Hydrophobins
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Dissertation

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Zusammenfassung

Ziel dieser Arbeit war es, die Rolle von Hydrophobinen beim Holzabbau und ihre Fähigkeit, Oberflächen – insbesondere Holzoberflächen - zu beschichten, zu untersuchen. Hydrophobine sind kleine amphipatische pilzliche Proteine, die dafür bekannt sind, die Oberflächenspannung von feuchten Oberflächen zu vermindern (Wasser/Luft-Grenzflächen), die Bildung von Lufthyphen durch Beschichtung mit einem hydrophoben Proteinfilm zu erleichtern und das Anhaften von pilzlichen Hyphen an hydrophobes Material zu unterstützen. Das SC3-Hydrophobin von *Schizophyllum commune* ist bisher das bestuntersuchte Klasse I-Hydrophobin. Ein weiteres Protein, SC15, unterstützt SC3 bei der Bildung von Lufthyphen und ihrer Anheftung an Oberflächen. In Abwesenheit des SC3-Hydrophobins verleiht es Hyphen eine geringe Hydrophobizität. Da von dem Weißfäulepilz *S. commune* SC3- und SC15-Hydrophobin-Mutanten existieren, wurde diese Art ausgewählt, um die Funktion dieser Proteine bei Holzbesiedelung und -abbau zu untersuchen. Versuche mit Holzblöcken und Sägemehl mit verschiedenen *S. commune*-Stämmen haben gezeigt, dass sowohl pilzliche Mono- als auch Dikaryen - einschließlich co-isogenen Wildtyp-Stämmen, Δ*Sc3*-Mutanten, Δ*Sc15*-Mutanten und Δ*Sc3*-Δ*Sc15*-Doppelmutanten - auf Buchen-, Birken- und Kiefernholz wachsen konnten. Die Ergebnisse zeigen, dass weder SC3 noch SC15 notwendig sind, damit *S. commune* in das Holz eindringen, das Holz abbauen und die Holzfestigkeit beeinflussen kann. Allerdings unterschied sich das Ausmaß der Myzelbildung an der Holzoberfläche in Abhängigkeit vom Vorhandensein von SC3-Hydrophobin. Meistens war der Holzabbau durch die Pilzstämme gering (Laubholz) oder fand nicht statt (Kiefernholz). Bei den Versuchen mit Holzblöcken und Sägemehl betrug der maximale Abbau durch *S. commune*-Stämme 4-5% (Buche und Birke) bzw. 9-16% (Buche und Kiefer). *S. commune*-Stämme entfärbten das Kiefernsägemehl, nicht aber das Buchen- und Birkensägemehl. Obwohl die Holzblöcke nur geringfügig oder gar nicht abgebaut wurden, wurde die Holzfestigkeit negativ durch die *S. commune*-Stämme beeinflusst. Der maximale Festigkeitsverlust durch *S. commune*-Stämme betrug bei Buche, Birke bzw. Kiefer 21, 52 bzw. 35%.

S. commune ist ein opportunistischer Krankheitserreger von geschwächten Bäumen. Die Besiedelung eines *Juglans ailantifolia*-Baumes durch den Pilz wurde während einer Zeitspanne von drei Jahren beobachtet. Der Pilz verursachte während dieser Zeit das Absterben mehrerer Äste. An mindestens einem Ast war *S. commune* nicht der einzige Pilz. Neben Fruchtkörpern von *S. commune* wurden solche von *Trametes hirsuta* gefunden. Beide Pilze wurden von diesem Baum isoliert und ihre Interaktion untersucht.

Interaktionsuntersuchungen von *S. commune* und *T. hirsuta* resultierten im „Deadlock"-Phänomen, bei dem sich eine Grenzzone zwischen den Pilzstämmen bildet, in der das Weiterwachstum der Opponenten verhindert wird. Mit fortschreitender Zeit verdrängte *T. hirsuta S. commune*. Als Stressreaktion während der Konkurrenzsituation war eine blaue Pigmentierung an der pilzlichen Interaktionszone zu beobachten. Alle *S. commune*-Stämme (monokaryotische und dikaryotische Wildtyp-Stämme und monokaryotische Hydrophobin-Mutanten) außer der dikaryotischen SC3-Hydrophobin-Mutante bildeten die Grenzzone und Pigmentierung in der Interaktionszone aus. Dies deutet darauf hin, dass das SC3-Hydrophobin nicht essentiell an der Stressreaktion von *S. commune* beteiligt ist.

Protein- und Polysaccharid-Produktion eines *S. commune*-Wildtyp-Stamms und einer co-isogenen Hydrophobin-Mutante in Flüssigkultur wurden verglichen. In Flüssigkultur wurde mittels FTIR-(Fourier-Transform-Infrarot)-Spektroskopie festgestellt, dass sich die Protein- und Polysaccharidgehalte im Myzel des Wildtyps und der Hydrophobin-Mutante signifikant unterscheiden. Der Proteingehalt des Wildtyps war höher als der in der Mutante, während der Polysaccharidgehalt der Mutante mit der Zeit höher war. Myzel von verschiedenen Wildtyp-Stämmen und co-isogenen Mutanten, das auf der Oberfläche von Buchenholz gewachsen war, wurde durch Clusteranalyse von FTIR-Spektren voneinander unterschieden. FTIR-Spektren von Myzel verschiedener Wildtyp-Stämme und co-isogener Mutanten, das im Holz gewachsen war, bildeten einen gemeinsamen Cluster. Die Flächen der Protein-„Peaks" der untersuchten Stämme unterschieden sich auf der Holzoberfläche nicht signifikant voneinander. Die Flächen der Polysaccharid-„Peaks" der dikaryotischen SC3-Hydrophobin-Mutante Δ*Sc3* 4-39 x Δ*Sc3* 4-40 waren auf der Holzoberfläche

hingegen signifikant höher als die der monokaryotischen Mutanten Δ*Sc3* 4-39 und Δ*Sc3* 4-40.

Das SC3-Hydrophobin von *S. commune* wurde aus dem Pilzmyzel mit einem Ertrag von 0.1 % (w/w, 0.5 mg an SC3-Hydrophobin aus 500 mg Myzel) aufgereinigt und zum Beschichten von hydrophobem und hydrophilem Material verwendet. Hydrophobes Teflon und Formwar-Filme wurden hydrophil und hydrophiles Glas wurde hydrophob nach Beschichtung mit SC3-Hydrophobin. Weiter wurden die amphipatischen Eigenschaften von Proteinfilmen auf Buchenholz getestet. Buchenholz wurde in Abhängigkeit von der verwendeten SC3-Konzentration hydrophil oder hydrophob. Niedrige SC3-Konzentrationen (50, 25, 15 µg/ml) bewirkten Hydrophobie und hohe Konzentrationen (100 bis 400 µg/ml) Hydrophilie bei Buchenholz. Oberflächen-Aktivitäten weiterer Klasse I-Hydrophobine von anderen Pilzarten wurden an Buchenholz untersucht. Circa 0,3 bis 0,1% (w/w) Hydrophobin ließ sich aus Myzel von *Coprinopsis cinerea* und *Pleurotus ostreatus*-Kulturen aufreinigen. Die Hydrophobine dieser beiden Pilze wiesen die gleichen Eigenschaften bezüglich der Oberflächenaktivität auf Holz auf wie das SC3-Hydrophobin. ATR-FTIR-(Abgeschwächte Totalreflexions-Fourier Transform Infrarot)-Spektren von mit Hydrophobin behandeltem Holz zeigten eine höhere „Peak-Intensität“ in dem Spektralbereich, der der Amid I-Bande von Proteinen zugeschriebenen wird, als unbehandeltes Holz, was für die Bindung der Hydrophobine an Holzkomponenten spricht.

Summary

The main aims of this research were to study the role of hydrophobins in wood decay processes and to test the ability of these proteins to coat surfaces, particularly wood surfaces. Hydrophobins are small amphiphatic fungal proteins that are known to decrease the surface tension of moist surfaces (water/air interfaces), to help in the formation of aerial hyphae by coating them with a protein film and making them hydrophobic and to support attachment of the fungal hyphae to hydrophobic materials. The SC3 hydrophobin from *Schizophyllum commune* is the best studied hydrophobin until now. Another protein, SC15, is known to act supportive to the SC3 hydrophobin in the formation of aerial hyphae and their attachment to surfaces and, in the absence of the SC3 hydrophobin, it mediates low levels of hyphal hydrophobicity and attachments. Due to the existence of several SC3 hydrophobin and SC15 mutant strains, the white rot fungus *S. commune* was selected for this study to study the functions of these proteins in wood colonization and decay. Wood block and saw dust tests with various *S. commune* strains showed that both fungal monokaryons and dikaryons, including co-isogenic wild type strains, *ΔSc3* mutants, *ΔSc15* mutants and *ΔSc3 ΔSc15* double mutants, were able to grow on beech, birch and pine wood. The obtained results indicate that neither the SC3 hydrophobin nor the SC15 protein are principally necessary for *S. commune* to enter into the wood, to decay the wood or to affect the strength to the wood. However, there were differences in the overall formation of surface mycelium in correlation of the presence of the SC3 hydrophobin. In most instances, the wood decay by the fungal strains were low (deciduous wood) or there was no decay (pine wood). In wood block and saw dust decay tests, the maximum decay caused by *S. commune* strains was 4-5% (beech and birch), and 9-16% (beech and birch), respectively. *S. commune* strains decolorized the pine saw dust but not beech or birch saw dust. Although there was only little or no decay in wood blocks, the strength of the wood was negatively affected by *S. commune* strains. The maximum strength loss caused by *S. commune* strains in beech, birch and pine wood was 21, 52 and 35 %, respectively.

S. commune is an opportunistic pathogen on weakened trees. An infestation of a *Juglans ailantifolia* tree by the fungus was observed over a period of 3 years. The fungus caused over the time die off of a number of branches. In at least one branch,

S. commune was not the only fungus. Next to *S. commune* fruiting bodies, carpophores of *Trametes hirsuta* were observed. Both fungi were isolated from the tree and studied in interactions. Furthermore, SC3 hydrophobin mutants and their co-isogenic wild type strains were included in the study.

Interaction studies of *S. commune* strains with *T. hirsuta* on agar plates resulted in the deadlock phenomenon in which a barrier is formed between the fungal strains hindering the opponents in further growth. Subsequently with time, the deadlock situation changed by the partial replacement of *S. commune* by *T. hirsuta*. As a stress reaction during the combat interaction, a blue pigmentation was observed at the fungal interaction zone. All the *S. commune* strains (monokaryotic and dikaryotic wild type strains and hydrophobin monokaryon mutants) except the SC3 hydrophobin mutant dikaryon showed the barrier formation and the pigmentation in the interaction zone, indicating that in principle the SC3 hydrophobin is not involved in the stress reactions of *S. commune*.

Further, differences between a *S. commune* wild type strain and a coisogenic hydrophobin mutant were studied in liquid cultures in relation to the produced protein and polysaccharide. In the liquid cultures, as determined by the FTIR (Fourier Transform Infrared) spectroscopy analysis, there were significant differences between the mycelium of the wild type strain and the mycelium of the hydrophobin mutant with respect to protein and polysaccharide contents. The protein content were higher in the wild type strain than in the mutant, whereas the polysaccharide content was higher over the time in the mutant strain. When growing on beech wood, the mycelium of different *S. commune* wild type strains and the co-isogenic hydrophobin mutants on the surface of the wood were discriminated by FTIR spectra submitted to a cluster analysis. Inside the wood, however, the FTIR spectra of the mycelia of the different *S. commune* wild type strains and hydrophobin mutants clustered together. There were no significant differences noted amongst the values from protein peak area values calculated from the FTIR spectra in between the tested strains on the surface of beech wood. On the surface of wood, the polysaccharide peak area value of the dikaryotic Sc3 hydrophobin mutant Δ*Sc3* 4-39 x Δ*Sc3* 4-40 was significantly higher than that of the monokaryotic mutants Δ*Sc3* 4-39 and Δ*Sc3* 4-40.

SC3 hydrophobin of *S. commune* was purified from fungal mycelium with yields of 0.1% (w/w, 0.5 mg of SC3 hydrophobin from 500 mg of mycelium) and used to coat hydrophobic and hydrophilic materials. Hydrophobic Teflon and form-war film turned hydrophilic and hydrophilic glass turned hydrophobic upon coating with SC3 hydrophobin. Further, the amphipathic nature of films of this protein was tested on solid beech wood. Beech wood turned hydrophilic or hydrophobic, depending on the concentration of SC3 hydrophobin applied. Lower concentrations (50, 25, 15 µg/ml) of SC3 resulted in hydrophobic behavior and higher concentrations (100 to 400 µg/ml) in hydrophilic behavior of beech wood. The surface activity of other class I hydrophobins from different fungal species were also tested on the beech wood. About 0.3 and 0.1% (w/w) purified hydrophobins were obtained from the mycelium of *Coprinopsis cinerea* and *Pleurotus ostreatus* cultures, respectively. The hydrophobins of both fungi followed the same pattern of surface activity on wood than the Sc3 hydrophobin. Results from ATR-FTIR (Attenuated Total Reflection - Fourier Transform Infrared) spectra of hydrophobin-treated beech wood showed a higher intensity in the peak associated with amide I band of proteins indicating the binding of hydrophobin protein to the wood components.

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Chapter 1

General Introduction

1. General Introduction

1.1 General background and aim of this thesis

Schizophyllum commune is a widely distributed wood inhabiting basidiomycete found throughout the tropical and temperate regions of the world (Raper 1958, James et al. 1999). It grows mainly on dead wood but can also be found as a pathogen on branches of living trees (Adaskaveg 1993, Latham 1970, Peddireddi et al. 2005). In the laboratory, the fungus is used as a model fungus for studying mating types and fruiting body development in the basidiomycetes (Raper & Fowler 2004). *S. commune* is further well known by the invention of small proteins called hydrophobins that were discovered as products of genes abundantly expressed during the emergence of fruiting bodies and aerial hyphae (Wessels 1994, Wessels 1997). Hydrophobins self-assemble into amphipathic films, coat the fungal surfaces, make them hydrophobic thereby enabling the fungal structures to grow into the air and protects these from adverse conditions (Wösten 2001, Walser et al. 2003). These proteins are well known for their remarkable biophysical properties (Wessels 1997). Coats of hydrophobins transform hydrophilic surfaces into hydrophobic and hydrophobic surfaces into hydrophilic (Wösten et al. 2000). This surprising property has caused much attention for applications in biotechnology such as in tissue engineering (increase in biocompatibility of medical implants and medical devices), dispersions and emulsions (e.g. drug delivery using oil vesicles stabilized with a hydrophobin), nanotechnology (e.g patterning molecules at a surface with a nanometric accuracy), as an intermediate for attaching molecules to a surface (e.g. immobilization of antibodies in a biosensor) (Scholtmeijer et al. 2001). Biologically, hydrophobins help the fungus in growth into the airspace by allowing hyphal tips to leave a liquid environment into the gaseous phase (Wösten et al.1999, Wösten 2001). In the multicellular fruiting bodies, hydrophobins have a water repellent function which avoids the airspaces in the structures to be filled with water (Lugones et al. 1999). It is however not known whether hydrophobins participate in fungal interactions such as in wood colonization and in the pathogenicity of *S. commune*. The work in this thesis is focused on basic research to study the role of hydrophobins in wood biology. In addition, the proteins were tested for their technical application in wood coatings.

1.2 The fungus *Schizophyllum commune*

S. commune is described as a severe wood destroyer in tropical regions and as a mild rot in temperate regions (Schmidt & Liese 1980). It is most commonly found growing on fallen trunks of deciduous trees and less often on conifers (Cooke 1961, Breitenbach & Kränzlin 1991). It also acts as a pathogen on standing trees (Adaskaveg et al. 1993, Latham 1970, Peddireddi et al. 2005). *S. commune* is often found growing on dead and living wood along with other fungi such as *Trametes hirsuta* (this study). Down- and up-regulation of hydrophobin genes were reported in the interaction of *Phlebiopsis gigantea* and *Heterobasidion parviporum* (Adomas et al. 2006). Interaction studies showed that *S. commune* is able to interact in form of the dead lock phenomenon with *T. hirsuta* or in form of dead lock followed by partial replacement by *T. hirsuta*. The outcomes of fungal interactions usually show either the dead lock phenomenon (where neither fungus makes ingress into the territory of the other), or replacement (where one fungus grows over through the other such that the latter was no longer recoverable by isolation), or partial replacement (where one fungus was recoverable from some but not all of the territory that it originally held) (Wald et al. 2004). The laboratory of Wessels has produced mutants in hydrophobin expression by deleting the hydrophobin encoding gene *Sc3* from the genome (Wösten et al. 1994). These mutants were used in comparative studies with wild type strains to define biological functions of hydrophobins. In confrontation studies, unlike a wild type dikaryon, the *S. commune* hydrophobin mutant dikaryon Δ*Sc3* 4-39 x Δ*Sc3* 4-40 did not show a strong dead lock reaction or the formation of barrage (barrier zone of hyphal growth) and pigmentation in interaction with *T. hirsuta* (this work). Dikaryons are secondary mycelia in the life cycle of higher basidiomycetes that form through mating of the primary mycelia, also known as homokaryons, that arise from germination of the sexual basidiospores. Dikaryons are the commonly found mycelia in nature that form the mushrooms in which the basidiospores are formed. Germinated mycelia in contrast are only short lived since as soon they meet a mating partner, i. e. another monokaryon (of different and thus compatible mating type) they will transform into a dikaryon (Kües 2000). Therefore, it is quite interesting to learn that the monokaryotic hydrophobin *S. commune* mutant strains Δ*Sc3* 4-39 and Δ*Sc3* 4-40 were able to form barrages and produce pigmentation in confrontation with *T. hirsuta*. The barrage formation of the *S. commune* dikaryon is often associated with the ability

of the fungus to resist an opponent. Barrier formation and pigmentation are seen as a stress reaction of a fungus caused by the presence of the opponent. Data presented in this thesis suggest a possible role hydrophobins during stress reactions of the fungus in the form of a dikaryon (see chapter 2 of this thesis for details).

1.3 *Schizophyllum commune* growth on wood

Many *S. commune* strains were shown to secrete enzymes such as xylanases, cellulases, laccases and peroxidases (Bray & Clark 1995, de Vries et al. 1986, Haddadin et al. 2002, Hegarty et al. 1987, Oku et al. 1993, Schmidt & Liese 1980, Senior et al. 1991, Thygesen et al. 2003) which are usually considered to play a key roles in the wood decay processes (Hoegger et al. 2007). However, there were also contradictory reports where there were no detectable enzyme activities in fungal cultures (Nsolomo et al. 2000, own unpublished results) and on wood (Boyle et al. 1992, own unpublished results). Although *S. commune* is regarded as a white rot fungus, wood decay tests in the laboratory in many instances did not show considerable weight losses of wood (Hegarty et al. 1987, Nsolomo et al. 2000, Schirp et al. 2003, Humar et al. 2001, 2002, Nilsson & Daniel 1983). To know whether the fungus needs hydrophobin coatings to penetrate into and grow inside the wood, we have used several hydrophobin mutants for wood decay experiments. Wood block tests and saw dust tests performed with *S. commune* resulted in low level decay of up to 5% and 15% mass loss, respectively (see chapter 3 of this thesis for details).

Disruption of the *Sc3* hydrophobin gene in *S. commune* affected the cell wall composition of the fungus. In contrast, compared to the wild type strain, the amount of mucilage increased considerably in the *Sc3* mutants (van Wetter et al. 2000, Walser et al. 2003). To further understand this process and to investigate the behavior of mycelium in the wood, we studied fungus infested wood with FTIR (Fourier transform infrared spectroscopy) and compared the relative protein and polysaccharide amounts by respective peaks in the FTIR spectra of a *S. commune* wild type and a hydrophobin mutant in liquid cultures. As shown previsously in the literature (van Wetter et al. 2001), the relative mycelial polysaccharide content was significantly higher in the hydrophobin mutant strain. In contrast, the mycelial protein content was higher in wild type strain (see chapter 4 of this thesis for details).

FTIR microscopy is a promising new technique to detect fungi in wood (Naumann et al. 2005, Naumann et al. 2007). To investigate the alterations in the mycelium due to the hydrophobin mutation while the fungus grows on wood, we measured FTIR spectra of the fungal mycelium both from the wild type and hydrophobin mutants of *S. commune* in and on wood. On the wood surface, the fungal strains differed as was shown by qualitative cluster analysis. In contrast, no significant differences were noted among the relative mycelial protein content between the wild type strains and the hydrophobin mutants on the surface of the wood, but at least in one instance, a wild type strain had lower polysaccharide content. The mycelial polysaccharide content of the hydrophobin mutants within wood was significantly lower compared to wild type (see chapter 5 of this thesis for details).

1.4 Application of hydrophobins with wood

Hydrophobins are known to be the most surface active proteins with remarkable biophysical properties. They were shown to change the nature of both natural and artificial surfaces (Scholtmeijer et al. 2001, Wösten 2001). Hydrophobic surfaces such as Teflon can be changed to hydrophilic and hydrophilic surfaces such as glass can be changed to hydrophobic by coating with hydrophobins. The coatings of hydrophobins differ from that of other proteins in that they can resist treatments such as with hot-SDS solutions. In consequence, they can only be removed by treatments with harsh acids like tri-fluoro-acetic acid or periodic acid (Wessels 1997). In this study, we purified and successfully tested the wood coating ability of Sc3 hydrophobin of *S. commune* (see chapter 7 of this thesis for details). The hydrophobins from *C. cinerea* and *P. ostreatus* were also tested, in concentration dependent manner; the hydrophobins either caused a water repellent or a water absorbent effect in the wood (see chapter 8 of this thesis for details).

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Chapter 2

Growth of *Schizophyllum commune* in natural environments and interaction with other fungi

2. Growth of *Schizophyllum commune* in natural environments and interaction with other fungi

2.1 Abstract

Schizophyllum commune was observed as an opportunistic pathogen on a *Juglans ailantifolia* tree. The fungus is found associated with other organisms on the same tree like the lichen *Xanthoria* and the white rot *T. hirsuta*. The infection of *S. commune* increased in a course of time towards the base of the branch as seen by the spreading of fruiting body formation towards the base of branches. *S. commune* strain ScJa 1 was isolated from the infected *Juglans* tree and another *S. commune* strain ScFs1 from a dead beech trunk. Wood decay test of *Juglans* wood with these and other *S. commune* strains resulted in mass loss up to 10%. *Sc3* hydrophobin mutants not producing hydrophobins as their parental wild type strains were able to decay *Juglans* wood. However the growth pattern of *Schizophyllum* strains varied on *Juglans* wood blocks. Wild type strains showed dense mycelium on the wood blocks and the *Sc3* hydrophobin mutants little formation of aerial hyphae. Interaction studies between two fungal species on agar plates showed that *S. commune* reacts with a dead lock phenomena with *T. hirsuta* ThJa 1. Further, only the hydrophobin mutant dikaryon *ΔSc3* 4-39 x *ΔSc3* 4-40 was unable to show the dead lock reaction or formation of a barrage and production of pigmentation in the interaction with *T. hirsuta* ThJa 1. In contrast, the hydrophobin monokaryotic mutant strains *S. commune* Δ*Sc3* 4-39 and Δ*Sc3* 4-40 strains were able to form barrages and pigmentation in the fungal interaction zone as their wild type parents. In conclusion, secretion of hydrophobins is not principally necessary for defense reactions against fungal opponents on the same substrate. On wood, *S. commune* was shown to invade the substrate first to be later replaced by *T. hirsuta*. In the natural situation, *S. commune* unlikely will therefore be able to defend itself on a wooden substrate. As a way out, abundant number of fruiting bodies might be timely produced that allow distribution of the fungus by basidiospores.

2.2 Introduction

Schizophyllum commune, the split gill mushroom, is one of the most widely distributed wood inhabiting basidiomycetes found throughout the tropical and temperate regions (Adaskaveg 1998, Raper et al. 1958, James et al. 1999, James and Vilgalys 2001, Schmidt and Liese 1980). The fungus is known by humans since the early times when primitive people used it as food or a type of chewing gum. It is still consumed today under specific common names such as virinche, parefi, soningan some, tukunw or buangi in countries like Congo, Peru, India (Assam) and Thailand (Cooke 1961).

The habitat of *Schizophyllum* is usually on wood such as beech, pine, birch, peach, balsa wood, *Ocotea sp.*, *Abies* sp., *Bambusa* sp., *Calamus* sp., *Citrus* sp., *Eucalyptus* sp., *Hevea* sp., *Juglans* sp., *Mangifera* sp., *Protium* sp., *Prunus* sp., *Pyrus* sp., *Quercus* sp., *Salix* sp., etc and also on herbaceous materials (Cooke 1961, Dai 2005, Nsolomo et al. 2000, Nicolotti et al. 1998, Brady et al. 2005). Apart from the above mentioned habitats, specimens of *S. commune* have been reported from more than 300 named plants in almost every geographical area in the world. For example, the plant pathologist Otto A. Reinking, during the early 1900s made 211 collections of *S. commune* from at least 47 host species of woody plants (Cooke 1961). The species is more commonly found on fallen trunks and on branches of deciduous trees and less often on conifers (Cooke 1961, Breitenbach and Kränzlin 1991).

