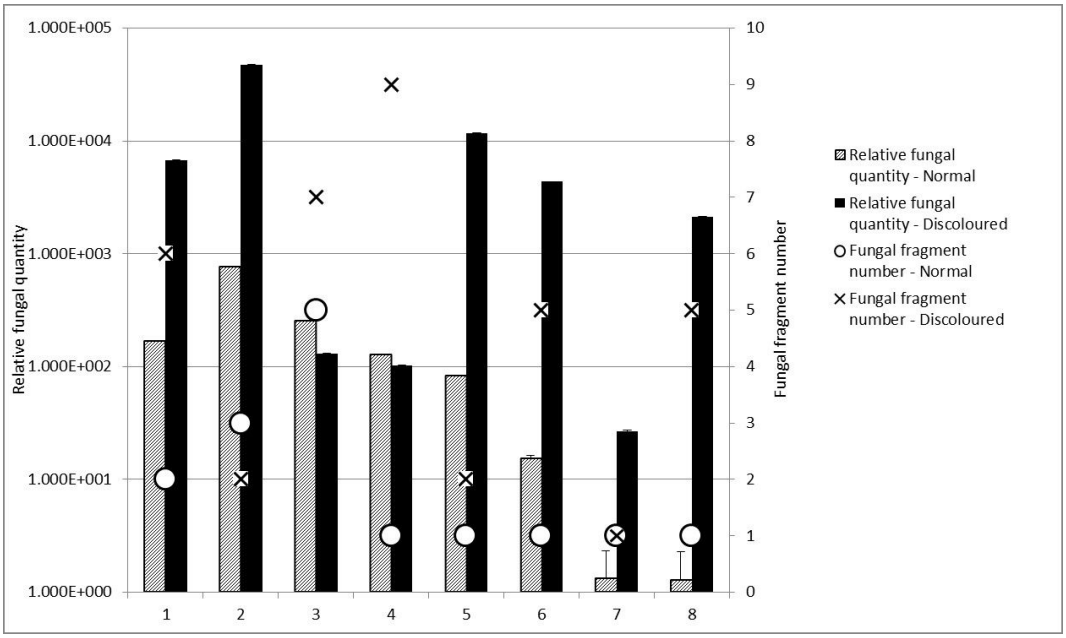


Fig. 2. Relative fungal quantity and fragment diversity in normal (non-discoloured) and discoloured wood. On x axis – sample tree number.

introduce bias by differential PCR amplification efficiencies between fungal species and genera (Bellemain et al. 2010, Anderson et al. 2003, Toju et al. 2012). In addition, the identification of fungal species is dependent on sequence comparisons to databases (Nilsson et al. 2012). The majority of the fungal sequences identified in this study were similar to uncultured or environmental fungal sequence accessions in databases, and it was often only possible to identify sequences to genus level. The sequencing of additional loci could improve the identification of fungi, as well as the use of additional databases. Sequencing also results in a level of redundancy, by sequencing multiple copies of identical fragments. The use of next-generation sequencing techniques can increase the number of sequences obtained, thus overcoming the problem of sequence redundancy, as well as enabling more accurate relative quantitation of different sequences (Lindahl 2013).

Molecular techniques were utilised to analyse total fungal quantity and diversity using the fungi-specific ITS1f/ITS4 primers (Manter & Vivanco 2007). This method has been used to quantify the powdery mildew pathogen *Golovinomyces orontii* (Castagne) V.P. Heluta in *Arabidopsis thaliana* (L.) Heynh. (Weßling & Panstruga 2012); however, there are no reports of the use of this technique to quantify total fungal DNA in tree species. In present study, the results of fungal DNA quantification were consistent with the results obtained using other studies (Nevalainen 2006, Przybyl 2001), indicating that fungal quantity and diversity in discoloured wood were higher as compared to the unaffected wood. However, the results obtained using this method need to be confirmed using a larger number of samples, and possibly utilising additional reference genes. A number of fungi and bacteria were isolated from discoloured wood, some of which have been previously reported to be present in discoloured wood (Schmidt 2006). However, we were not able to detect any microorganism species that was present in all discoloured samples. This suggests that there is no single species that causes wood staining, but rather that discolouration can be caused by the presence of a number of different microorganisms. Although it is necessary to examine much larger number of samples for revealing any tendencies in the composition of fungal populations in birch wood, fungal isolation and DNA amplification with ITS and 18S primers revealed a few fungal taxons similar between different sample sets for each method. *Cadophora* sp., *Pholiota* sp. and *Phialophora* sp. have been detected in wood associated with discolouration and decay (Shortle et al. 1978, Hallaksela & Niemisto 1998, Bellewille et al. 2011).

Each of the utilised techniques in this study – culturing, sequencing of the PCR fragments, DGGE and direct estimation of fungal diversity by fragment length heterogeneity, suggests that fungal diversity in birch wood might be too various for determination of specific causal agent for wood discoloration. Comparison of different taxa of fungi found in discoloured and non-discoloured living wood between individuals did not reveal a single prevalent fungal species or genus that was uniquely found in discoloured wood. In addition, the relative quantitation of the total fungal DNA in discoloured and non-discoloured wood showed that in most cases the total fungal amount is higher in discoloured wood. This indicates that the discolouration of birch wood is not a response to a particular fungal species, but rather a physiological response to the presence of microorganisms in wood, as suggested previously (Sorz & Hietz 2008). The fact that wood discolouration does not affect physical properties of birch wood (Giroud et al. 2008) also suggests that wood discolouration may not negatively affect wood of living birch trees in terms of susceptibility to wind or snow breakage.

Further investigation into host responses to fungal colonisation may indicate the physiological basis of wood discolouration. Perhaps it might be reasonable to investigate different chemotypes of *B. pendula* because of different constitutive profiles of many phenolics and other compound classes within birch wood

affected by its genotype. Hiltunen et al. (2006) observed two strongly coloured compounds in *B. pendula* wood extractives – sinapyl and coniferyl aldehydes, which different levels might be connected with certain genotype and variations in the growing site. Considering that significant variation in wood discoloration was observed between half-sib families, this could also present an opportunity to investigate genetic components of this trait as well as breeding for lower incidence of discoloured wood in birch breeding programs.

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