S. commune is reported to be a typical white rot (Hegarty et al. 1987). It is regarded as a saprobe but also as an opportunistic wound pathogen on living trees. The fungus colonizes aggressively the trees under stress causing white rot of the sapwood (Adaskaveg 1998). Various studies describe the fungus as a pathogen on standing trees, amongst them are *Mangifera indica* (mango tree), *Malus* sp., and *Prunus* sp. (Adaskaveg et al. 1993, Brady 2005, Dai 2005, Latham 1970). In case of *Pyrus malus* (apple), all parts of the tree were found to be infected including the fruits (Cooke 1961). Next to infesting branches of living trees, *S. commune* is also found growing on the mature leaves e.g. of *Tectona grandis* (teak) which is one of the most valuable timber resources in the tropical regions (Chareprasert et al. 2006). As described for peach, symptoms caused by this fungus on trees include fresh leaves turning necrotic

or chlorotic and dry, wood discoloration and white sapwood rot (Dai 2005). Recent reports state that the species can also grow on the bale of grass silage on farms in the Irish midlands (Brien et al. 2005, Brady et al. 2005, Brien et al. 2007). In the tropical world, *S. commune* is found throughout wherever sugar cane is grown. Fruiting bodies are regularly seen on internodes of *Saccharum officinarum* (Cooke 1961). Singer (1949), reported *S. commune* growing on stolons of strawberries in Europe.

Generally, *S. commune* is a severe wood destroyer in tropical regions and a mild rot in temperate regions (Schmidt and Liese 1980). The species can tolerate a wide range of temperatures up to 50^{o}C. Laboratory tests showed that the species can also tolerate high saline conditions of up to 70 g/l and hence it is referred as a euryhaline species. It can grow at salinities higher than that of sea water and is thus also found growing on open sites exposed to seawater as well as on branches of living *Casuarina littoralis* trees in the tropical coastal forest of Papua New Guinea (Castillo and Demoulin 1997).

Surprisingly by the various observations in nature, wood decay tests with *S. commune* in the laboratory in most instances did not result in considerable mass loss of wood (Hegarty et al. 1987, Nsolomo et al. 2000, Humar et al. 2001, 2002, Schmidt and Liese 1980). In nature, *S. commune* is found growing along with other organisms (Fig. 1a and 7). Chareprasert et al. (2006) for example reported that *S. commune* is growing as an endophyte on teak leaves along with *Fusarium*, *Penicillium* and Xylariaceae members. Toole (1951) reported *Stereum complicatum* and *S. commune* as the first fungi on fire wounds of trees. Heptig (1941) noted *S. commune*, *Panus stipticus*, *Daldinia concentrica*, *Nummularia* sp., *Stereum* sp., and *Polyporus* sp. growing on fire wounds of oak one year after the fire (Shigo 1967).

The development of fungal communities in woody species is complex with multidimensional pathways (Heilmann-Clausen and Boddy 2005). Usually, the substrate is occupied by several different decomposing organisms, eventually dominated by the wood decaying fungi (Woods et al. 2005). Interspecific antagonistic fungal interactions have been studied since the early 1980s. In natural environments, the interspecific antagonistic fungal interactions can be for the space rather than

directly for nutrients (Heilmann-Clausen and Boddy 2005). Antagonistic interactions are common when mycelia of different individuals meet. These interactions appear to be important in determining the community patterns among wood decay fungi and may affect the overall decay rate of wood. The term combative is used to describe the active antagonistic interspecific mycelial interactions among filamentous fungi. The outcome of combative interactions can be either replacement or deadlock. In the replacement pattern, one fungus gains the territory of the other whereas in the deadlock situation as a mutual exclusion reaction neither species is capable of taking dominance over the other. Recent studies also showed that the interspecific interactions can alter the functions of the mycelium such as mycelial search patterns, distribution and reallocation of nutrients within mycelia and respiration (Boddy 2000, Owens et al. 1994, Wald et al. 2004). Morphology changes of mycelia are common during interactions which take place by the formation of stationary barrages resisting the invasion of the opponent, invasion by mycelial fronts, mycelial fans and linear organs like cords and rhizomorphs. Such changes are often correlated with differences in physiology, metabolism and enzyme production (Wald et al. 2004). Interactions can result in inter- and intra-cellular pigment formation, changes in enzyme activities and in marked differences in hydrophobic metabolites (Boddy 2000, Wald et al. 2004). Mycelial interactions in wood were reported where complete replacement of one species by another occurred without any interaction zones in wood after 152 days (Wells and Boddy 2002). Internal spread of different fungi such as *Bjerkandera adusta*, *Chondrostereum purpureum*, *Coriolus versicolor*, *Daedaleopsis confragosa*, *Hypholoma fasciculare*, *Phlebia merismoides*, *Pseudotrametes gibbosa* and *Stereum hirsutum* individually and in combination inoculated into hardwood stumps of beech (*Fagus sylvatica*), birch (*Betula pendula*) and oak (*Quercus robur*) were studied previously in the laboratory and colonization patterns were found to vary for different fungus-tree combinations (Rayner 1979). The outcome of fungal interactions varied depending on the supplied resources, e.g. water potential and the gaseous regime, and to a lesser extent on temperature, size and quality of resource. Fungi occupying larger wood resources have a relatively higher success in combat than those occupying smaller resources when challenged with the same species (Boddy 2000). Interactions of *S. commune* with other fungi like *Pleurotus ostreatus* and *Trametes versicolor* were studied previously by Tsujiyama and Minami (2005) in agar tests (malt extract

2%, agar 1.5% at 28°C) where the former was overgrown by the latter. Strong activities of phenol-oxidising enzymes were detected in dual cultures of *S. commune* and *P. ostreatus* (Tsujiyama and Minami 2005).

In this chapter, studies on growth of *S. commune* in natural environments, antagonistic behaviour towards *Trametes hirsuta* and the possible role of hydrophobins in these interactions are presented.

2.3 Materials and Methods

2.3.1 *Juglans* tree

A tree of *Juglans ailantifolia* growing near to the Göttingen Zentrum für Molekulare Biowissenschaften (GZMB) was moved by a few meters in 2003 due to construction works. There was no visible fungal infection e.g. by fungal fruiting bodies before the tree was moved. Fruiting bodies of *S. commune* were first noticed in spring (May) 2004.

2.3.2 Isolation of fungal strains from wood

Fruiting bodies were collected from the *Juglans* tree as well from fallen branches of a *beech* tree in the forest Billingshäuser Schlucht of Göttingen. A piece of the tissue aseptic taken from inside of the fruiting bodies were placed on the Maloy agar [2% malt extract (Oxoid, Hampshire, England), 4mg/l benlate as a fungicide working against ascomycetes 50% WP (Dupont, Paris, France), 1% agar (Serva, Heidelberg, Germany); after autoclaving 100 mg/l streptomycin was added against bacteria]. Plates were incubated at 25°C under continuous light. Grown mycelia were further transferred onto fresh 2% malt extract plates. Clamps at hyphal septa were observed with a Zeiss Axiophot photomicroscope (Zeiss, Göttingen, Germany) equipped with a soft imaging colour view II Mega pixel digital camera (Soft Imaging System, Münster, Germany) that was linked to a computer equipped with analySIS® software programme (Soft Imaging System, Münster, Germany).

2.3.3 Fungal strains

S. commune dikaryon ScJa 1 and *T. hirsuta* dikaryon ThJa 1 were isolated from the living *Juglans* tree and *S. commune* dikaryon ScFs 1 from a fallen beech branch. The

co-isogenic *S. commune* monokaryons 4-39 (*MATA41 MATB41,* CBS 341.81), 4-40 (*MATA43 MATB43,* CBS 340.81), the corresponding *Sc3* hydrophobin mutants 72-3 (*ΔSc3 MATA41 MATB41*), *ΔSc3* 4-40 (*ΔSc3 MATA43 MATB43*) and the homokaryon (multiple nuclei share one common cytoplasm as it is found in hyphal cells or mycelium and all nuclei are genetically identical), *S. commune* Acon Bcon fbf (selected for our studies because of high mRNA abundance for Sc3 protein) recessive natural mutant for fruiting were kindly provided by Prof. Wösten, Utrecht, The Netherlands. Dikaryotic *S. commune* strains 4-39 x 4-40 (*MATA41 MATB41* x *MATA43 MATB43*), *ΔSc3* 4-39 x *ΔSc3* 4-40 (*MATA41 MATB41* x *MATA43 MATB43*) were produced by mating the corresponding monokaryons. Wild type *S. commune* monokaryon ScLs 48 and dikaryon ScLs 79 were obtained from the own institute's collection. *S. commune* dikaryon ScFs 2 was kindly provided by Prof. Holdenrieder, ETH Zurich, Switzerland, and *P. ostreatus* N001 (dikaryon) kindly by Prof. Ramirez, Universida Publica de Navarra, Pamplona, Spain.

2.3.4 Growth conditions

All strains were grown at 25 ^{o}C in continuous light. For wood decay tests, *S. commune* strains were cultivated on *S. commune* minimal medium (20 g glucose, 1.5 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ x $7H_2O$, 1 g yeast extract, 0.12 mg thiamine-HCl, 0.1 mg pyridoxine HCl, 0.005 mg biotin, 0.2 mg $CuSO_4$ x $5H_2O$, 0.08 mg $MnCl_2$ x $4H_2O$, 0.4 mg cobaltous chloride hexahydrate, 1.2 mg calcium nitrate tetrahydrate per 1 liter H_2O; Dons et al. 1979), *T. hirsuta* on BSM [Basidiomycete standard medium; 5 g glucose monohydrate, 0.65 g L- asparagine 1 g KH_2PO_4, 0.5 g $MgSO_4$ x $7H_2O$, 0.5 g KCl, 0.5 g yeast extract, 10 g agar, 50 ml stock solution I (0.2 g $FeSO_4$ x $7H_20$ per liter), 50 ml stock solution II (0.16 g $Mn(CH_3COO)_2$ x $4H_2O$, 0.04 g $Zn(NO_3)_2$ x $4H_2O$, 1 g $Ca(NO_3)_2$ x $4H_2O$, 0.06 g $CuSO_4$ x $5H_2O$) per 1 liter H_2O, pH 4.5; Hüttermann and Volger 1973] and *P. ostreatus* on SMY medium (10 g of sucrose, 10 g of malt extract, 4 g of yeast extract, per 1 liter H_2O; pH 5.6; Peñas et al. 2002). For interaction studies, all strains were grown on BSM.

2.3.5 *Juglans* decay test

Non-infected branches of *Juglans* were cut into 3 x 1 x 0.5 cm^3 (longitudinal to the axis x tangential to the axis x radial to the axis) blocks and used for the decay test.

Wood was not sorted either from the outer or inner part of the branch and was dried in an oven at 100°C for two days. Initial weights of the dried wood blocks were determined before soaking the samples overnight in water and autoclaving. Each six wood blocks were used in 9 cm Petri plates. To avoid the direct contact of the wood blocks with the agar, sterile steel grids were used beneath the wood blocks. Wood blocks were transferred onto the steel grids when the mycelium of the growing fungi covered half of the Petri plates. Mycelium was inoculated in the middle of the plates by placing a block of agar with mycelium that was cut with a cork borer (1cm Ø). Petri plates were sealed with Parafilm (PECHINEY, Chicago, USA) and incubated at 25°C both for initial mycelial growth as well as for further incubation, once the wood blocks were placed onto the grids. To check decay by the strains, harvests were performed after 8, 12, 16 and 20 weeks of incubation. Plates without fungi but with wood blocks were used as negative controls. After incubation, the mycelium attaching to the wood blocks was separated by using a scalpel. Wood blocks were then dried at 100°C for 3 days and weighed to note the final (dried) weight. For decay test with mixed infections of *S. commune* and *T. hirsuta* each 2 wood blocks of three Petri plates were used and harvest was performed after 10 weeks of incubation. Due to limited availability of *Juglans* wood, controls (wood without fungal infections) were not included in the mixed infection experiment. Mass loss of wood was calculated as (initial wt – final wt)/initial wt x 100.

2.3.6 Interaction studies

Pre-cultures were prepared on 1% agar BSM medium for all the strains. 9 cm Ø Petri dish cultures were incubated 7 to 10 days until the mycelium was fully grown in the plate. Blocks of agar were cut with a cork borer (1cm Ø) from the edge of the plate of each strain. Using aseptic conditions, two agar blocks with mycelium (one from each species) were placed 4 cm apart in 9 cm diameter Petri dishes. All the *S. commune* strains were paired against *T. hirsuta* ThJa 1 and 3 replicate dishes were prepared for each combination. Petri dishes were sealed with parafilm and incubated for 6 weeks.

2.3.7 Statistical analysis

Statistics was performed by using SPSS software release 9.0.0, standard version (SPSS Inc.). Data sets were compared using Duncan multiple range test up to 95% confidence level ($p \leq 0.05$).

2.4 Results

2.4.1 *S. commune* as a pathogen on a *Juglans ailantifolia* tree

In the spring (May) 2004, a *S. commune* infection on a *J. ailantifolia* tree growing next to the Göttingen Zentrum für Molekulare Biowissenschaften (GZMB) was noted by the formation of numerous white fruiting bodies on the sunny side of two large branches of the tree (Fig. 1). The tree was therefore further observed over the time (Fig. 2) and the infection monitored from time to time. Due to an increase in fungal infection, the tree appeared to weaken with time (Fig. 3a). Infected branches in May 2004 had a low outgrowth of leaves compared to branches appearing to be still healthy. In November 2004, masses of fruiting bodies were seen on the two infected branches (Fig. 2a, 3a).

In May 2005, the tree produced numerous leaves on all healthy branches but not on branches infected with *S. commune* (Fig. 2b, 3b and 3c). In June 2005, healthy branches were covered with foliage except the two *S. commune* infested branches where formation of a leave was an exception (Fig. 2c, 4a, 4b). In June 2005, the two weakened and nearly dead branches were then cut from the tree, resulting in an even foliage appearance on the remaining tree (Fig. 2d).

Nevertheless, on a new branch in September and October 2005 new fruiting bodies appeared (Fig. 2d branch 3 and Fig. 4c). The infection did however not hamper leave formation in spring 2006 (Fig. 2f) and spring 2007 (Fig. 2i), even though, judging from fruiting body formation, the infection spread further over the branch over the time from top to bottom (Fig. 2d to 2i, 4c). Over the time, compared between the years 2005 and 2006, there was an increase in the number of fruiting bodies on the third and later infected branch (Fig. 5a, 5b). There was an increase in fruiting body formation over a distance of about 30 cm per year at the lower part of the branch (Fig. 5c).

Probably, the *Juglans* tree became weak due to its movement in 2003 from one place to another, and *S. commune* appeared to be an opportunistic pathogen on this tree. As described above, fruiting bodies of *S. commune* initially appeared on two branches and the infection passed on to a third branch in course of time, although the two earlier infected branches were removed in June 2005 (Fig. 2e). Importantly, on all the three branches, the fruiting bodies of *S. commune* faced their gills towards a south-west direction, consistent with other reports from the literature that *S. commune* requires sun to produce fruiting bodies on branches (Breitenbach and Kränzlin 1991).

It is possible that the branches of the tree weakened by the transfer were sun burned in the very hot summer 2004 resulting in cracks in the bark and allowing the fungus to enter the tree through these cracks (Fig. 6). However, on the third branch showing *S. commune* fruiting bodies in the year 2005, we observed the mushrooms to brake through the bark that was overgrown with epiphytic lichens (*Xanthoria sp.,* Fig. 7). The infection obviously caused severe cracking of the bark of the branch by fruiting body formation over the time (Fig. 8). Emerging fruiting bodies appeared newly in cracks at the lower part of the branch but also in cracks where senescent fruiting bodies from the year before still resided (Fig. 8g).

Further in March 2007, new fruiting bodies of *S. commune* were noticed at the cut surface of the branch number 2 trimmed in the year 2005 (Fig. 9). Also here, fruiting bodies initiated in cracks but not of the bark but of the dead wood. From the observations we have, we however cannot decide whether the fruiting bodies were from the old infection noted first in the branch in the year 2004 or from a new infestation that made use of cracks in the aging wood.

Since *S. commune* is reported to be usually not aggressive in wood degradation (see introduction) it was an obvious thought to audit the branches for other infections. The fungus was at least on one branch (Fig. 2a branch 1) not the only basidiomycete, as also fruiting bodies of the white rot species *T. hirsuta* were discovered on the branch (Fig. 1a). On the third branch showing *S. commune* fruiting bodies first in the year

2005 (Fig. 5a, 5b) we did not notice another fungal species, but only the harmless epiphytic lichens (Fig.7).

With cutting down the infected branches in year 2005, there was a chance to inspect the wood underneath places with *S. commune* fruiting body formation. At such places, there were clear signs of white rot (Fig. 10a, 10b). The place of decay supports that *S. commune* should have some decaying ability although in the branch infested in addition by *T. hirsuta*, the second fungus might support growth of *S. commune* due to more aggressive wood decay.

2.4.1.1 Fungal isolations

Fruiting bodies of *S. commune* as well as fruiting bodies of *T. hirsuta* were collected in November 2004 from the *J. ailantifolia* (walnut) tree (Fig. 7b) for strain isolation (*S. commune* ScJa 1, *T. hirsuta* ThJa 1). Furthermore, *S. commune* ScFs 1 was isolated from a fruiting body from a dead *Fagus sylvatica* (beech) branch collected in the Billingshäuser Schlucht, Göttingen in May 2004 (Fig. 7a). Isolated mycelia were observed under a microscope. Clamp cells were discovered on hyphae of all isolates indicating that they are dikaryotic. ITS sequences confirmed for *S. commune* ScJa 1, ScFs 1 and *T. hirsuta* ThJa 1 the species designation (P. J. Hoegger, personal communication).

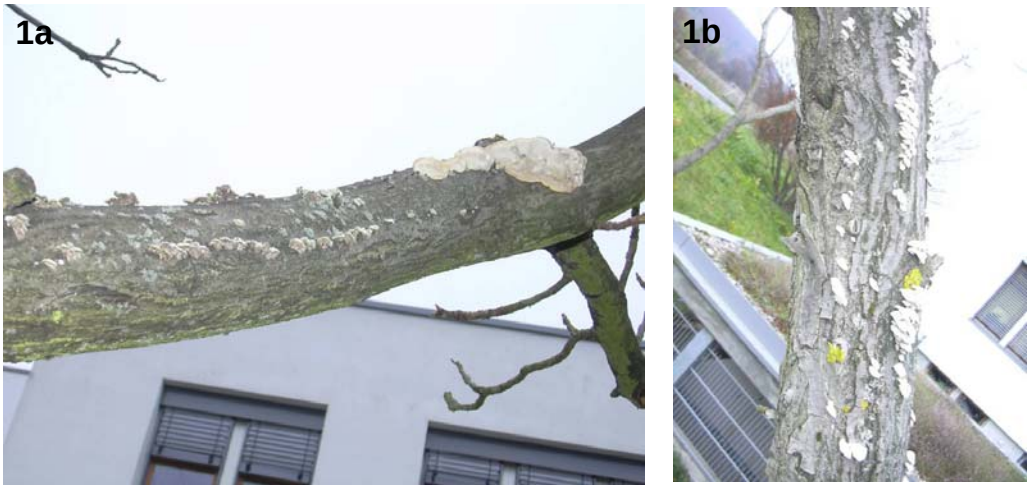
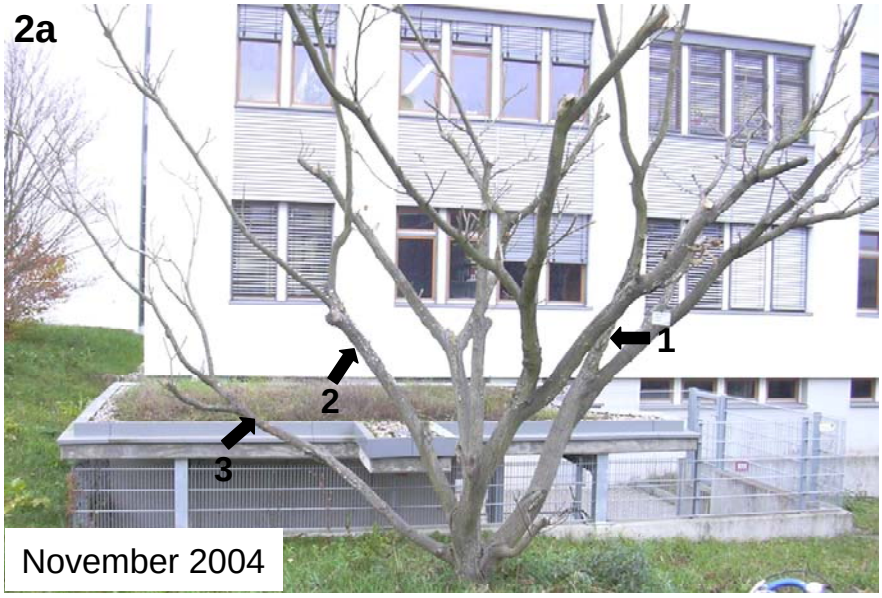
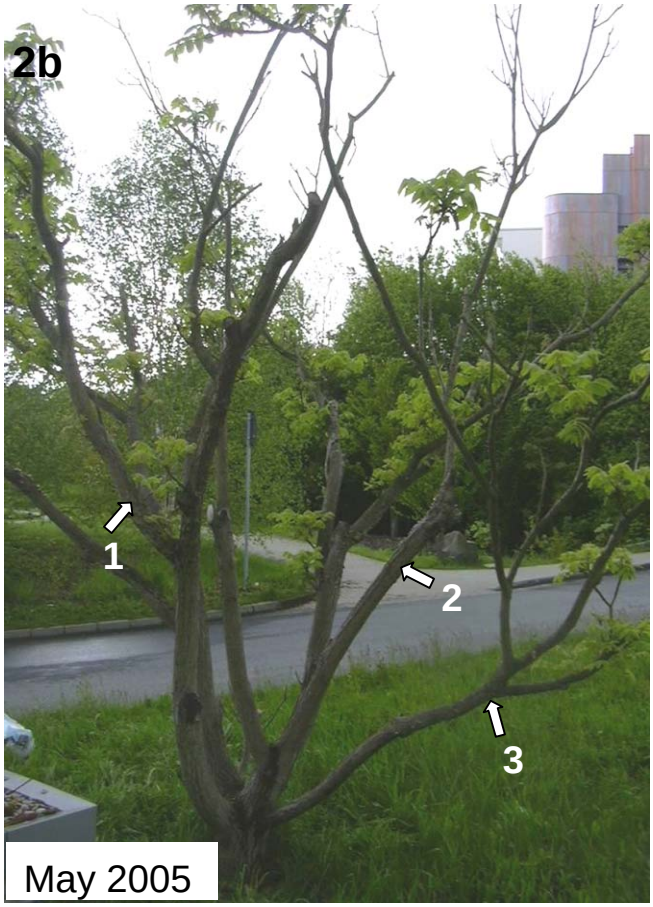


Fig. 1 Formation of numerous white fruiting bodies on the upper sunny side of two large branches of a *Juglans ailantifolia* tree. Enlarged view of branch 1 showing fruiting bodies of

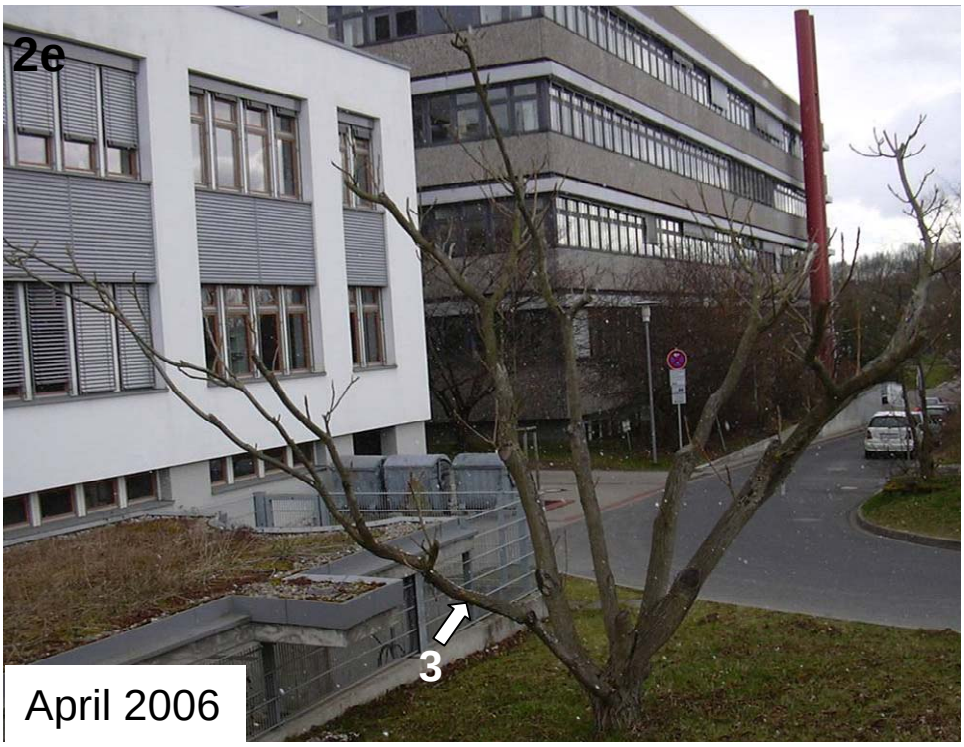
S. commune and *T. hirsuta* **(1a)** and enlarged view of branch 2 showing only fruiting bodies of *S. commune* **(1b)**.

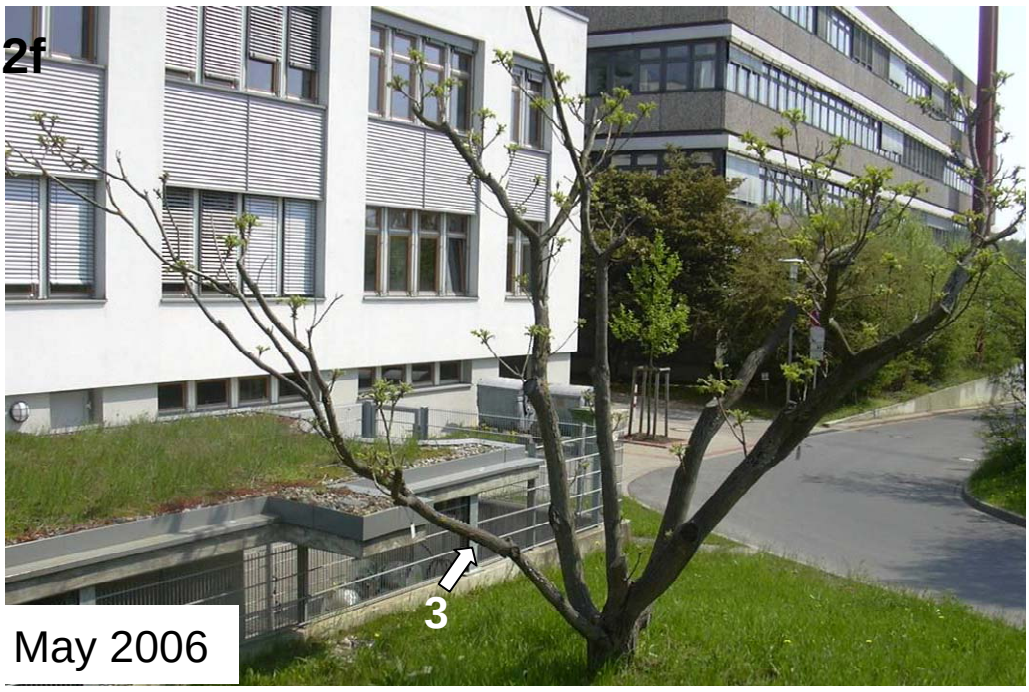


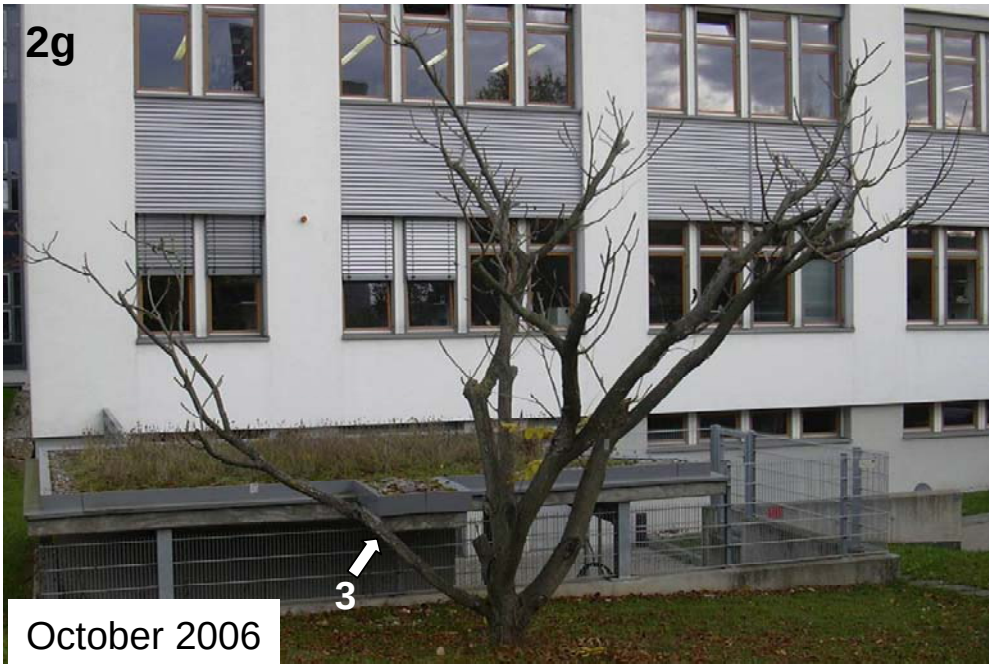

2b
1
2
3
May 2005


2c
2
1
3
June 2005


2d
3
October 2005


2e
3
April 2006


2f
3
May 2006


2g
3
October 2006


2h
3
March 2007

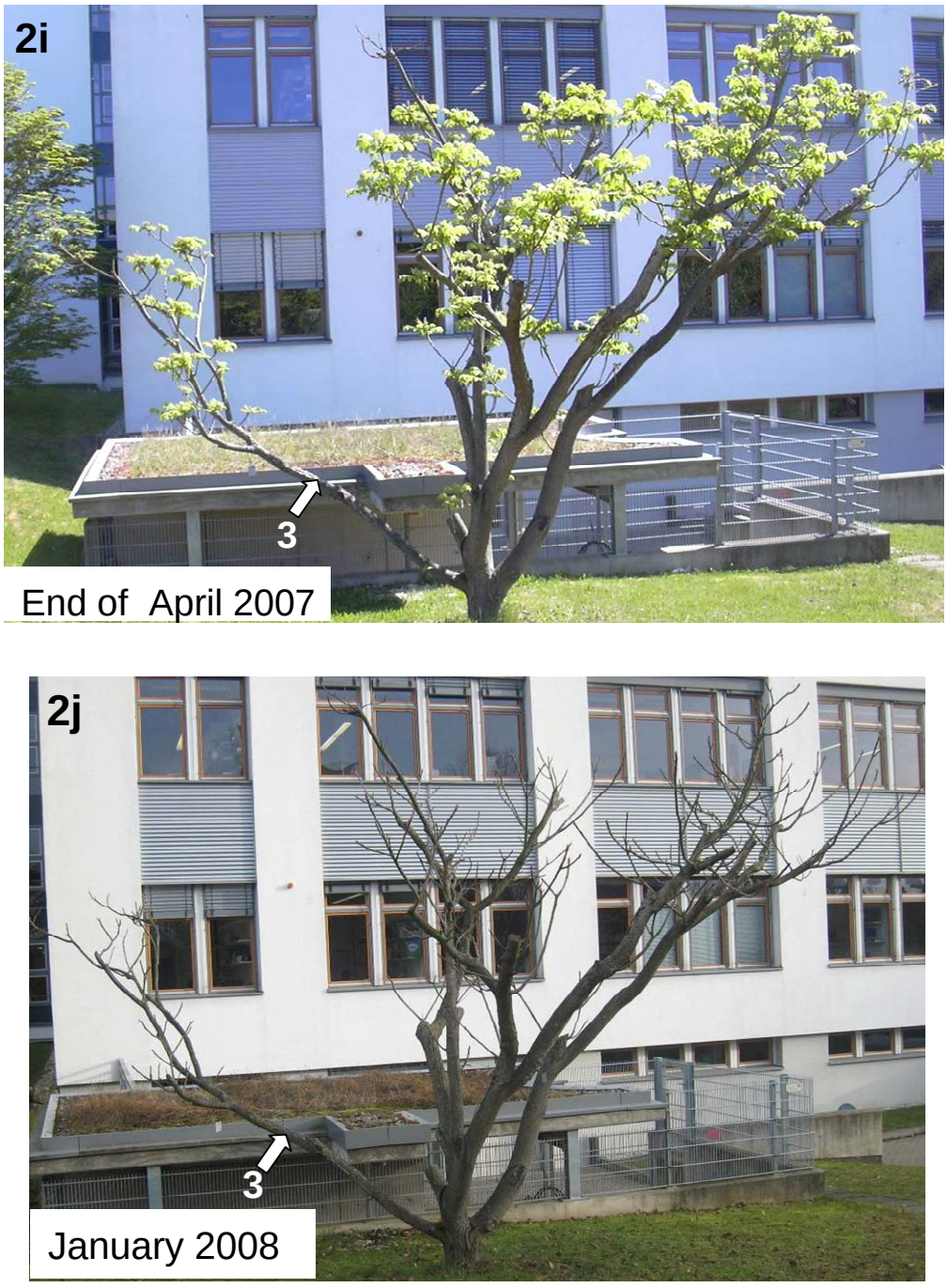


Fig. 2 Living *Juglans ailantifolia* tree infected with *S. commune* in a course of time. Infected branches are marked with numbers 1, 2 and 3. An infection on branch 3 was first noted in autumn 2005.

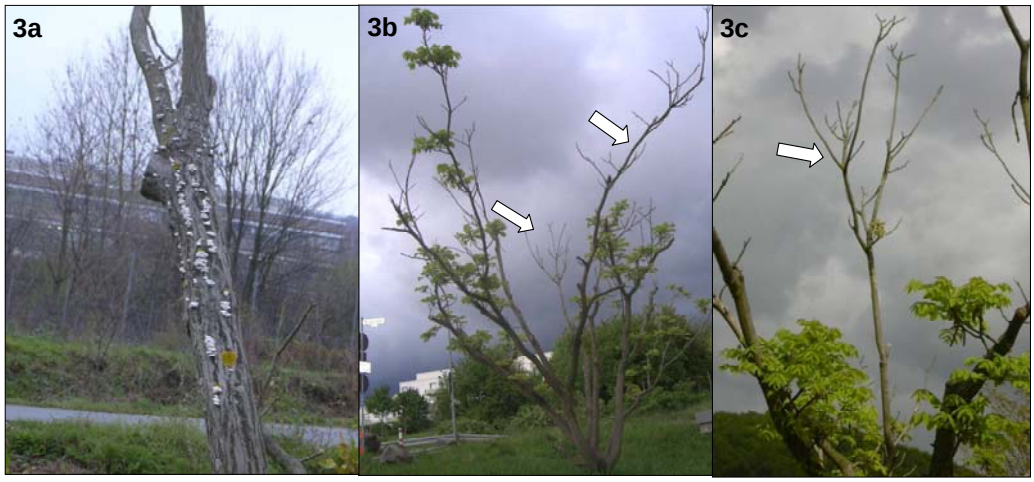


Fig. 3 Symptoms of *S. commune* infection on a *J. ailantifolia* tree. Numerous *S. commune* fruiting bodies were formed on the infected branch of *J. ailantifolia* in November 2004 **(3a)**. Healthy branches showing leaves in May 2005 but not the branches infected with *S. commune* (indicated with arrows in **3b, 3c**).

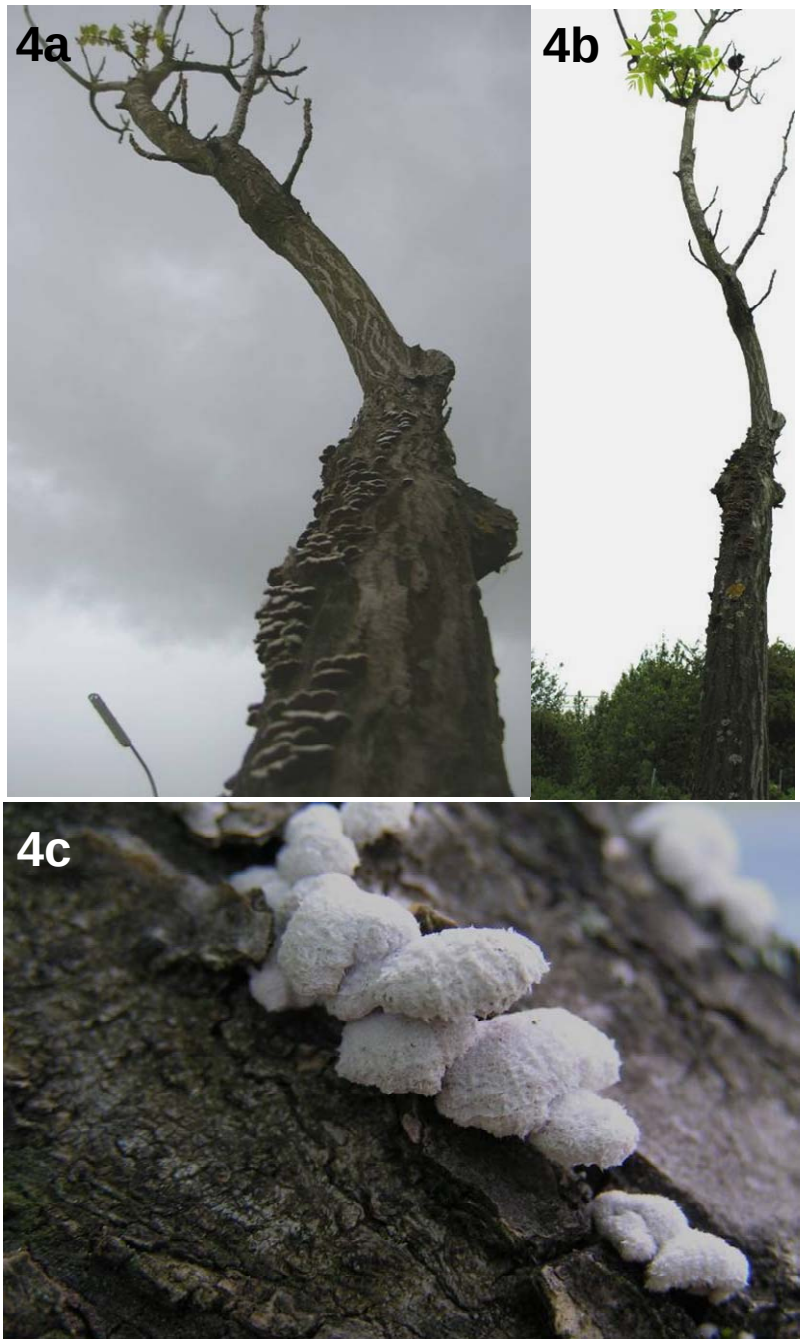


Fig. 4 Branches of *J. ailantifolia* infected with *S. commune*. Branches producing in June 2005 only a few exceptional leaves (compare Fig. 2d) unlike the other yet healthy branches of the tree **(4a, 4b)** and formation of new fruiting bodies on branch 3 (compare Fig. 2e) in September and October 2005 **(4c)**.

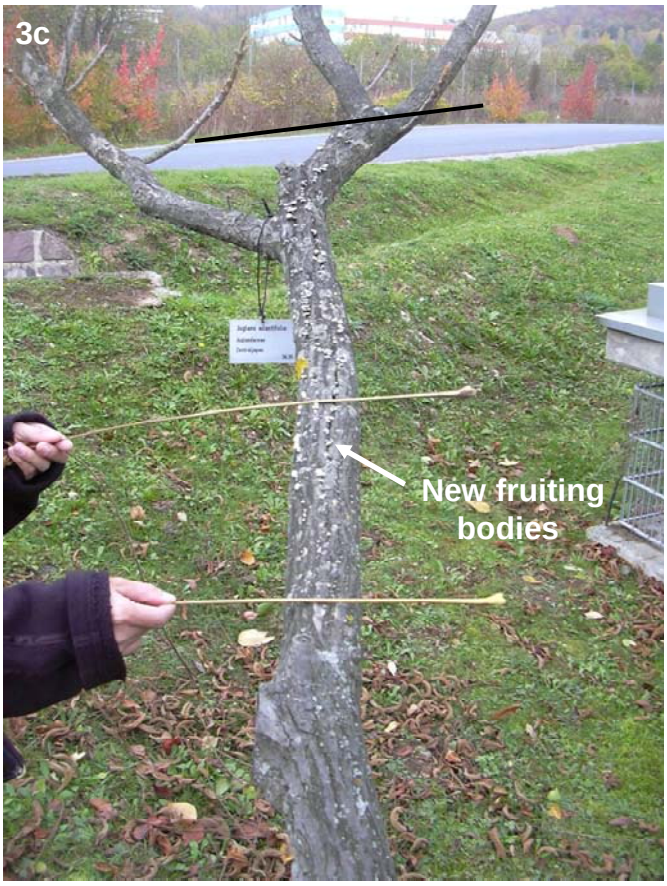


Fig. 5 Spread of infection of *S. commune* in a branch of a living *Juglans ailantifolia* tree (branch 3 in Fig. 2e to 2k) as visualized by the formation of fruiting bodies. The length of the yearly spread between 2005 and 2006 is indicated by the two sticks. The lower points to the maximum spread of fruiting body formation in 2006, upper to the maximum spread of fruiting body formation in 2005.

Fig. 6 Cracks in the bark of branch 1 are used for fruiting bodies of *S. commune* to break to the surface of the branch. It is possible that the cracks originate from sun burning in the hot summer of the year 2004, allowing *S. commune* to enter the branch of *J. ailantifolia*. Photo was taken in June 2005.

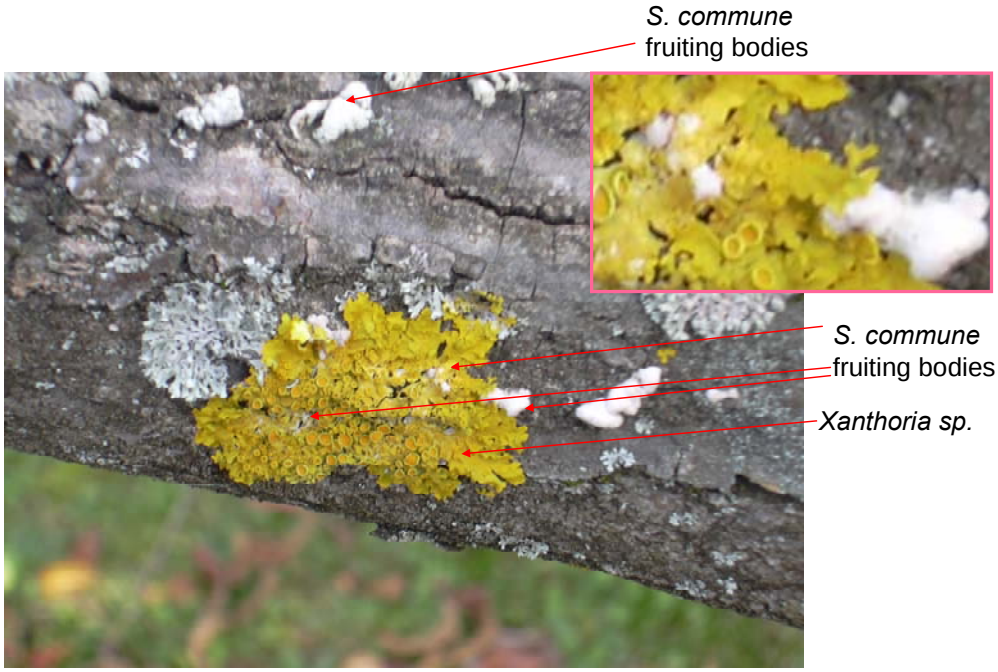


Fig. 7 *S. commune* growing along with lichens (*Xanthoria parietina*) on branch 3 (see Fig. 2h to 2k) of a living *Juglans* tree. Photo was taken on 31st of October 2006.

September 2005

October 2005

April 2006

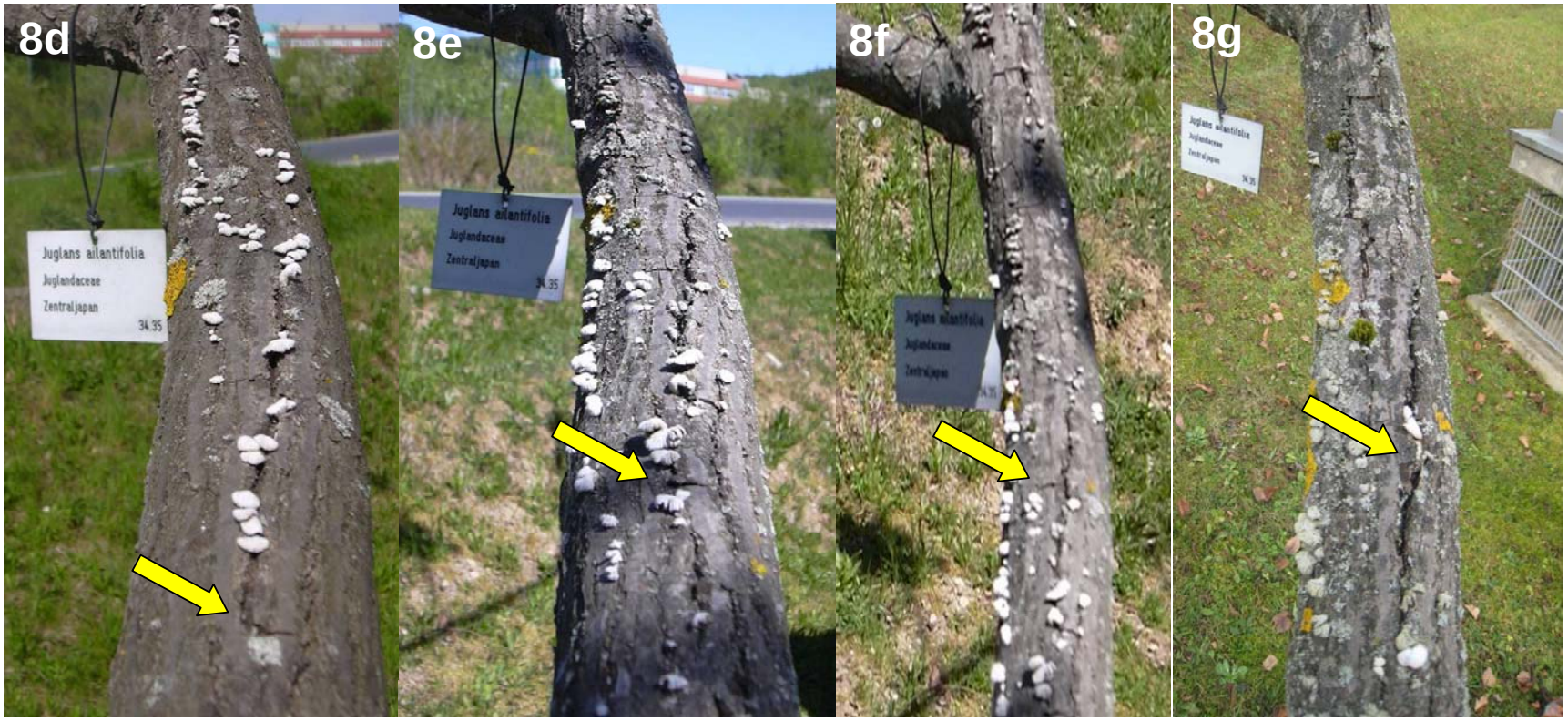


Fig. 8 Weakened branch of a *Juglans ailantifolia* tree (branch 3 in Fig. 2e to 2k) in a course of time due to a *S. commune* infection. Note the severity of the cracks on the branch which is possibly due to the formation of fruiting bodies. Alternatively, the cracks may help the fungus to emerge onto the surface of the branch. Arrows indicate same position on the branch.

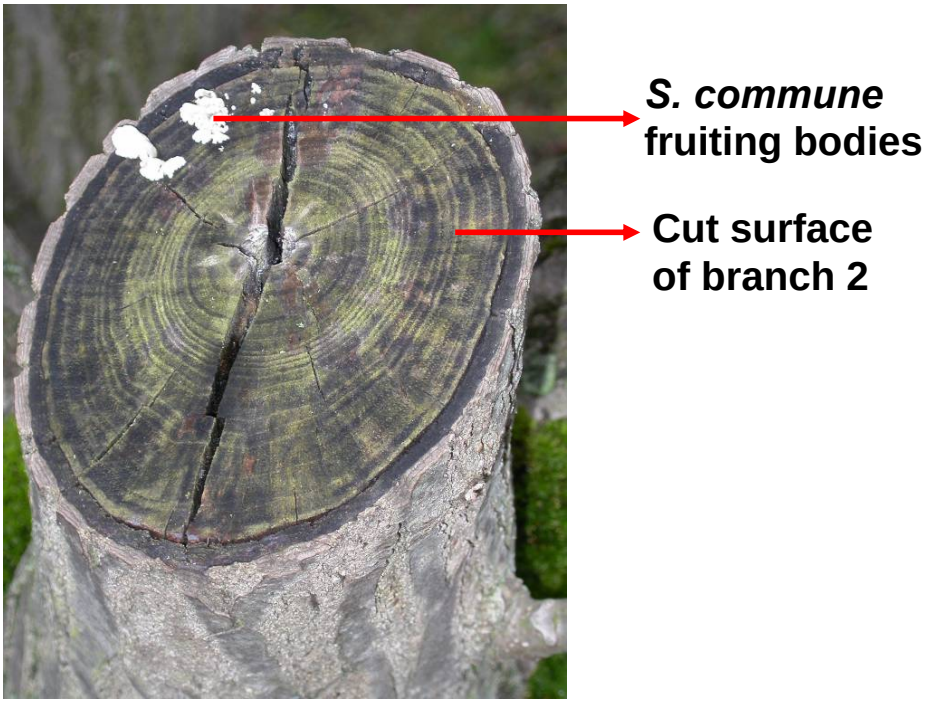


Fig. 9 Formation of new *S. commune* fruiting bodies at the cut surface of the stump of branch number 2 from the living *Juglans* tree shown in Fig. 2a to 2d (photo taken in March 2007).

Fig. 10 Discs from a *S. commune* infected branch of *J. ailantifolia* (branch 2 in Fig. 2a to 2d) cut at places of fruiting body formation. Note the loosened structure and white colour of the wood underneath the fruiting bodies suggesting white rot to happen and note the dark stained portion of the fungal infection demarcating the still healthy wood.

Fig. 11 *S. commune* fruiting bodies growing on fallen branch of *Fagus sylvatica* **(11a)**, and *T. hirsuta* and *S. commune* growing close to each other on a branch of a living *Juglans ailantifolia* tree **(11b)**.

2.4.2 Wood decay test

The newly isolated *S. commune* strains as well as strains from the institutes collection (ScLs 79 and ScLs 48) and fungal collections of Prof. Holdenrieder from Zurich (*S. commune* ScFs 2) and of Prof. Wösten from Utrecht [*S. commune* 4-39 (*MATA41 MATB41,* CBS 341.81), 4-40 (*MATA43 MATB43,* CBS 340.81), 72-3 (*ΔSc3* 4-39, *MATA41 MATB41*), *ΔSc3* 4-40 (*ΔSc3 MATA43 MATB43*), the self compatible homokaryon *S. commune* Acon Bcon fbf (*MATA41mut MATB41mut fbf* CBS 341.81*)* characterized by mutations in the mating type loci *A41* and *B41* and by a defect in gene *fbf* that completely blocks fruiting and leads to a faster growth rate, and *S. commune* dikaryons 4-39 x 4-40 (*MATA41 MATB41* x *MATA43 MATB43*), and *ΔSc3* 4-39 x *ΔSc3* 4-40 (*MATA41 MATB41* x *MATA43 MATB43*) produced by mating from corresponding monokaryons] were tested for growth on *J. ailantifolia* wood blocks. The white rot *P. ostreatus* N001 and *T. hirsuta* ThJa 1 were used as a positive control for growth on wood (Fig. 12 & 13).

The new *S. commune* isolate ScJa 1 from *J. ailantifolia* wood showed a stronger formation of aerial mycelium (Fig. 12d) than the isolate ScFs 1 from beech wood (Fig.12e) and all other *S. commune* strains (Fig. 12f, 12g, 12h) suggesting that this strain can easily attack the wood. However, the overall effect on mass loss over the time did not distinguishd from any of the other tested *S. commune* strains (Fig. 14, Table 1, 2). Most interestingly, the growth pattern of the coisogenic *S. commune* wild type strains 4-39 (*MATA41 MATB41,* CBS 341.81), 4-40 (*MATA43 MATB43,* CBS 340.81) and the respective hydrophobin mutants *ΔSc3* 4-39 and, *ΔSc3* 4-40 and the self-compatible homokaryotic fruiting mutant *S. commune* Acon Bcon fbf (*MATA41 mut MATB41 mut fbf*) strain varied dramatically on the *Juglans* wood blocks. The wildtype strains showed a denser mycelium on the wood blocks whereas the mycelium was very thin in case of the hydrophobin mutants, especially in case of *ΔSc3* 4-39 when comparing it to the wildtype parental strain 4-39 (Fig. 13a, 13b, 13c, 13d, 13e, 13f). Despite the thin mycelial growth of the hydrophobin mutants and the homokaryon Acon Bcon fbf, there was no significant difference ($p \leq 0.05$) in mass loss with little increase over the time for the different samples (Fig. 14, Tables 1 and 2). Generally, mass loss of *Juglans* wood caused by *S. commune* strains varied from 3 to 10%, whereas *T. hirsuta* and *P. ostreatus* strongly decayed *Juglans* resulting in up

to 45% loss of mass (Fig. 14, Table 1). The comparable low mass loss caused by the *S. commune* strains suggests that easy accessible wood compounds might have been degraded rather than that there was an aggressive attack on the lignocellulose. Furthermore, since over the time there was no clearly visible, respectively statistically significant increase in mass loss upon incubation with any of the *S. commune* strains, the variations between single samples (either for a given strain over the time, or for different strains at the same or different time points) have to be considered to reflect statistical variations in the test system. Statistical variations are influenced by the fact that only 2 or 3 wood blocks could be analysed per strain due to the limited total amount of *J. ailantifolia* wood that was available for this study. Furthermore, these wood blocks might have shown variations in quality (e.g. being from the outer or the inner part of the branch they came from, this possibly representing nutrient richer sapwood or nutrient low heart wood).

On the whole, statistically significant values of mass loss were encountered with *S. commune* probes against the non-treated wood (case **a** in Table 2) likely due to easily degradable organic matter in the wood blocks (Fig. 14 and Tables 1 and 2). Furthermore, statistically significant differences were seen when comparing *S. commune* treated wood samples with those of *P. ostreatus* N001 and *T. hirsuta* ThJa 1 treated samples (Fig. 14 and Tables 1 and 2). This is easily be explained by the aggressive wood decay performed by the latter two species. At the end of the incubation after 20 weeks, apart from the light coloured wood blocks decayed by *T. hirsuta* and *P. ostreatus* N001, all the wood samples incubated with *S. commune* closely resembled in look the control samples (Fig. 15).

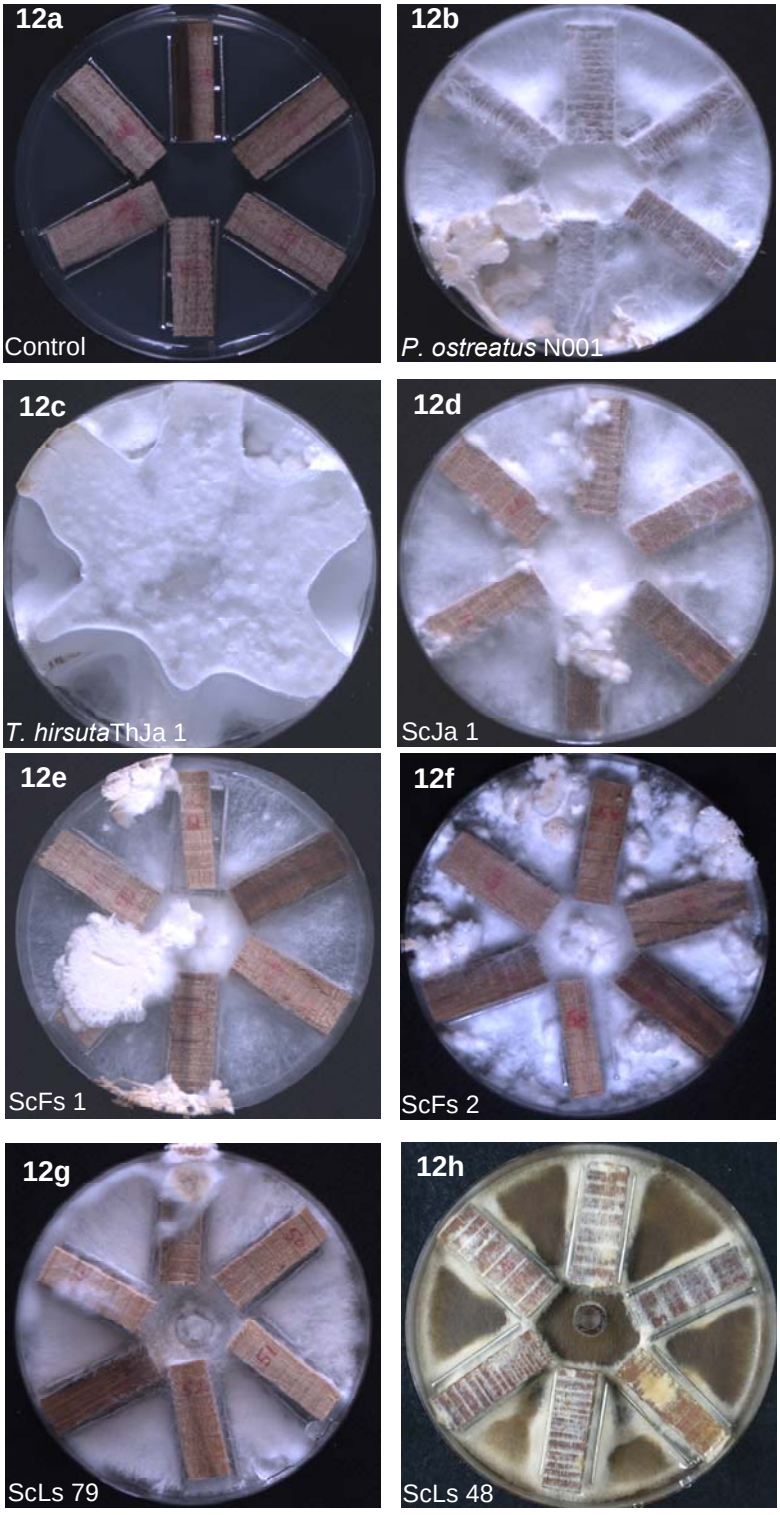


Fig. 12 Growth morphology of basidiomycetes on *J. ailantifolia* wood blocks after 8 weeks of incubation at 25 °C in light. Negative control without fungus **(a)**, *P. ostreatus* N001 **(b)**, *T. hirsuta* ThJa 1 **(c)**, *S. commune* strains ScJa 1 **(d)**, ScFs 1**(e)**, ScFs 2 **(f)**, ScLs 79 **(g)**, and ScLs 48 **(h)**. Note that strains ScJa 1, ScFs 1, ScFs 2 and *P. ostreatus* N001 (Fig 12d, 12e, 12f & 12b) formed primordia and fruiting bodies on the plates.

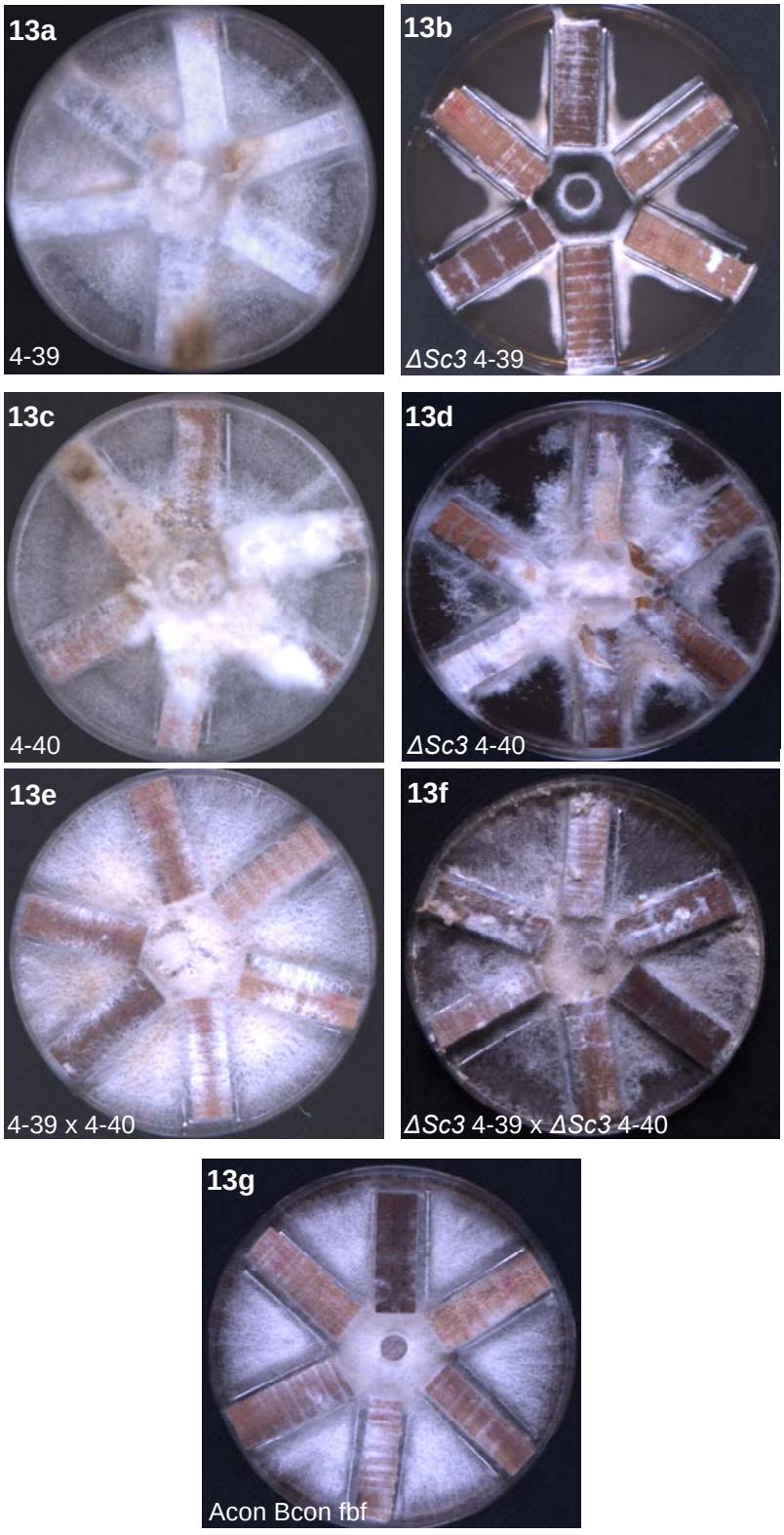


Fig. 13 Growth morphology of wildtype and hydrophobin mutants after 8 weeks of incubation at 25°C in light on *J. ailantifolia* wood blocks. *S. commune* strains 4-39 **(a)**, Δ*Sc3*

4-39 **(b)**,4-40 **(c)**, Δ*Sc3* 4-40 **(d)**, 4-39 x 4-40 **(e)**, Δ*Sc3* 4-39 x Δ*Sc3* 4-40 **(f)**, and Acon Bcon fbf *MATA41mut MATB41mut fbf* **(g)**.

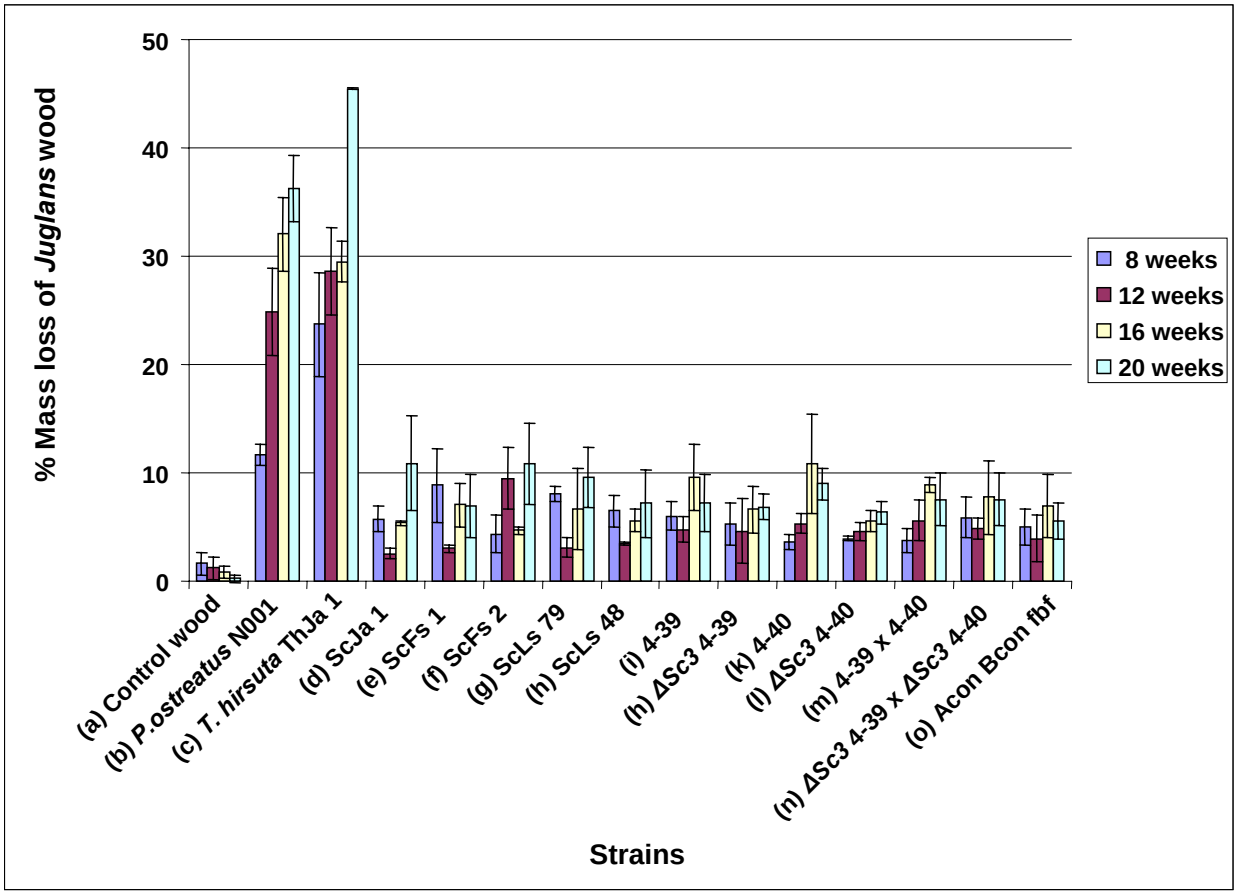


Fig. 14 Decay of *Juglans ailantifolia* wood caused by *S. commune* strains (wild type and hydrophobin mutants) after 8, 12, 16 and 20 weeks of incubation. Values are averages of 3 wood blocks, respectively 2 wood blocks (compare Table 1). **(a)** to **(o)** indicate the labelling used for the statistical analysis presented in Table 2.

Table. 1 Percentage of mass loss values of *Juglans ailantifolia* wood blocks caused by *S. commune* strains after 8, 12, 16 and 20 weeks.

Fungal	***Juglans ailantifolia* decay after**			
Strain	**8 weeks**	**12 weeks**	**16 weeks**	**20 weeks**
Control (wood without fungi)	1.6 ± 1.05	1.20 ± 1.05	0.86 ± 0.58	0.25 ± 0.35
P. ostreatus N001(+)	11.64 ± 1.0	24.84 ± 4.97*	32.05 ± 3.39	36.22 ± 3.04
T. hirsuta ThJa 1(+)	23.69 ± 4.79	28.66 ± 4.02	29.51± 1.92*	45.45 ± 0.09*
ScJa 1	5.75 ± 1.14	2.53 ± 0.49	5.40 ± 0.21	10.89 ± 4.33
ScFs1	8.86 ± 3.39	3.01 ± 0.31	7.07 ± 2.01	6.97 ± 2.93
ScFs 2	4.36 ± 1.69	9.48 ± 2.88*	4.66 ± 0.36*	10.81 ± 3.78
ScLs 79	8.10 ± 0.69	3.09 ± 0.89	6.62 ± 3.76	9.57 ± 2.78
ScLs 48	6.50 ± 1.48	3.48 ± 0.09	5.60 ± 1.08*	7.19 ± 3.13
4-39	6.01 ± 1.28	4.79 ± 1.20	9.64 ± 3.04	7.26 ± 2.66
Δ*Sc3* 4-39	5.30 ± 1.94	4.64 ± 3.03	6.63 ± 2.12	6.86 ± 1.14*
4-40	3.63 ± 0.65*	5.30 ± 0.90	10.83 ± 4.68	8.96 ± 1.51
Δ*Sc3* 4-40	3.95 ± 0.18	4.55 ± 0.86	5.57 ± 0.97	6.35 ± 1.06
4-39 x 4-40	3.71 ± 1.11	5.58 ± 1.88	8.86 ± 0.66	7.53 ± 2.43
Δ*Sc3* 4-39 x Δ*Sc3* 4-40	5.90 ± 1.87	4.86 ± 0.99	7.72 ± 3.43	7.5 ± 2.42
Acon Bcon fbf	4.99 ± 1.70	3.95 ± 2.21	6.89 ± 2.92	5.50 ± 1.68

Values are averages of 3 wood blocks. * indicates averages from two values. (+) *P. ostreatus* N001 and *T. hirsuta* ThJa 1 served as positive controls.

Table 2 Statistical data (calculated by using Duncan multiple range test) indicating the significant differences ($p \leq 0.05$) in the mass loss of *Juglans* wood caused by different fungal strains during different time periods (compare Table 1 and Fig. 14).

Sample number	Fungal strains	*	8 weeks decay different with	12 weeks decay different with	16 weeks decay different with	20 weeks decay different with
1	**ScJa 1**	d	**a**, *b, c*, f, i, k, m	*b, c,* f	*b, c,* k	*b, c*
2	**ScFs 1**	e	**a**, *c,* f, i, k, m	*b, c,* f	**a**, *b, c*	*b, c*
3	**ScFs 2**	f	**a**, *b, c,* e	**a**, *b, c,* d, e, g, h, o	*b, c,* k	*b, c*
4	**ScLs 79**	g	**a**, e, *b, c*	*b, c,* f	**a**, *b, c*	*b, c*
5	**ScLs 48**	h	**a**, *b, c*	*b, c,* f	*b, c*	*b, c*
6	**4-39**	i	*b, c,* e	**a**, *b, c*	**a**, *b, c*	*b, c*
7	***ΔSc3* 4-39**	j	*b, c*	**a**, *b, c*	**a**, *b, c*	*b, c*
8	**4-40**	k	*b, c,* e	**a**, *b, c*	**a**, *b, c*, f	*b, c*
9	**Δ*Sc3* 4-40**	l	*b, c,* e	**a**, *b, c*	*b, c*	*b, c*
10	**4-39 x 4-40**	m	*b, c,* e	**a**, *b, c*	**a,** *b, c*	*b, c*
11	**Δ*Sc3* 4-39 x Δ*Sc3* 4-40**	n	*b, c*	**a**, *b, c*, f	**a**, *b, c*	*b, c*
12	**Acon Bcon fbf**	o	*b, c*	*b, c,* f	**a**, *b, c*	*b, c*
13	**Control wood**	**a**	*b, c*, e, g, h, i	*b, c,* f, i, j, k, l, m, n	*b, c,* e, i, j, k, m, n, o	*b, c*
14	***P. ostreatus* N001**	*b*	**a**, d, f, g, h, i, j, k, l, m, n, o	**a**, d, e, f, g, h, i, j, k, l, m, n, o	**a**, d, e, f, g, h, i, j, k, l, m, n, o	**a**, *c,* d, e, f, g, h, i, j, k, l, m, n, o,
15	***T. hirsuta* ThJa 1**	*c*	**a**, *b,* d, e, f, g, h, i, j, k, l, m, n, o	**a**, *b*, d, e, f, g, h, i, j, k, l, m, n, o	**a**, d, e, f, g, h, i, j, k, l, m, n, o	**a**, *b,* d, e, f, g, h, i, j k, l, m, n, o

(*) For an easy understanding, the fungal strains were indicated by the alphabetical letters.

Fig. 15 *Juglans* wood blocks after the 20 weeks decay test by different *S. commune* strains at 25 °C in continuous light.

2.4.3 Fungal interactions

Since there is the possibility, that growth of *S. commune* in wood in nature is promoted by the presence of other, possibly stronger wood decaying fungi, reactions of *S. commune* strains in dual inoculations on agar BSM medium plates with *T. hirsuta* were observed. Dual inoculations resulted in interactions with deadlock

formation followed by replacement of *S. commune* by *T. hirsuta* over the time (Fig. 16, 17 & 18). *S. commune* strains except the hydrophobin mutant dikaryon *ΔSc3* 4-39 x *ΔSc3* 4-40 formed barrages (formation of dense mycelia between both the fungi) with *T. hirsuta*. There was also pigment formation (coloured zone) at the interaction region after 1 week of incubation compared to the other *S. commune* strains (Fig. 16, 17, 18).

The pigmentation was more prominently dark or broad in case of ScFs2 (Fig. 16a). *S. commune* strain ScFs2 formed numerous primordia/fruiting bodies in the single culture (Fig. 19), whereas there was no primordia formation in presence of *T. hirsuta* (Fig. 16a). Only the *S. commune* dikaryon *ΔSc3* 4-39 x *ΔSc3* 4-40 did not form either a barrage or produced pigmentation (Fig. 18c, 18f). The growth rate of *ΔSc3* 4-39 x *ΔSc3* 4-40 strain was much lower in the dual cultures in the presence of the *T. hirsuta* strain ThJa 1 than in the single culture (Table 3). From this confrontation tests, it appears that *S. commune* is generally weaker than *T. hirsuta*, at least under conditions on BSM agar Petri dishes that offers easily accessible nutrients.

In contrast, in the interaction study on *Juglans* wood blocks, *S. commune* strain ScJa 1 appeared to be the first invader of the *J. ailantifolia* wood whereas at a later stage after 10 days *T. hirsuta* ThJa 1 overgrow the wood and completely replaced the *S. commune* ScJa 1 (Fig. 20c, 20f). The mixed infection of *S. commune* ScJa 1 and *T. hirsuta* ThJa 1 caused a mass loss of up to 46% which was similar to the decay caused by *T. hirsuta* alone whereas *S. commune* ScJa 1 alone reduced in this experiment a mass loss of wood by about 16.51 ± 1.93 % (Fig. 21). The higher decay in the dual infection was probably due to complete replacement of *S. commune* ScJa 1 by *T. hirsuta* ThJa 1 over the time. By visual inspection, the wood blocks after 10 weeks of decay infected with *T. hirsuta* ThJa 1 closely resembled the wood blocks treated with mixed infection of *S. commune* ScJa 1 and *T. hirsuta* ThJa 1 (Fig. 22). Comparison of the wood blocks decayed by *S. commune* ScJa 1 from experiment 1 and 2 revealed that few replicate samples in experiment two appeared whiter but on a whole is not very different from the first experiment (Fig. 23).

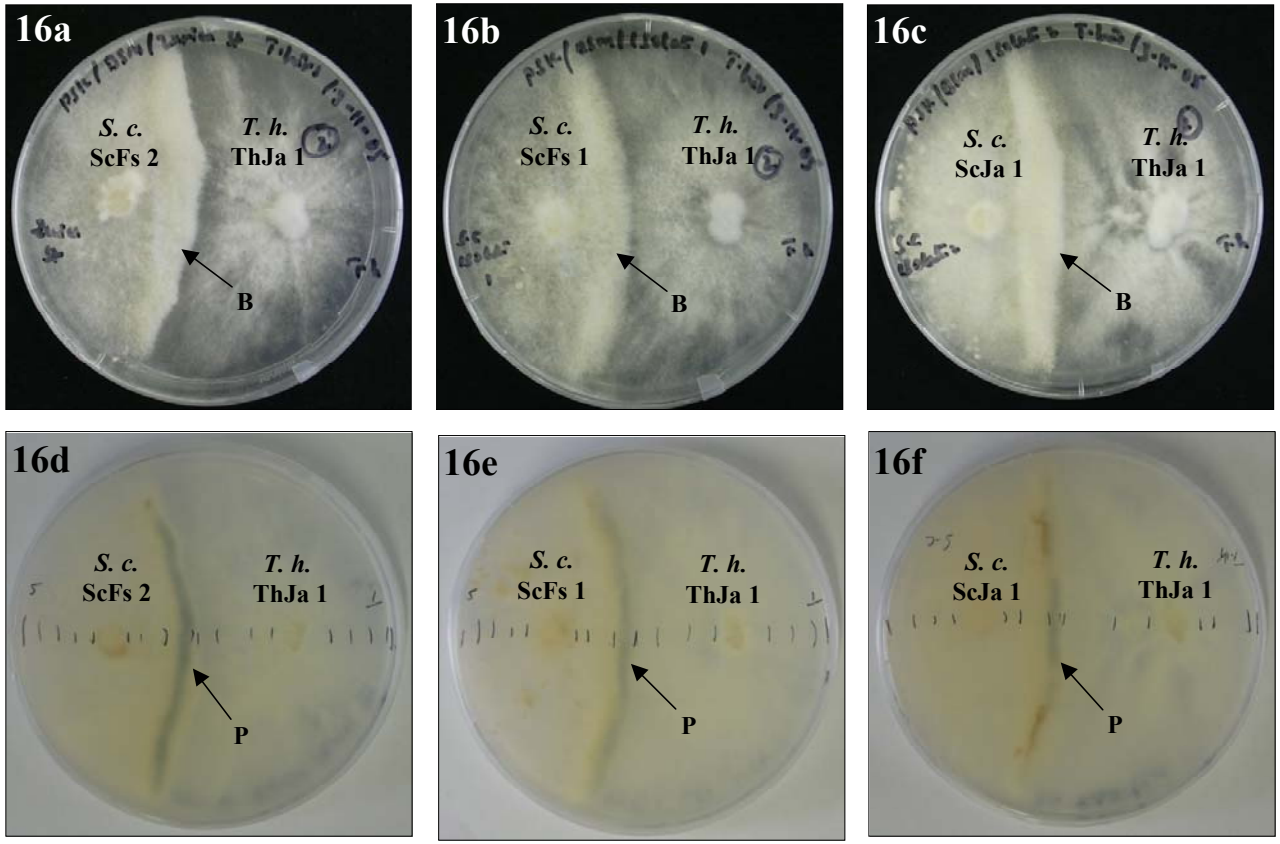


Fig. 16 Interactions between *T. hirsuta* ThJa 1*(T. h*), and *S. commune* ScFs 2, ScFs 1, ScJa 1 (*S. c*) on BSM medium under incubation in constant light at a temperature of 25°C. Barrier formation (B) is followed by the subsequent replacement of *S. commune* ScFs 2, ScFs 1, and ScJa 1 by *T. hirsuta* ThJa 1 (a, b, c). The lower row (d, e, f) shows the plates photographed from the bottom to present the pigmentation (P) occurring at the interaction zone of the two species.

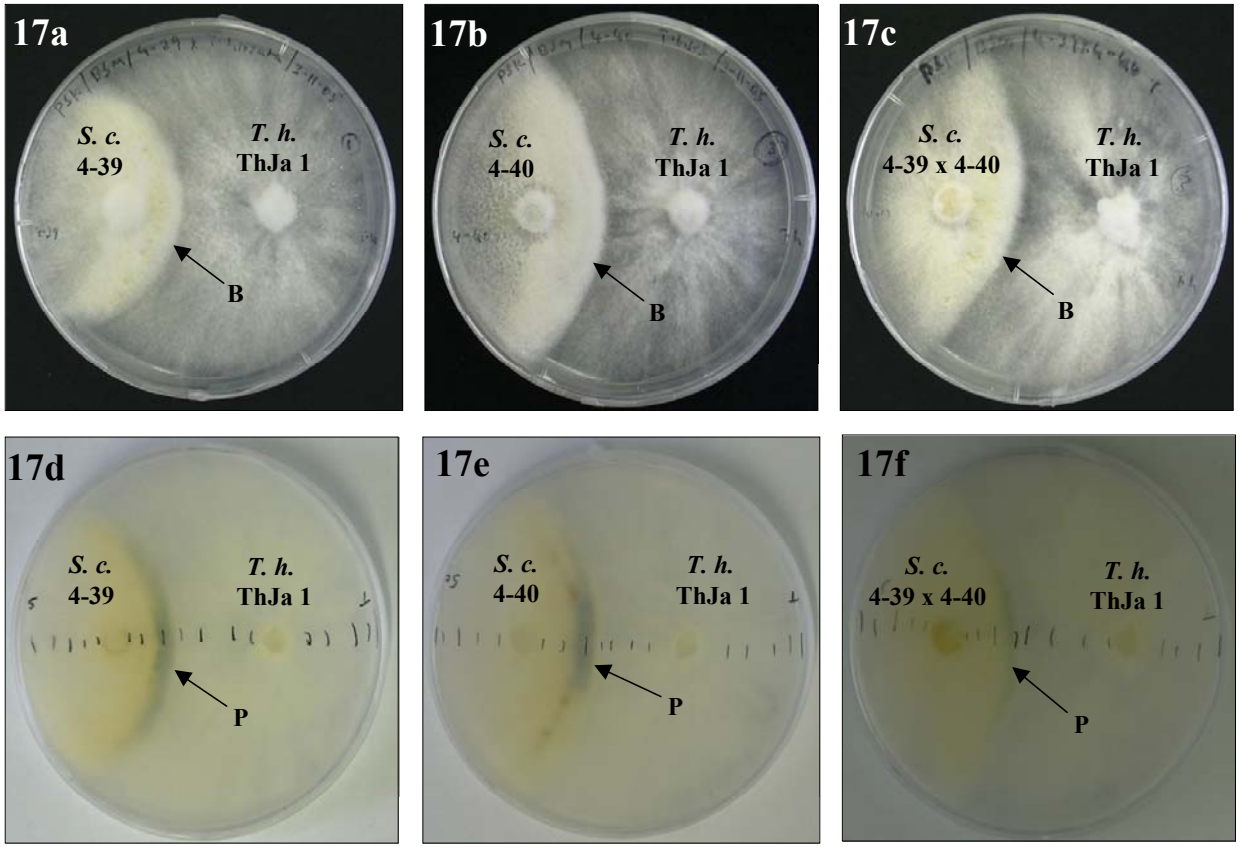


Fig. 17 Interactions between *T. hirsuta* ThJa 1(*T. h*), and *S. commune* (*S. c*) 4-39 (*MATA41 MATB41*), 4-40 (*MATA43 MATB43*) and 4-39 x 4-40. Barrier formation on BSM medium under incubation in constant light at a temperature of 25 °C. Barrier formation (B) is followed by the subsequent replacement of *S. commune* strains by *T. hirsuta* ThJa 1 (a, b, c). The lower row (d, e, f) shows the plates photographed from the bottom to present the pigmentation (P) occurring at the interaction zone of the two species.

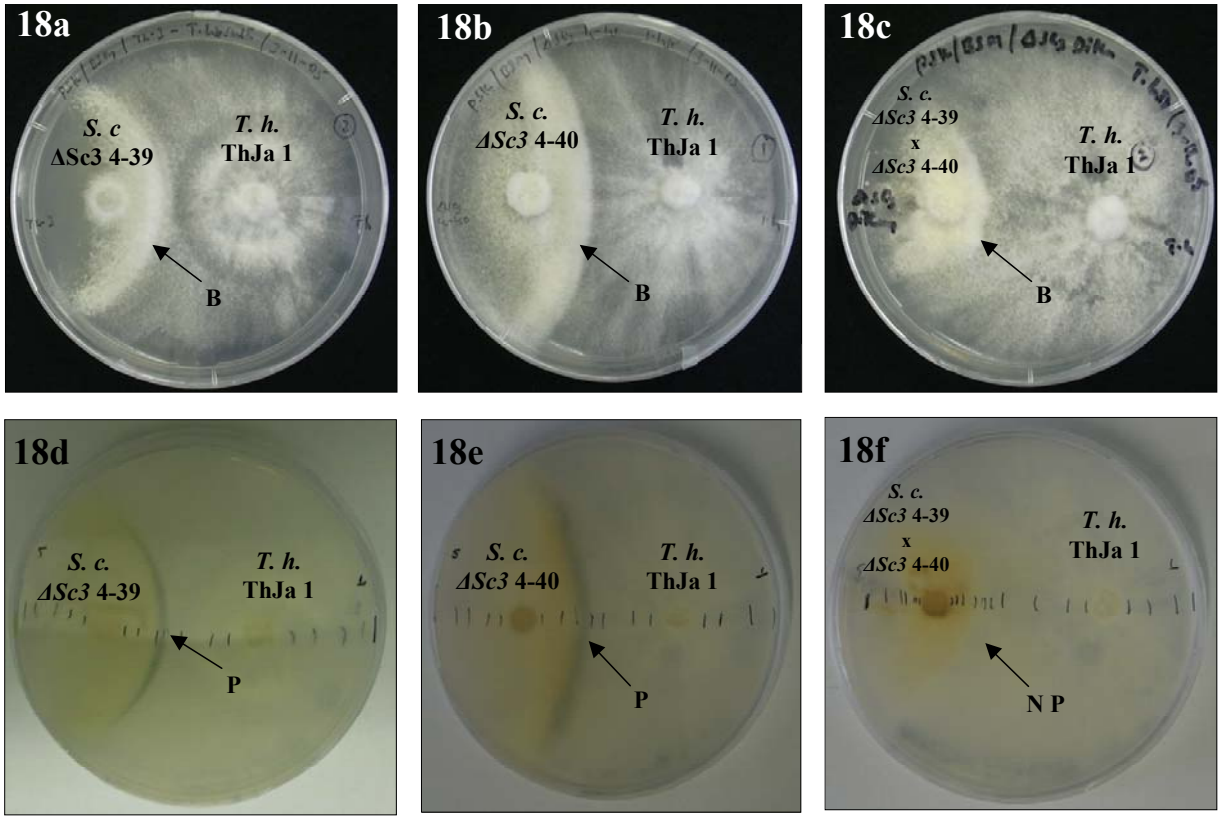


Fig. 18 Interactions between *T. hirsuta* ThJa 1 (*T. h*), and *S. commune* hydrophobin mutants (*S. c*) ΔSc3 4-39 (*MATA41 MATB41*), Δ*Sc3* 4-40 (*MATA43 MATB43*), *ΔSc3* 4-39 x *ΔSc3* 4-40 on BSM medium under incubation in constant light at a temperature of 25°C. Barrier formation (B) is followed by the subsequent replacement of *S. commune* by *T. hirsuta* (a, b, c). The lower row (d, e) shows the plates photographed from the bottom to present the pigmentation (P) at the interaction zone of the two species. Note the insignificant barrier and no pigmentation (NP) formed between *S. commune* strain *ΔSc3* 4-39 x *ΔSc3* 4-40 and *T. hirsuta* ThJa 1(c, f).

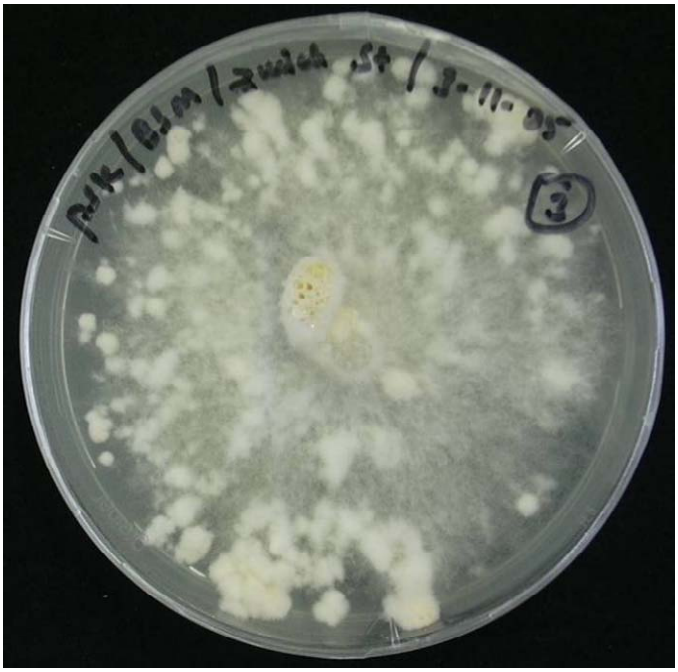


Fig. 19 Numerous mycelial tufts formed (fruiting body primordia) in the single culture of *S. commune* ScFs 2 under continuous light at a temperature of 25 ^{o}C on BSM medium.

Table 3 Effect of opposing colonies of *S. commune* to *T. hirsuta* ThJa 1 with mean extension rate (mm/day) on BSM medium compared with controls, and growth towards and away from opponents incubated under constant light illumination at a temperature of 25°C.

Strains	**Extension of opponent (*S. commune*)**				**Extension of *T. hirsuta* ThJa 1**			
	Towards *T. h.*	Away *T. h.*	Diam. in interaction < control	Towards and away significant difference	Towards *S. c.*	Away *S. c.*	Diam. in interaction < control	Towards and away significant difference
4-39	3.78 ± 1.30	3.83 ± 1.17	ns	ns	5.60 ± 1.34	4.13 ± 1.73	ns	ns
ΔSc3 4-39	3.89 ± 1.53	4.28 ± 1.52	ns	ns	5.00 ± 2.00	4.00 ± 2.00	ns	ns
4-40	4.00 ± 1.40	4.63 ± 1.84	**S**	ns	4.00 ± 1.67	4.25 ± 2.05	**S**	ns
ΔSc3 4-40	3.75 ± 1.03	4.33 ± 1.00	ns	ns	4.00 ± 2.30	5.60 ± 2.34	**S**	ns
4-39 x 4-40	4.67 ± 1.36	4.22 ± 1.09	ns	ns	4.38 ± 2.06	3.71 ± 1.80	**S**	ns
ΔSc3 4-39 x *ΔSc3* 4-40	2.00 ± 1.06	1.89 ± 1.05	**S**	ns	6.22 ± 2.53	3.63 ± 1.77	ns	**S**
ScFs 2	4. 00 ± 1.65	4.22 ± 0.66	ns	ns	5.13 ± 2.79	3.33 ± 1.94	ns	ns
ScFs 1	5.33 ± 1.63	3.67 ± 1.80	ns	ns	5.00 ± 2.23	3.29 ± 2.36	ns	ns
ScJa 1	6.00 ± 2.54	4.25 ± 2.25	ns	ns	4.25 ± 4.20	4.14 ± 2.54	**S**	ns

ns indicate non-significance and **S** significant ($p \leq 0.05$) stimulation

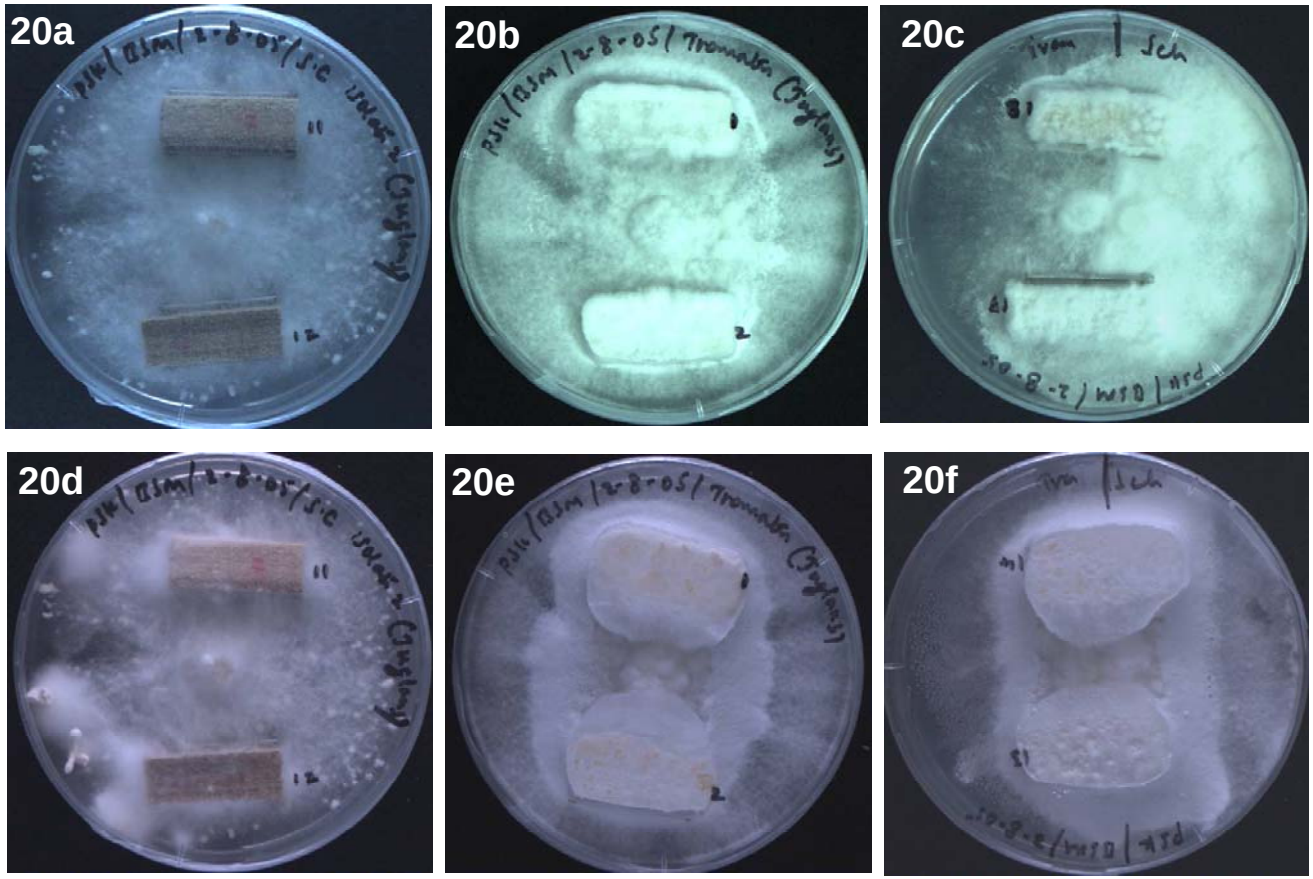


Fig. 20 Wood decay test of *Juglans* with mixed infections. *S. commune* ScJa 1 (a, d), *T. hirsuta* ThJa 1 (b, e) and mixed culture of *S. commune* ScJa 1 and *T. hirsuta* ThJa 1 (c, f). Pictures shown are 3 weeks and 7 weeks old on upper and lower row, respectively. Incubated at 25 °C in continuous light on BSM medium.

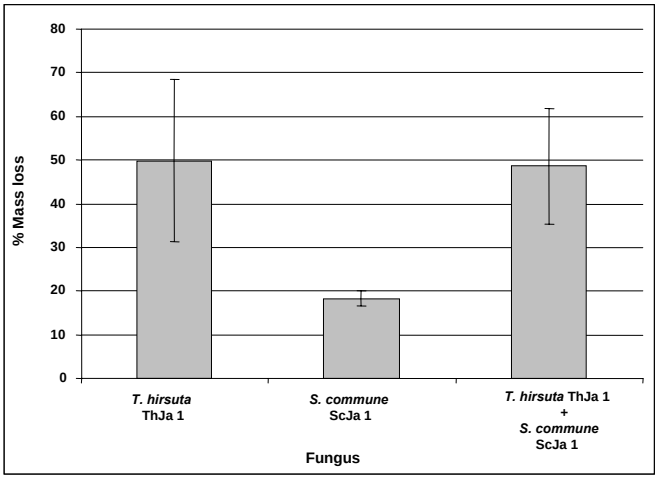


Fig. 21 Decay of *Juglans ailantifolia* wood caused by *T. hirsuta* ThJa 1, *S. commune* strain ScJa 1 and mixed infection of ThJa 1 and ScJa 1 after 10 weeks of incubation. Values are averages of 6 wood blocks.

Fig. 22 *Juglans* wood blocks after 10 weeks decay test by *T. hirsuta* ThJa 1, *S. commune* ScJa 1 and mixed infection of *S. commune* ScJa 1 + *T. hirsuta* ThJa 1 strains at 25°C in continuous light.

Fig. 23 Comparison of *Juglans* wood blocks after decay by *S. commune* ScJa 1 from experiment 1 (upper row) and experiment 2 (lower row).

2.5 Discussion

The cosmopolitan fungus *S. commune* grows in very diverse environmental conditions on various types of dead wood (Cooke 1961, Schmidt & Liese 1980, Raper & Krongelb 1958, James & Vilgalys 2001, James et al. 1999). It has been described previously as a wound parasite and in a few cases it was recorded as an opportunistic pathogen (Adaskaveg 1993, Shigo 1967, Cooke 1961, Dai 2005, Latham 1970). The previous studies are supported by our observations when *S. commune* fruiting bodies were found growing on a fallen beech branch (Fig. 11a) and in the other case on the living *Juglans* tree that was weakened by the movement of the tree from one place to another (Fig. 11b). *S. commune* as a pathogen is reported to cause chlorotic, necrotic symptoms of *Prunus* leaves, wood discoloration and sap wood rot of *Prunus* (Dai 2005). On the other hand it was also reported to occur as an endophyte on teak leaves (Chareprasert et al. 2006). However, wood decay tests in the laboratory with *Schizophyllum* previously reported on *pine, beech* and *picea* wood samples caused a mass loss only of up to 5%. Hong (1982) reported a mass loss of *Hevea brasiliensis* (rubber tree) by *Schizophyllum* as high as 47.7%. No wood decay tests were

performed previously in the laboratory in the combination of *Schizophyllum* and *Juglans* wood. In our study, the mass loss of *Juglans* wood caused by *Schizophyllum* strains varied from 3 to 10% in 20 weeks incubation time (Fig. 14). *Schizophyllum* strain ScJa 1 originally isolated from the same *Juglans* tree caused in the first experiment mass loss of 10.89 ± 4.33 % being highest of all the *Schizophyllum* strains used in this study. Analysis of the values over the time in this series of experiments however did not reveal much statistical differences in the ability of *S. commune* strains to degrade wood. Thereby, the *Sc3* hydrophobin mutant's Δ*Sc3* 4-39, Δ*Sc3* 4-40 and Δ*Sc3* 4-39 x Δ*Sc3* 4-40 were able to cause mass loss of *Juglans* to a similar level than the wild type strains in a range from 4 to 7%, although there was a morphological difference in the growth pattern of the *S. commune* wild type and *Sc3* hydrophobin mutants while growing on *Juglans* wood blocks. The *T. hirsuta* strain ThJa 1 which was isolated from the same branch closely growing to *S. commune* ScJa 1 in contrast decayed *Juglans* strongly with mass losses of up to 45%. In a second set of experiments set up to test dual infections on wood (Fig. 21), the mass loss by *S. commune* ScJa 1 was slightly higher with 16 ± 5.09 % supporting that the strains had indeed a low level activity of wood decay. This suggestion is in accordance with the observation on *J. ailantifolia* branches where white rot occurred directly under places in the bark where *S. commune* fruiting bodies broke through (Fig. 10).

Interactions between different fungi can be seen not only in cultures as demonstrated in this study (Fig. 15 to 17) but also when the fungi are growing in decaying wood in nature (Iakovlev and Stenlid 2000). The competitive interactions in the agar based systems in many instances were found to correlate with that of the natural systems (Boddy & Rayner 1983, Holmer et al. 1997, White and Boddy 1992, Woods et al. 2005). Growing the strains *S. commune* ScJa 1 and *T. hirsuta* ThJa 1 obtained from the same branch of a living *Juglans* tree resulted in barrier formation and pigmentation on the agar system and with overgrowth of *S. commune* by *T. hirsuta* (Fig. 15c, 15f). Like wise on wood where *S. commune* was first active, in the end *T. hirsuta* overgrow the other species (Fig. 20). These observations suggest that also in nature, *S. commune* might be overtaken by *T. hirsuta*.

The mechanisms involving the confrontive interactions between two different species might affect the synthesis and release of hydrophobic metabolites from the hyphae and their conversion to free radicals and polymers in the presence of enzymes (Iakovlev and Stenlid 2000). The formation of barrages is often associated with the ability of the fungus to resist the invasion by the opponent (Boddy 2000, Iakovlev & Stenlid 2000, Wald et al 2004). In our study, all the *S. commune* strains except the *Sc3* mutant dikaryon (*ΔSc3* 4-39 x *ΔSc3* 4-40) have shown an ability to produce a barrage with pigmentation when confronted to *T. hirsuta*. Since also the *Sc3* hydrophobin mutant monokaryon strains 72-3 (*ΔSc3 4-39*) and *ΔSc3 4-40* were able to form a clear barrier against *T. hirsuta* ThJa 1, a principle requirement for the hydrophobins forming hydrophobic layers on the surface of hyphae (Wösten 2001, Walser 2003) is not obvious, although the mutant dikaryon behaved differently. In a recent study analysing interactions between *Phlebiopsis gigantea* and *Heterobasidion parviporum* it was found by molecular approaches that a specific hydrophobin gene of *H. parviporum* was up-regulated whereas other two hydrophobin genes were down-regulated at the interaction zone during the nonself-competitive interaction between *P. gigantea* and *H. parviporum* (Adomas et al. 2006). It was also shown that hydrophobins are involved in cell wall assembly and that hydrophobin monomers can act as toxins or elicitors (Talbot 2001).

Although from our results it is evident that hydrophobins are not principally necessary for the defense reactions in *S. commune*, it is not clear why the SC3 hydrophobin mutant dikaryon did not produce pigmentation and barrier formation towards *T. hirsuta* ThJa 1. It has been shown that some hydrophobins have lectin (a carbohydrate-binding protein) specificities that might be involved in interaction with the exposed environments (Wösten 2001, Walser et al. 2003). In particular, lectin activity has been shown for hydrophobin SC4 (van Wetter et al. 2000a). Since in contrast to the wildtype dikaryon, this hydrophobin is the only one being expressed in the *Sc3* hydrophobin mutant dikaryon (van Wetter et al. 2000a, Wösten 2001), it is possible that in the case of lack of gene *Sc3* the dikaryon-specific SC4 hydrophobin might have a different interaction process with *T. hirsuta* Thja 1. SC3 and SC4 are known to be able to replace each other in function, but not completely. For example transformed *ΔSc3* monokaryons expressing SC4 are not as water-repellent as the co-

isogenic monokaryon expressing the SC3 hydrophobin (van Wetter et al. 2000a). Hydrophobins help to secrete proteins from the cells (Talbot et al. 1996) and to retain proteins in the cell walls (Talbot et al. 1996, van Wetter et al. 2000b). If one or both functions are impaired in the case of *Sc3* knockout dikaryon, enzymes or metabolites for production of pigments might be missing providing the outcome in the interaction between *S. commune* and *T. hirsuta* from the first organisms. However, this would not explain the different reaction between *ΔSc3* monokaryons and the *ΔSc3* dikaryon. Likely, the interaction process between *S. commune* and *T. hirsuta* is much more complex as can be foreseen at the current time.

2.6 References

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Chapter 3

Wood decay by *Schizophyllum commune*

3. Wood decay by *Schizophyllum commune*

3.1 Abstract

Wood decay tests with beech, birch and pine wood were performed to test the decay ability of *Schizophyllum commune*. In wood block tests, the maximum decay caused by *S. commune* strains was 4-5% whilst the control strains *P. ostreatus* and *T. versicolor* showed mass loss up to 30 and 50%, respectively. Overall, the mass loss caused by different *S. commune* strains including the SC3 hydrophobin and SC15 mutants, regardless of being a monokaryon or a dikaryon, were not significantly ($p<0.05$) different from each other. In saw dust decay tests of beech, birch and pine, the decay varied from 9 to 16% amongst the *S. commune* strains; *P. ostreatus* and *T. versicolor* decayed 20 to 30% and 30 to 40%, respectively of the wood samples. *S. commune* strains decolorized the pine saw dust which was not observed in case of *P. ostreatus* and *T. versicolor*. Regardless lack of visible decay, the strength of wood was negatively affected by *S. commune*. Maximum strength losses caused by *S. commune* strains in beech, birch and pine wood blocks were 21, 52 and 35%, respectively. Amongst the *S. commune* strains, there were no significant differences in the strength losses of wood. *P. ostreatus* N001 caused 36, 123 and 188% strength loss of beech, birch and pine wood, respectively, whereas *T. versicolor* reduced the strength of beech, birch and pine wood by 43, 34 and 94%, respectively.

3.2 Introduction

Schizophyllum commune as one of the most widely distributed wood inhabiting basidiomycetes is usually considered to be a white rot fungus. However, wood decay tests in the laboratory in most instances did not result in considerable weight losses (Hegarty et al. 1987, Nsolomo et al. 2000, Humar et al. 2001, 2002, Schirp et al. 2003). Schmidt and Liese (1980), Nilsson and Daniel (1983) and Leithoff and Peek (2001) measured between 1 to 6% weight loss for different strains and concluded the fungus is a rather weak wood-destroyer. Similar results were obtained when growing *S. commune* strains on *J. ailantifolia* wood (Chapter 2 of this thesis). In rare cases, mass losses of nearly 50% were observed, mainly due to lignin degradation and to a low degree due to cellulose degradation (Hong 1982). Although there might be no or little weight loss in wood decay tests, toughness of wood can be negatively affected. 32% strength loss has been measured (Schirp et al. 2003). In flake board tests with *S. commune*, weight loss of 9.6% occurred together with strength loss (67.5% modulus of rupture loss; Hadi et al. 1995). A monokaryon of *S. commune* was shown to some extent to demethylate lignin. This was not due to production of laccases, peroxidases or ligninases (Trojanowski et al. 1986). Poor lignin degradation in another study was thought to result from the inability of the fungus to solubilize lignin (Boyle et al. 1992). On wheat straw, some tropical isolates were shown to degrade lignin (up to 15%) and to cause simultaneous mass loss of up to 23.5%. Another strain showed even higher mass loss (26.7%) but no lignin decay (Capelari and Zadrazil 1997). Lignin in olive pomace was efficiently degraded by a *S. commune* isolate (up to 52.7% of lignin breakdown). During pomace degradation, high laccase activities were recorded (Haddadin et al. 2002). Many *S. commune* strains have xylanase and cellulase activities (Schmidt and Liese 1980, Clarke and Yaguchi 1986, Hegarty et al. 1987, Bray and Clarke 1995, Haltrich et al. 1995, Thygesen et al. 2003). Most of them have also laccase and peroxidase activities (Schmidt and Liese 1980). de Vries et al. (1986) described laccase activity specific to the dikaryotic state of *S. commune* whilst in the parental monokaryons, they could not detect this activity. Phenoloxidase activities were not found in other studies, also not upon treatment with phenolic inducers (Boyle et al. 1992, Nsolomo et al. 2000). By the well established xylanase and cellulase activities of the fungus, it has been suggested that the main role in nature is to recycle carbon by breaking down cellulose and xylans in fallen wood (Raper and

Fowler 2004). In conclusion, the ability of *S. commune* to degrade wood remains to be a puzzle.

S. commune is used as a model fungus to study mating types and fungal development in basidiomycetes (Raper and Fowler 2004). Well known is the discovery of hydrophobins in this fungus, small-secreted fungal proteins of about 100 to 140 amino acids with 8 conserved cysteine residues. Upon secretion, hydrophobins self-assemble into amphipathic stable films that cover fungal cells and make their surfaces hydrophobic. Therefore, these films enable fungal structures to grow into the air and protect them from adverse environmental conditions. Due to the hydrophobin coating, aerial hyphae are repellent towards water. Within mushrooms, hydrophobin films coat the air channels and prevent them from filling with water. In total, four different hydrophobins have been described in *S. commune* (SC1, SC3, SC4 and SC6). SC3, the best characterized hydrophobin, is expressed in the vegetative mycelium of monokaryons and dikaryons. The other three, SC1, SC4 and SC6, are dikaryon specific. SC4 is low expressed in the mycelium and well in mushroom tissues. SC1 and SC6 are specific to fruiting stages (Wösten 2001, Walser et al. 2003). Another important secreted protein, SC15 (not a hydrophobin), mediates formation of aerial hyphae and attachment in the absence of the SC3 hydrophobin by making the aerial hyphae hydrophobic (Lugones et al. 2004).

So far, it was not known whether hydrophobins participate also in wood colonization and pathogenicity of *S. commune* and in degradation of lignin as a hydrophobic component (Wösten et al. 1994). Is it necessary that the hyphae have a hydrophobic surface when growing in wood? First tests of SC3 hydrophobin mutants on *J. ailantifolia* wood gave no indications for a requirement of this small hydrophobic proteins (see chapter 2 of this thesis). In this chapter, the participation of SC3 and SC15 proteins in the decay process and their affect on the strength of beech, birch and pine wood are discussed by making use of knockout mutants of *S. commune* (van Wetter et al. 1996, Lugones et al. 2004, de Jong 2006) that lack either the *Sc3* gene, the *Sc15* gene or both genes. Monokaryons lacking the *Sc15* gene or both genes are characterized by a strong decrease in formation of aerial hyphae and surface hydrophobicity (Wösten et al. 1994, van Wetter et al. 1996) whereas monokaryons

lacking the *Sc15* gene behave like the wildtype (Lugones et al. 2004). Most interesting are the double mutants. They form no aerial mycelium and are fully hydrophilic (Lugones et al. 2004).

3.3 Materials and methods

3.3.1 Fungal strains

The co-isogenic *S. commune* monokaryons 4-39 (*MATA41 MATB41,* CBS 341.81), 4-40 (*MATA43 MATB43*, CBS 340.81), their corresponding *Sc3* hydrophobin mutants 72-3 (Δ*Sc3 MATA41 MATB41),* Δ*Sc3* 4-40 (Δ*Sc3 MATA43 MATB43),* and the corresponding *Sc15* protein mutants Δ*Sc15 4-39 (*Δ*Sc15 MATA41 MATB41),* Δ*Sc15 4-40 (*Δ*Sc15 MATA43 MATB43)* and the *Sc3Sc15* double mutant Δ*Sc3*Δ*Sc15 (MATA41 MATB43)* and Δ*Sc3*Δ*Sc15 (MATA43 MATB41)* were kindly provided by Prof. Wösten, Utrecht, The Netherlands. Dikaryotic *S. commune* strains 4-39 x 4-40 (*MATA41 MATB41* x *MATA43 MATB43*), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (*MATA41 MATB41* x *MATA43 MATB43*), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 *(MATA41 MATB41* x *MATA43 MATB43)*, Δ*Sc3*Δ*Sc15 MATA41B43* x Δ*Sc3*Δ*Sc15 MATA43B41* were produced by mating the corresponding monokaryons on *S. commune* minimal medium (Dons et al. 1979). *Trametes versicolor* strain 6 originated from the institute's collection. *Pleurotus ostreatus* N001 was kindly provided by Prof. Ramirez, Universida Publica de Navarra, Pamplona, Spain.

3.3.2 Growth conditions

All strains were grown at 25 ^{o}C in continuous light. *S. commune* strains were cultivated on *S. commune* minimal medium (20 g glucose, 1.5 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ x $7H_2O$, 1 g yeast extract, 0.12 mg thiamine-HCl, 0.1 mg pyridoxine HCl, 0.005 mg biotin, 0.2 mg $CuSO_4$ x $5H_2O$, 0.08 mg $MnCl_2$ x $4H_2O$, 0.4 mg cobaltous chloride hexahydrate, 1.2 mg calcium nitrate tetrahydrate per 1 liter H_2O; Dons et al. 1979), *T. versicolor* 6 on BSM [Basidiomycete standard medium; 5 g glucose monohydrate, 0.65 g L-asparagine 1 g KH_2PO_4, 0.5 g $MgSO_4$ x $7H_2O$, 0.5 g KCl, 0.5 g yeast extract, 10 g agar, 50 ml stock solution I (0.2 g $FeSO_4$ x $7H_20$ per liter), 50ml stock solution II (0.16 g $Mn(CH_3COO)_2$ x $4H_2O$, 0.04 g $Zn(NO_3)_2$ x $4H_2O$, 1 g $Ca(NO_3)_2$ x $4H_2O$, 0.06 g $CuSO_4$ x $5H_2O$) per 1 liter H_2O, pH 4.5;

Hüttermann and Volger 1973] and *P. ostreatus* on SMY medium (10 g of sucrose, 10 g of malt extract, 4 g of yeast extract, per 1 liter H_2O; pH 5.6; Peñas et al. 2002).

3.3.3 Wood decay tests

3.3.3.1 Wood block test

Three types of wood – quality selected beech, birch and pine blocks of 10 cm x 1 cm x 0.5 cm (longitudinal to the axis x tangential to the axis x radial to the axis) were used. From each type of wood, 8 replicate wood blocks were used for each harvest per each fungal strain. The wood blocks were dried in an oven at 60^{o}C for two days. Initial weights of the dried wood blocks were determined before soaking the samples overnight in water and autoclaving. Each four wood blocks were used per 18 cm Petri plate filled with 70 to 75 ml *S. commune* minimal agar medium on which mycelium was grown. To avoid the direct contact of the wood blocks with the agar, sterile steel grids were used beneath the wood blocks. Wood blocks were transferred onto the steel grids when the mycelium of the growing fungi covered half of the Petri plates. Mycelium was inoculated in the middle of the plates by placing a block of agar with mycelium that was cut with a cork borer (1cm Ø). Petri plates were sealed with Parafilm (PECHINEY, Chicago, USA) and incubated at 25^{o}C both for initial mycelial growth for 7 to 10 days as well as for further incubation, once the wood blocks were placed onto the grids. To check decay by the strains, harvests were performed after 16 and 20 weeks of incubation with the wood blocks. Plates without fungi but with wood blocks were used as negative controls. After incubation, the mycelium attaching to the wood blocks was separated by using a scalpel. Wood blocks were then dried at 104°C for 3 days and weighed to note the final (dried) weight. Mass loss of wood was calculated as (initial wt – final wt/initial wt) x 100.

3.3.3.2 Saw dust decay test

Three types of saw dust, namely beech, birch and pine, were used. The amount of saw dust used per 500 ml flask was 6 g. 5 replicates were used for each strain for each type of saw dust. In each flask, 50 ml of *S. commune* minimal medium was added. The saw dust was exposed to air and not completely immersed in the medium. 10 ml homogenised inoculum was added for each replicate. To check the decay by the strains, harvest was done after 20 weeks incubation at 25^{o}C. The decayed saw dust

was dried at 104^{o}C for 3 days and weighed to note the final (dried) weight. Mass loss of the saw dust was calculated as mentioned above for the wood block decay test.

3.3.4 Bending strength test of wood

After calculating the mass loss, wood blocks have been used for determining the bending strength (ability to absorb shock with some permanent deformation and more or less injury to the wood specimen). Before measurement, the wood blocks were oven dried at 104^{o}C for 1 day. The wood blocks were kept perpendicular to the force applied, calculated according to the European standard EN 310 (Wood-based panels; Determination of modulus of elasticity in bending and of bending strength; DK 674.03: 620.172.225: 620.174; 1993) with a slight modification. Bending strength was calculated as:

$f_m = 3\ F_{max}\ L_1 / 2bt^2$

F_{max} = absolute strength (N)

L = length of the sample holder

b = width

t = thickness

f_m = relative bending strength (N/mm^2)

3.4 Results

3.4.1 Wood block decay tests

All *S. commune* strains, both monokaryons and dikaryons including the *ΔSc3* and *ΔSc15* mutants and the *ΔSc3ΔSc15* double mutant, were able to grow on beech, birch and pine wood (Fig. 1, 2, 3). There was however a difference in the overall formation of surface mycelium. The wild type monokaryons and the wild type dikaryon formed abundant aerial mycelium on the agar surface of the cultures and on all wood samples (Fig. 1, 2, and 3, samples 4, 5, 6). Similarly, the *ΔSc15* strains were able to produce abundant aerial mycelium (Fig. 1, 2, and 3, samples 10, 11, 12). In contrast, the *ΔSc3* monokaryons lacking the hydrophobin SC3 had hardly any aerial mycelium both on the agar medium as on the surface of the wood blocks (Fig. 1, 2, and 3, samples 7 and 8), confirming previous results by van Wetter et al. (1996) that protein SC3 is required for abundant formation of aerial mycelium on the monokaryon. The dikaryon

lacking the *Sc3* gene in both its nuclei possessed some aerial mycelium both on the agar and on the wood surface (Fig. 1, 2 and 3, sample 9) in accordance with that hydrophobin SC4 takes partially over the function of SC3 in formation of aerial mycelium on the dikaryon (van Wetter et al. 2000). Finally, monokaryons with the double mutations had no aerial mycelium on agar and wood surface whereas the dikaryon resulting from crosses of the two double mutants produced low levels of aerial mycelium on agar and wood (Fig. 1, 2 and 3, samples 13, 14, 15) which is again consistent with the observation that hydrophobin SC4 can partially take over the function of SC3 (van Wetter et al. 2000). Mass loss of all wood samples was determined after 16 and 20 weeks of cultivation. After five months incubation, mass loss of the control samples without fungi of beech wood, birch wood and pine wood was 6.4 ± 1.8%, 5.7 ± 0.4% and 6.7 ± 1.4%, respectively (Fig. 4, 5, 6). The highest mass loss of samples treated with *S. commune* strains in comparison was 11.6 ± 3.8%, 10.5 ± 1.2% and 9.5 ± 0.4%, respectively. When substracting the value for mass loss of the control samples, the highest mass loss of samples treated with *S. commune* strains were 5.2 ± 1.7 %, 4.8 ± 0.5 % and 3.2 ± 2.2 % in beech, birch and pine wood respectively. As determined by statistical Duncan multiple range test of SPSS software, no significant differences were observed in the amount of decay caused by the *S. commune* wild types and the hydrophobin and the *ΔSc15* mutants, regardless of being a monokaryon or a dikaryon (data not shown). Mass loss of wood samples treated with *P. ostreatus* N001 and *T. versicolor* 6 were in between 30 and 50% for the differed types of wood (Fig. 4, 5, 6) indicating that the decay experiment in principle worked.

Bending strength was tested from all wooden samples at the end of the experiment (after 4 and 5 months of incubation, respectively). All *S. commune* strains were found to be able to affect the strength of wood (Fig. 7, 8, 9). The maximum strength loss caused by *S. commune* strains in beech, birch and pine wood was 21, 52 and 35%, respectively (Fig. 7, 8, 9). Highest strength loss of about 188% was caused by *P. ostreatus* N001 in pine (Fig. 9). *T. versicolor* 6 was able to cause strength loss of pine wood up to 95% (Fig. 9) compared to control (wood without fungi). As determined by statistical Duncan multiple range test of SPSS software, no significant differences were observed in the strength loss caused by the *S. commune* wild types,

hydrophobin and the *ΔSc15* mutants, regardless of being a monokaryon or a dikaryon (data not shown).

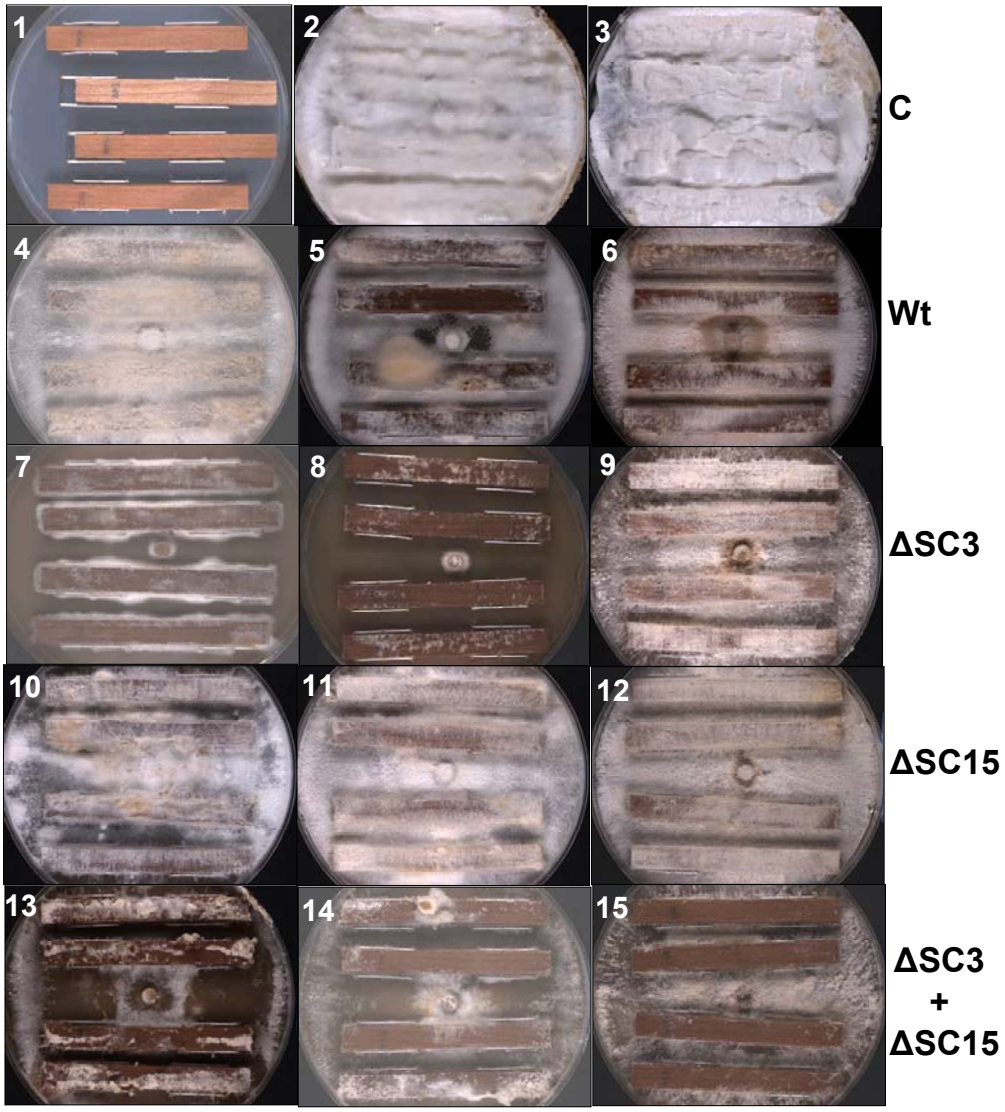

Fig. 1 Growth morphology of *S. commune* strains on beech wood blocks. Controls (C) beech wood without fungi (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3); *S. commune* wild type strains (Wt) 4 -39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* Sc3 mutant strains (ΔSc3) Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* Sc15 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Plates were photographed after 20 weeks of incubation with wood blocks at a time point when effects of senescence on the mycelia were already obvious.

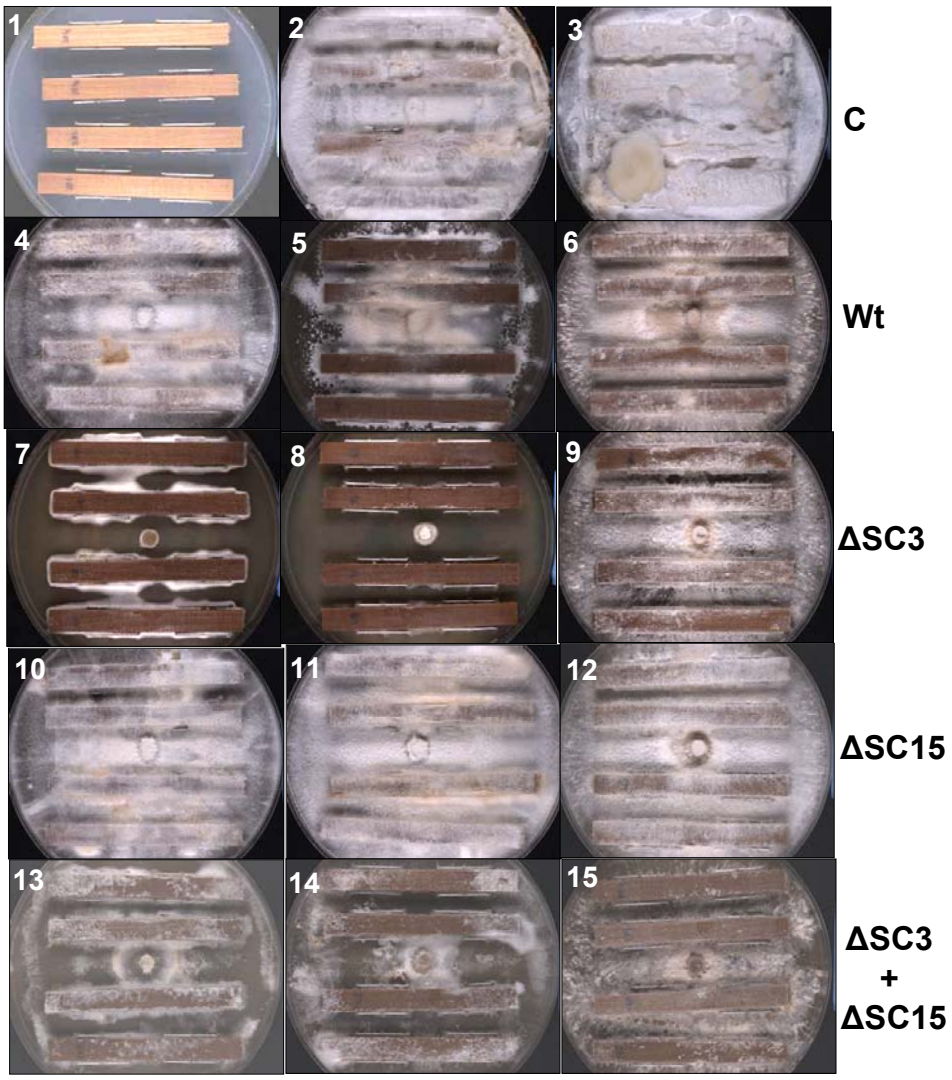

Fig. 2 Growth morphology of *S. commune* strains on birch wood blocks. Controls (C) birch wood without fungi (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3); *S. commune* wild type strains (Wt) 4 -39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* Sc3 mutant strains (ΔSc3) Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* Sc15 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Plates were photographed after 20 weeks of incubation with wood blocks at a time point when effects of senescence on the mycelia were already obvious.

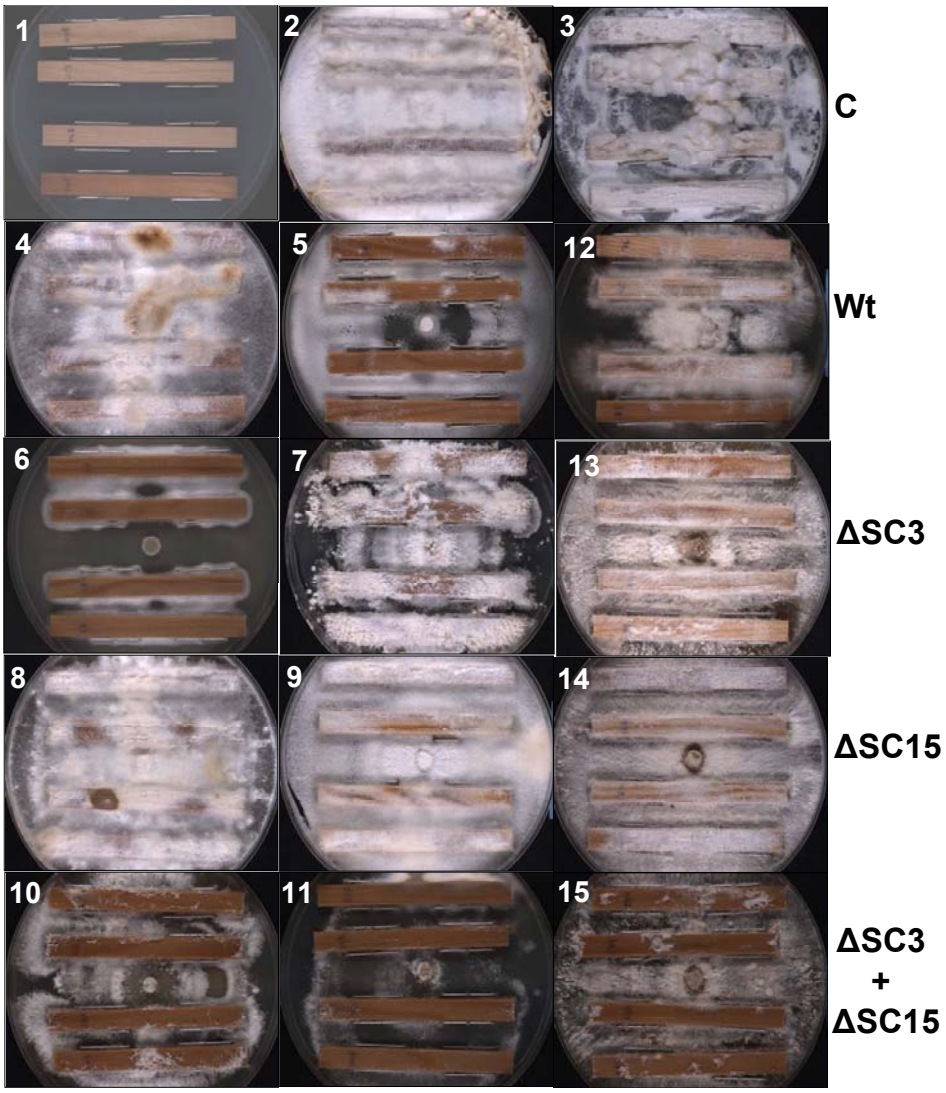

Fig. 3 Growth morphology of *S. commune* strains on pine wood blocks. Controls (C) pine wood without fungi (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3); *S. commune* wild type strains (Wt) 4 -39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* Sc3 mutant strains (ΔSc3) Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* Sc3 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* X Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Plates were photographed after 20 weeks of incubation with wood blocks at a time point when effects of senescence on the mycelia were already obvious.

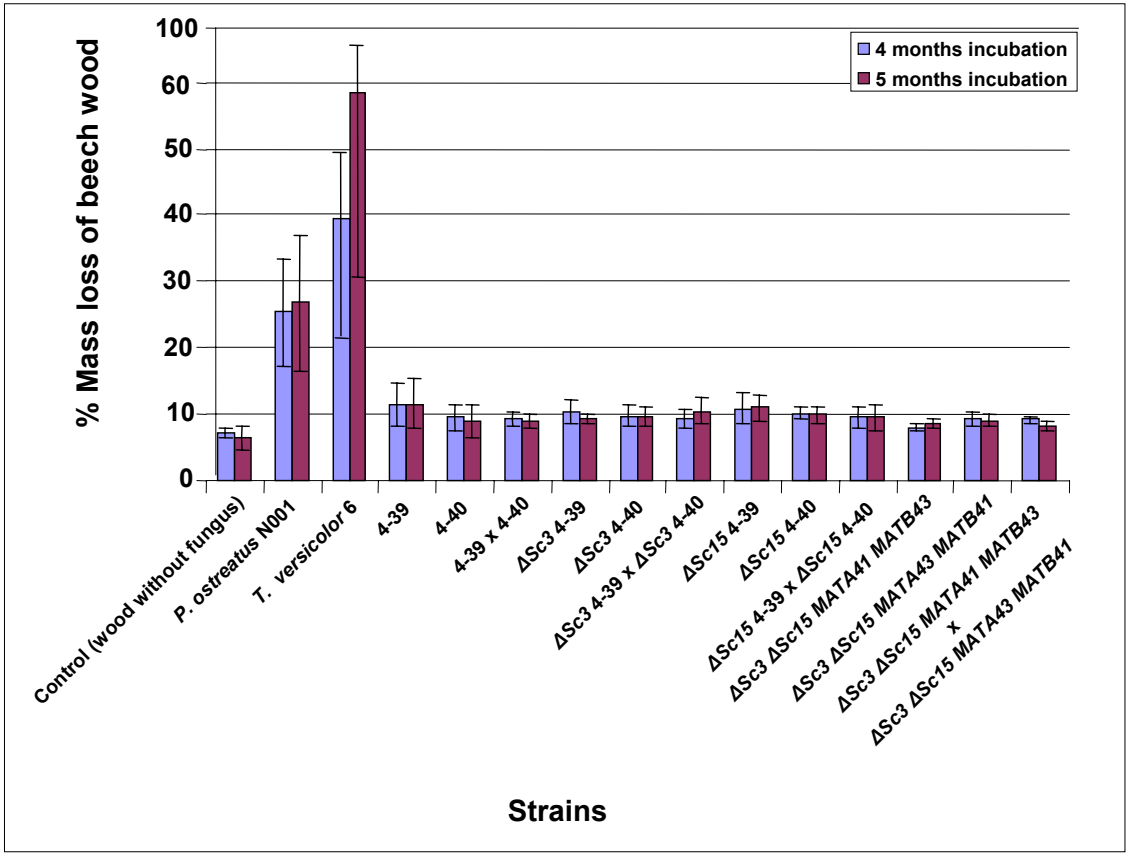

Fig. 4 Beech wood decay by *S. commune* wild type and hydrophobin and SC15 mutants. Strains were grown on *S. commune* minimal medium at 25°C in continuous light. Values shown were averages of mass losses of 7 wood blocks.

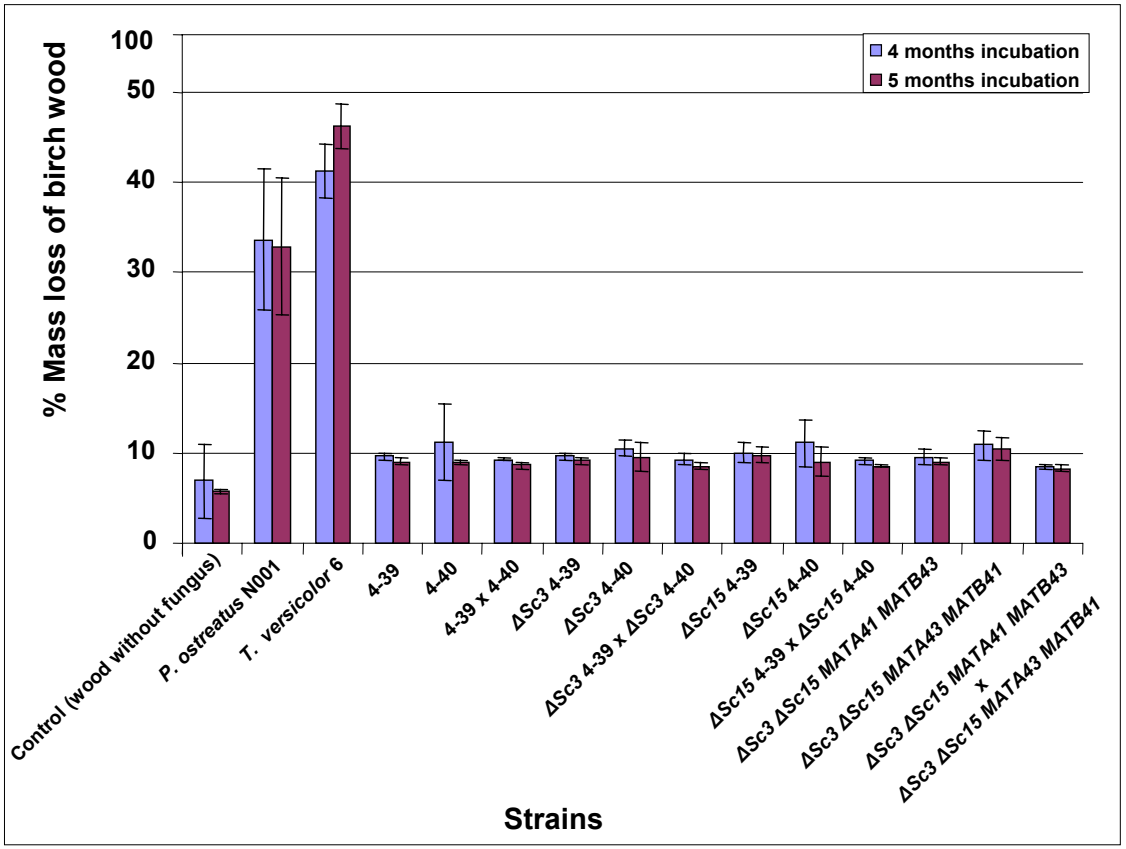

Fig. 5 Birch wood decay by *S. commune* wild type and hydrophobin and SC15 mutants. Strains were grown on *S. commune* minimal medium at 25°C in continuous light. Values shown were averages of mass losses of 7 wood blocks.

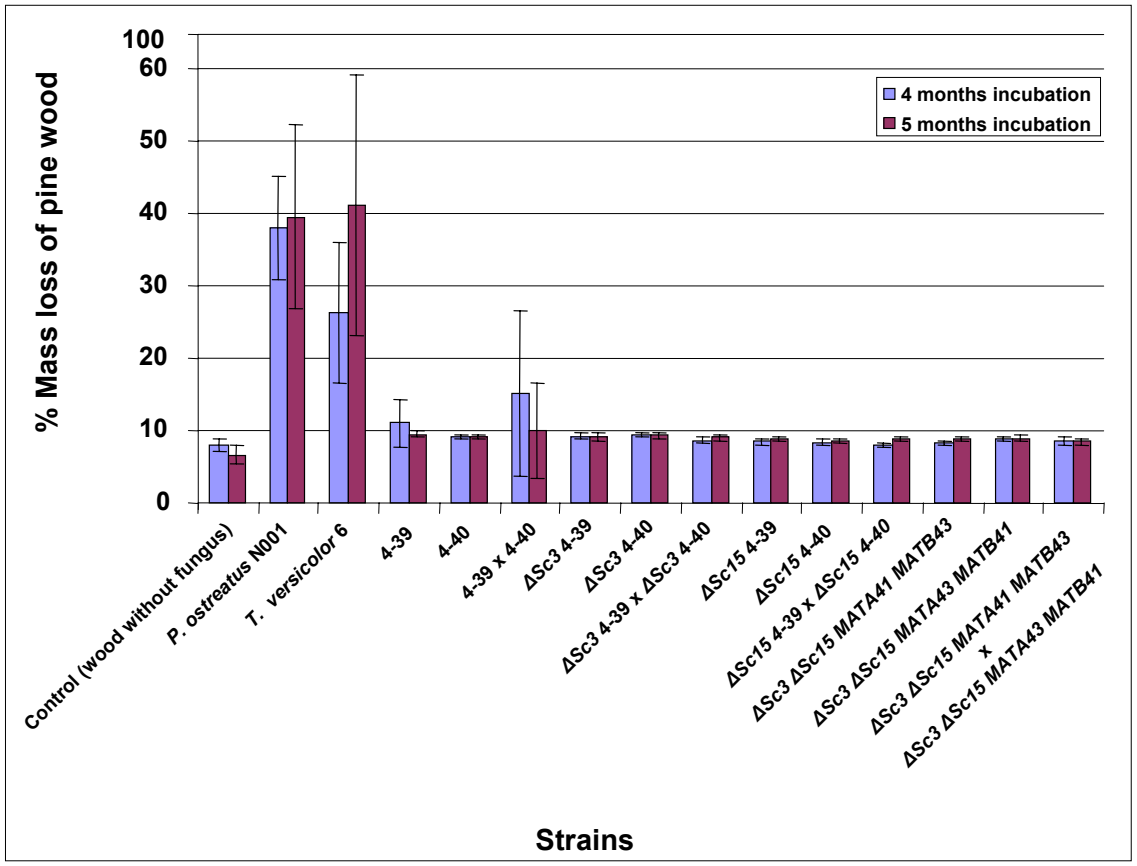

Fig. 6 Pine wood decay by *S. commune* wild type and hydrophobin and SC15 mutants. Strains were grown on *S. commune* minimal medium at 25^{o}C in continuous light. Values shown were averages of mass losses of 7 wood blocks.

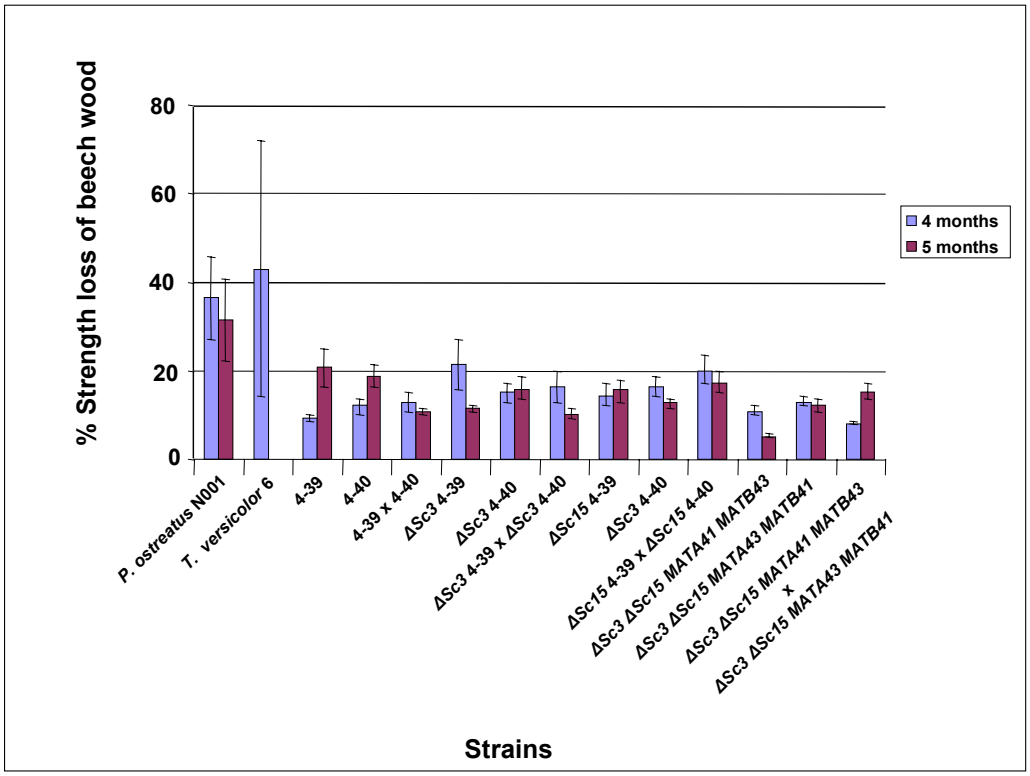

Fig. 7 Bending strength loss of beech wood caused by *S. commune* strains (wild type strains, hydrophobin and SC15 mutants). Percentages were calculated from the average values of bending strength obtained from 7 different wood blocks with fungi (control wood without fungus absolute value – sample wood with fungus absolute value) divided by the average of 7 values obtained from control samples without fungi and multiplied with 100. In case of *T. versicolor* strain 6 treated wood blocks after 5 months of incubation, no value was determined since the decay proceeded so far that the samples were brittle and could therefore not anymore be measured. Note that the standard deviations in case of the *P. ostreatus* and the *T. versicolor* samples are very high (*P. ostreatus* 36 ± 9% after 4 months and 31 ± 9% after 5 months of incubation; *T. versicolor* 43 ± 29% after 4 months of incubation) since the structure of the wood was severely affected resulting in difficulties of measuring exact values.

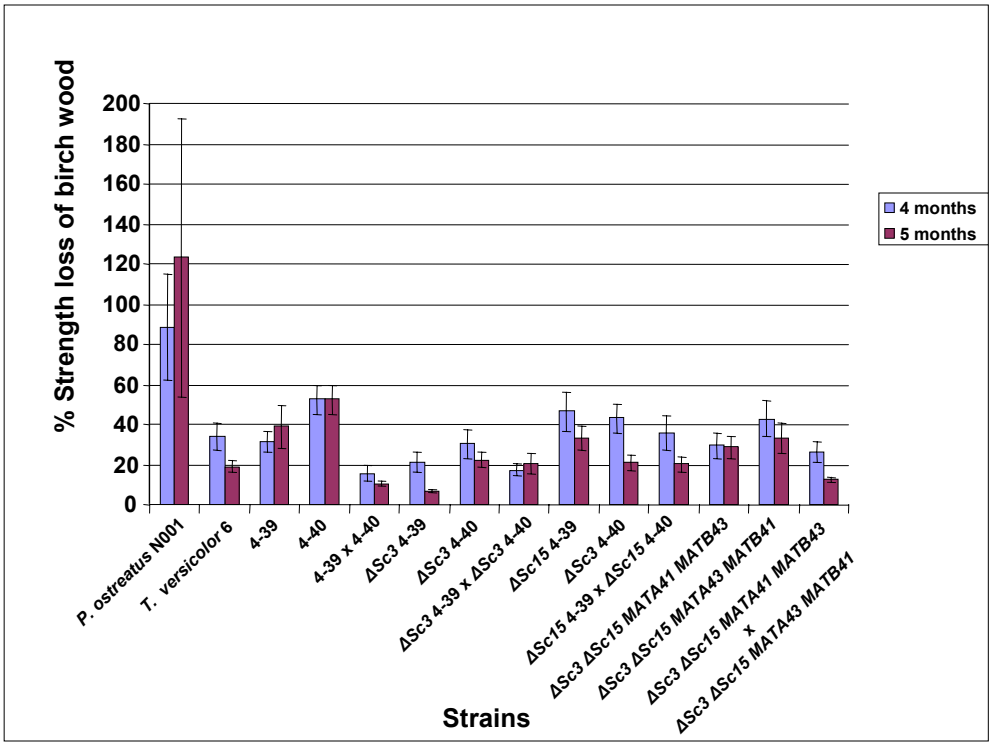

Fig. 8 Bending strength loss of birch wood caused by *S. commune* strains (wild type strains, hydrophobin and SC15 mutants). Percentages were calculated from the average values of bending strength obtained from 7 different wood blocks with fungi (control wood without fungus absolute value – sample wood with fungus absolute value) divided by the average of 7 values obtained from control samples without fungi and multiplied with 100. Note that the standard deviations in case of the *P. ostreatus* and the *T. versicolor* samples are very high (*P. ostreatus* 88 ± 26% after 4 months and 123 ± 69% after 5 months of incubation; *T. versicolor* 34 ± 6% after 4 months and 19 ± 3% after 5 months of incubation) since the structure of the wood was severely affected resulting in difficulties of measuring exact values.

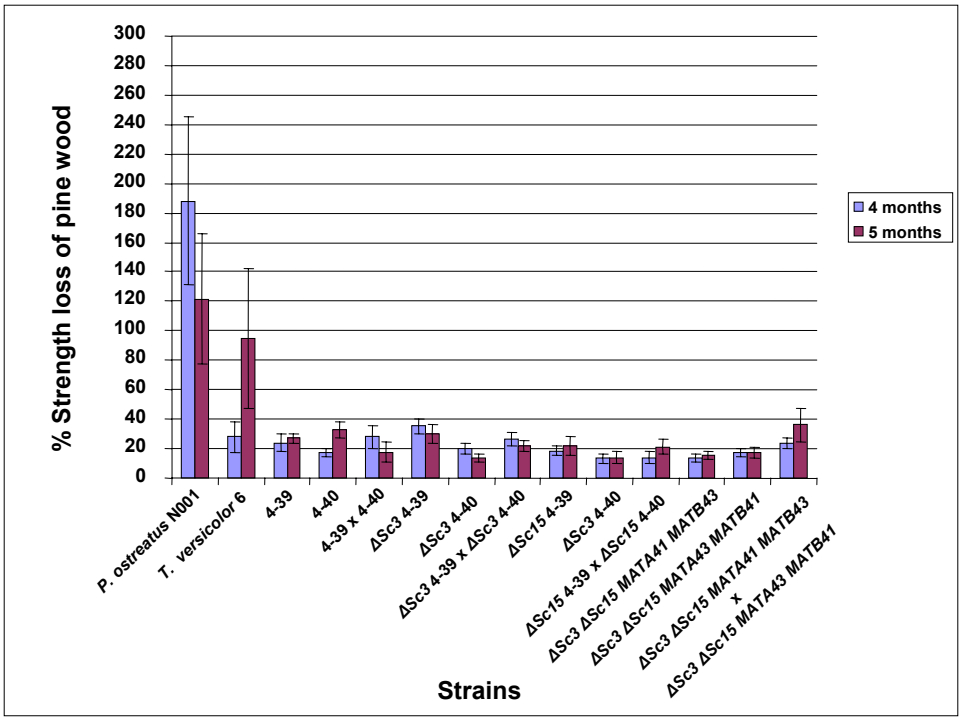

Fig. 9 Bending strength loss of pine wood caused by *S. commune* strains (wild type strains, hydrophobin and SC15 mutants). Percentages were calculated from the average values of bending strength obtained from 7 different wood blocks with fungi (control wood without fungus absolute value – sample wood with fungus absolute value) divided by the average of 7 values obtained from control samples without fungi and multiplied with 100. Note that the standard deviations in case of the *P. ostreatus* and the *T. versicolor* samples are very high (*P. ostreatus* 188 ± 56% after 4 months and 121 ± 44% after 5 months of incubation; *T. versicolor* 28 ± 10% after 4 months and 94 ± 47% after 5 months of incubation) since the structure of the wood was severely affected resulting in difficulties of measuring exact values.

3.4.2 Decay of saw dusts from different tree species by *S. commune* strains

To further elucidate the decay abilities of *S. commune* strains, the fungi were grown on different types of saw dusts, offering the fungi a large surface area and a better aeration for their growth. All *S. commune* mono- and dikaryons including the Sc3 and Sc15 mutants grew on beech, birch and pine saw dusts (Fig. 10, 11, 12). However, the morphological appearances of the different strains grown on the saw dust samples varied. Mainly two types of surface growth were observed. On beech saw dust, as expected, the SC3 monokaryotic mutants *ΔSc3* 4-39 and *ΔSc3* 4-40 as well as the SC3

and SC15 double mutants *ΔSc3 ΔSc15 MATA41 MATB 43* and *ΔSc3 ΔSc15 MATA43 MATB 41* and the SC3, SC15 dikaryotic double mutant *ΔSc3 ΔSc15 MATA41 MATB 43* x *ΔSc3 ΔSc15 MATA43 MATB 41* had a relatively thin mycelium (Fig. 10, samples 7, 8, 13, 14, 15 respectively). Furthermore, the *S. commune* strain 4-39 and its SC15 mutant *ΔSc15* 4-39 grow in a thinner manner (Fig. 10, samples 4, 10), unlike all the other strains (Fig. 10, samples 5, 6, 9, 11, 12). Similarly on birch saw dust, the *S. commune* strain 4-39 and its mutants (the SC3 mutant *ΔSc3* 4-39, the SC15 mutant *ΔSc15* 4-39, the SC3, SC15 monokaryotic double mutants *ΔSc3 ΔSc15 MATA41 MATB 43* as well as the 4-40 mutant *ΔSc3 ΔSc15 MATA43 MATB 41,* Fig. 11, samples 4, 7, 10, 13, 14) had a relatively thin mycelium on the surface of unlike other strains (Fig. 11, samples 5, 6, 8, 9, 11, 12, 15), whereas on pine saw dust, most of the strains grew with a stronger mycelium (Fig. 12, samples 5, 6, 8, 9, 11, 12).

It thus appears (i) that with the much larger wooden surfaces, effects caused by the deletion of the SC3 hydrophobin gene are less severe in causing a different phenotype compared to wild type than on surfaces of very limited area and (ii) that differences in the genetic backgrounds other than the hydrophobin genes can have stronger effects on the fungal growth on the wood. Since monokaryons 4-39 and 4-40 are co-isogenic, such differences, if present, must be closely linked to the mating type genes, being the only known differences between the two strains. Such growth effects encountered for the strains might not be effective on all types of wood saw dust: on pine saw dust, aerial mycelium was present in all cultures to a more or less extent but not in the culture of the *ΔSc3* mutant *ΔSc3* 4-39 (Fig. 12). It can however not be excluded that the relative often occurring *thin* mutation happened during cultivation. The *thin* mutation, as the name says, gives rise to thin mycelium (Fowler and Mitton 2000). All *S. commune* strains were found to decolorize the pine saw dust indicating likely a decay action by the fungi (Fig. 15). This decolorization affect was however not observed in case of beech and birch saw dust (Fig. 13, 14). Nevertheless, mass loss was encountered for all *S. commune* strains on all three types of saw dust (Fig. 16, 17, 18). Mass losses varied by the *S. commune* strains from 9 to 16% in 4 months incubation time. As determined by Duncan multiple range test, there were some significant differences in the decay caused by the *S. commune* strains including the *Sc3* and *Sc15* mutants but there was no particular pattern observed among the three

types of substrates (Table 1). In these series of experiments, *P. ostreatus* N001 decayed 26, 32 and 20% of beech, birch and pine saw dust, respectively, and *T. versicolor* strain 6 39, 40 and 28% of beech, birch and pine saw dust, respectively. This indicates the principle functionality of the experiment. Unlike the *S. commune* strains, *P. ostreatus* N001 did however not decolorize either beech, birch or pine saw dust (Fig. 13, 14, 15 sample 2). *T. versicolor* strain 6 decolorized beech but not birch or pine saw dust (Fig. 13, 14, 15 sample 3).

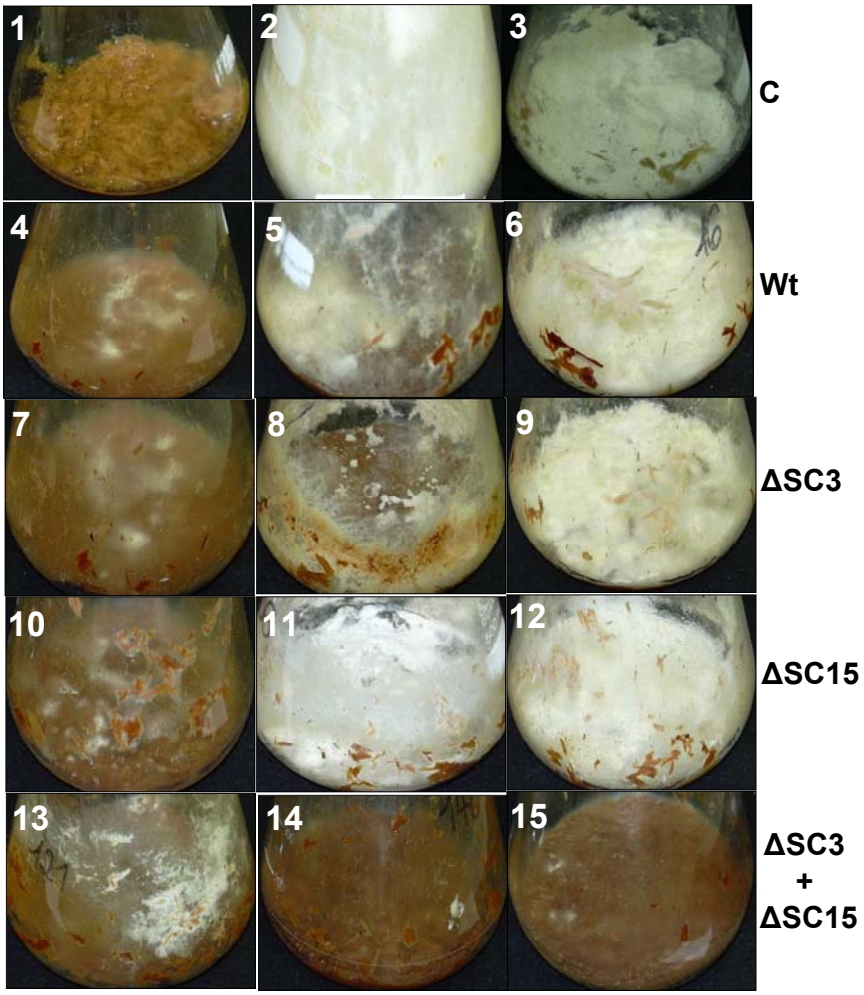

Fig. 10 Growth morphology of *S. commune* strains on beech saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutant strains Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Cultures were photographed after 16 weeks of incubation at 28°C under constant illumination. Note that in

sample 8, the amount of aerial mycelium might appear to be more intensive due to the fact that the fungi grew on the surface of the glass flask, limiting full view on to the surface growth on the saw dust.

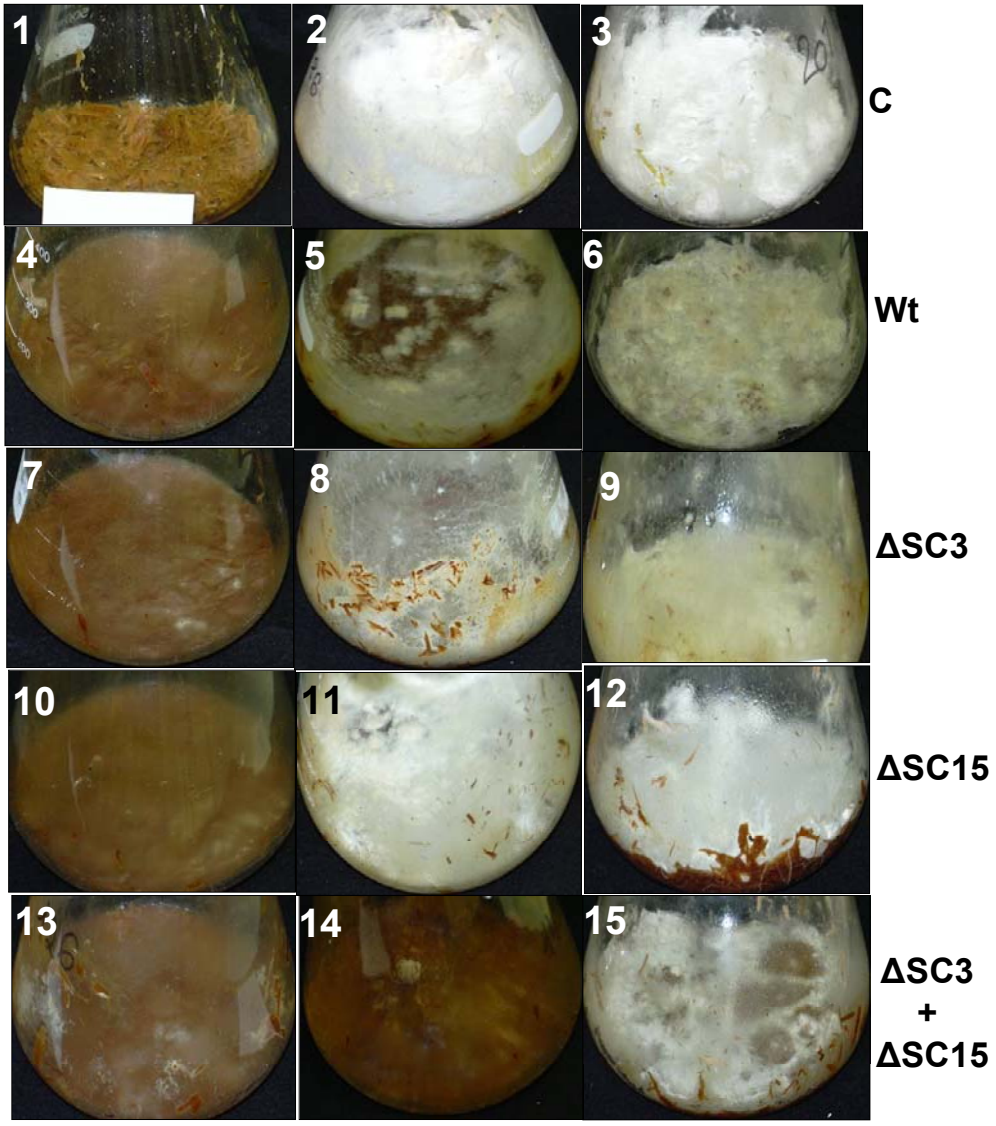

Fig. 11 Growth morphology of *S. commune* strains on birch saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutant strains Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Cultures were photographed after 16 weeks of incubation at 28°C under constant illumination.

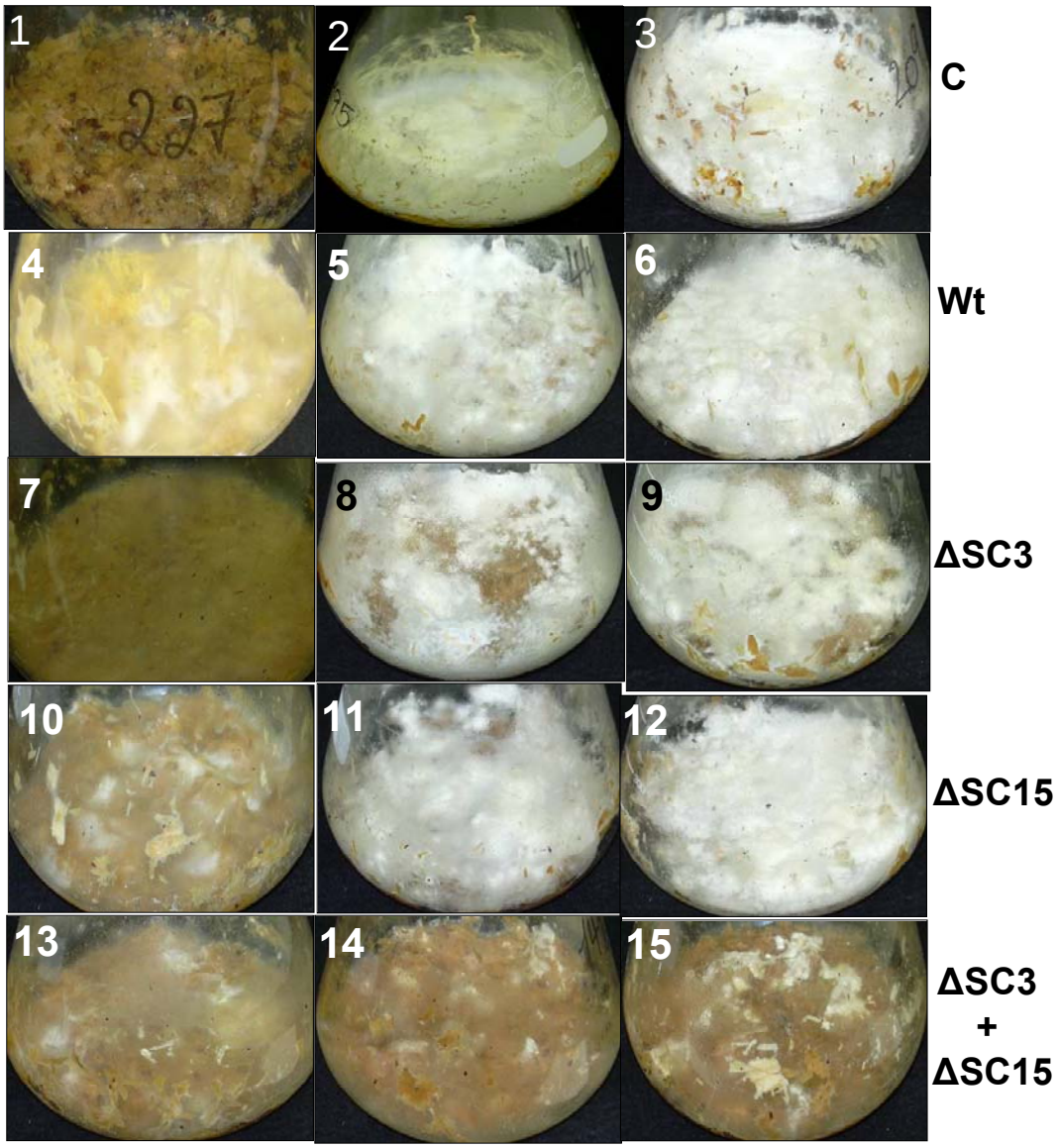

Fig. 12 Growth morphology of *S. commune* strains on pine saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor 6* (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutant strains Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutant strains Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 and SC15 mutant strains (ΔSC3 + ΔSC15) Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3* Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Cultures were photographed after 16 weeks of incubation at 28°C under constant illumination.

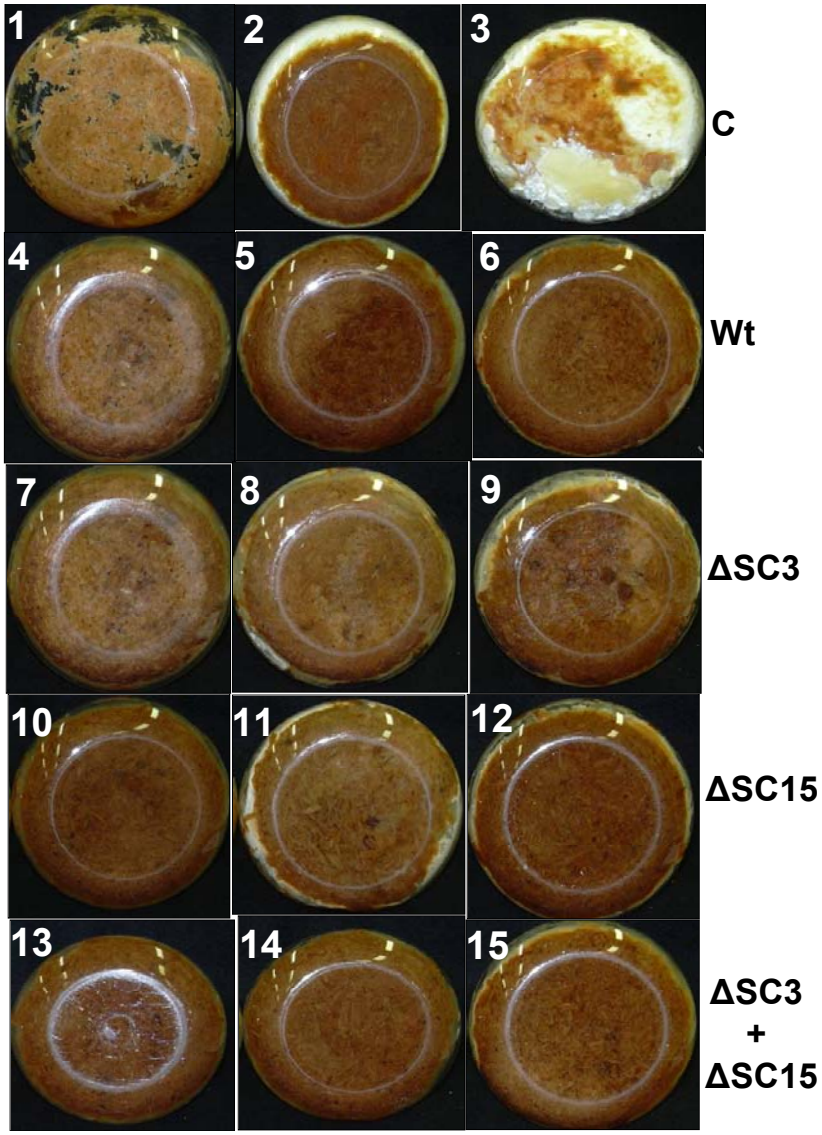

Fig. 13 *S. commune* strains do not decolorize beech saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutants Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutants Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 SC15 mutants Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3*Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Flasks were photographed from beneath after 16 weeks of cultivation at 25°C under constant illumination.

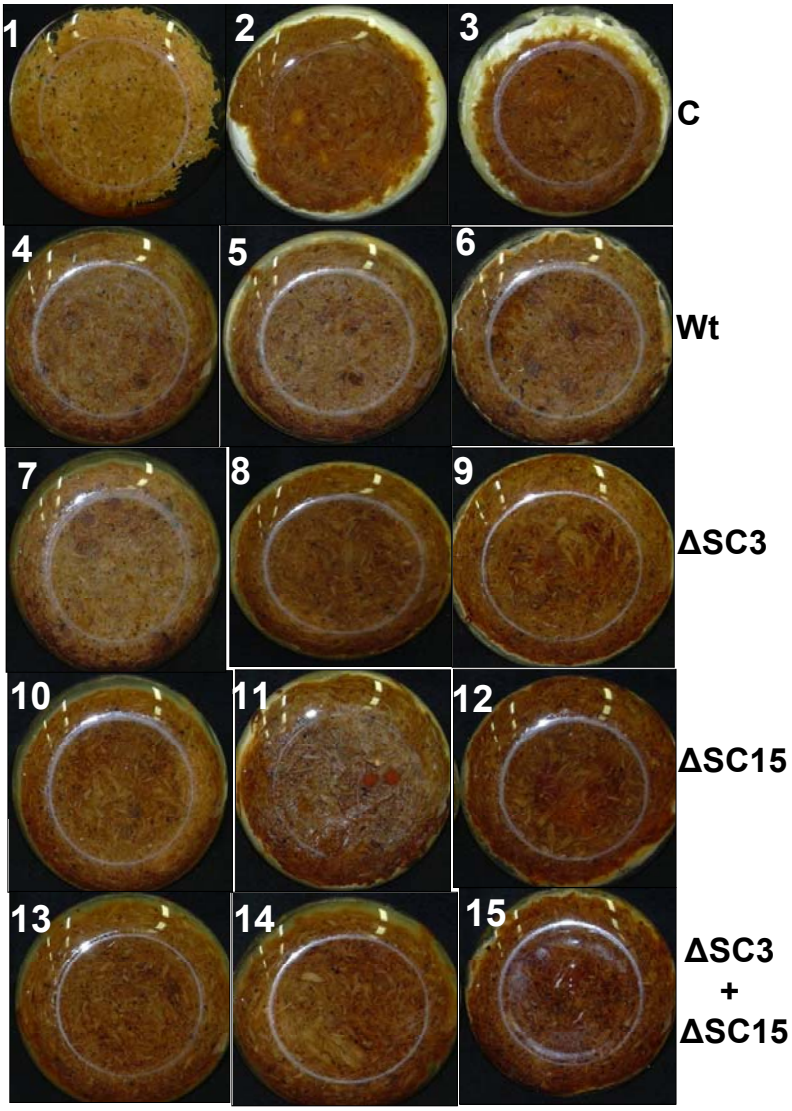

Fig. 14 *S. commune* strains do not decolorize birch saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutants Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutants Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 SC15 mutants Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3*Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Flasks were photographed from beneath after 16 weeks of cultivation at 25°C under constant illumination.

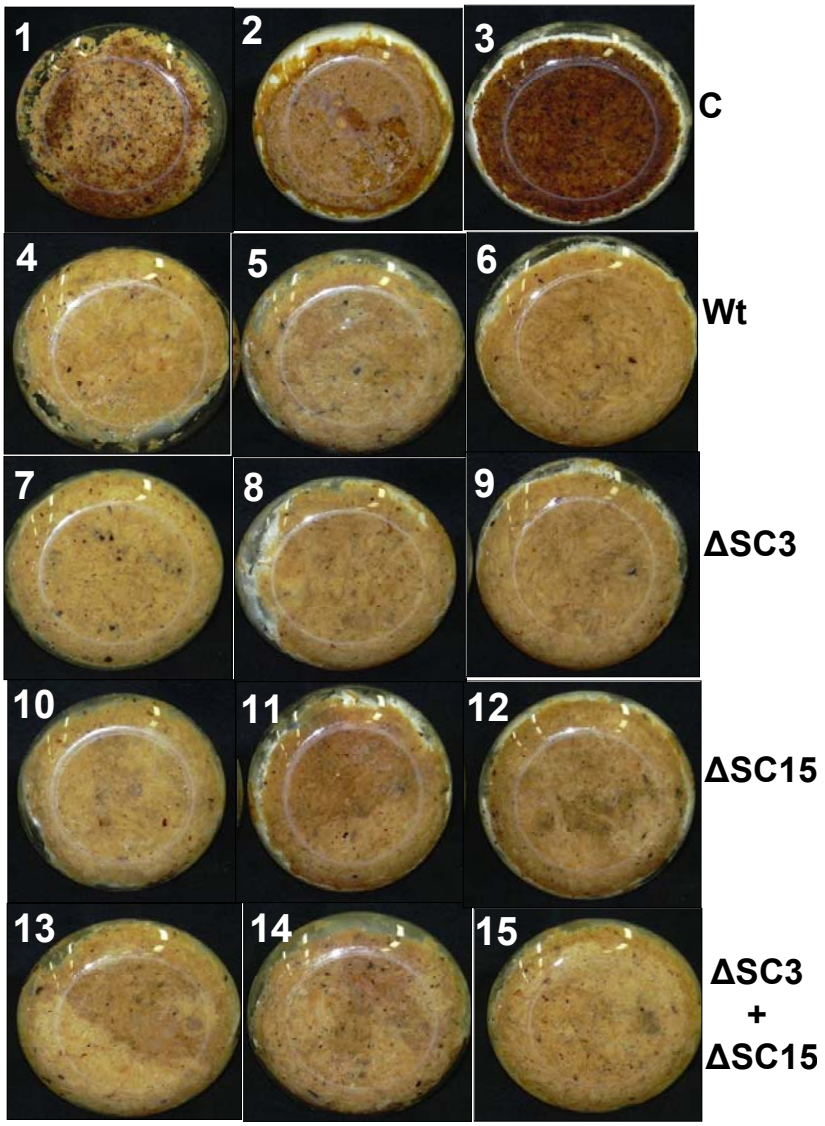

Fig. 15 *S. commune* strains decolorize pine saw dust. Control (saw dust without fungi) (1), *P. ostreatus* N001 (2), *T. versicolor* 6 (3), *S. commune* wild type strains 4-39 (4), 4-40 (5), 4-39 x 4-40 (6); *S. commune* SC3 mutants Δ*Sc3* 4-39 (7), Δ*Sc3* 4-40 (8), Δ*Sc3* 4-39 x Δ*Sc3* 4-40 (9); *S. commune* SC15 mutants Δ*Sc15* 4-39 (10), Δ*Sc15* 4-40 (11), Δ*Sc15* 4-39 x Δ*Sc15* 4-40 (12); *S. commune* SC3 SC15 mutants Δ*Sc3* Δ*Sc15 MATA41 MATB43* (13), Δ*Sc3*Δ*Sc15 MATA43 MATB41* (14) and Δ*Sc3*Δ*Sc15 MATA41 MATB43* x Δ*Sc3* Δ*Sc15 MATA43 MATB41* (15). Flasks were photographed from beneath after 16 weeks of cultivation at 25°C under constant illumination.

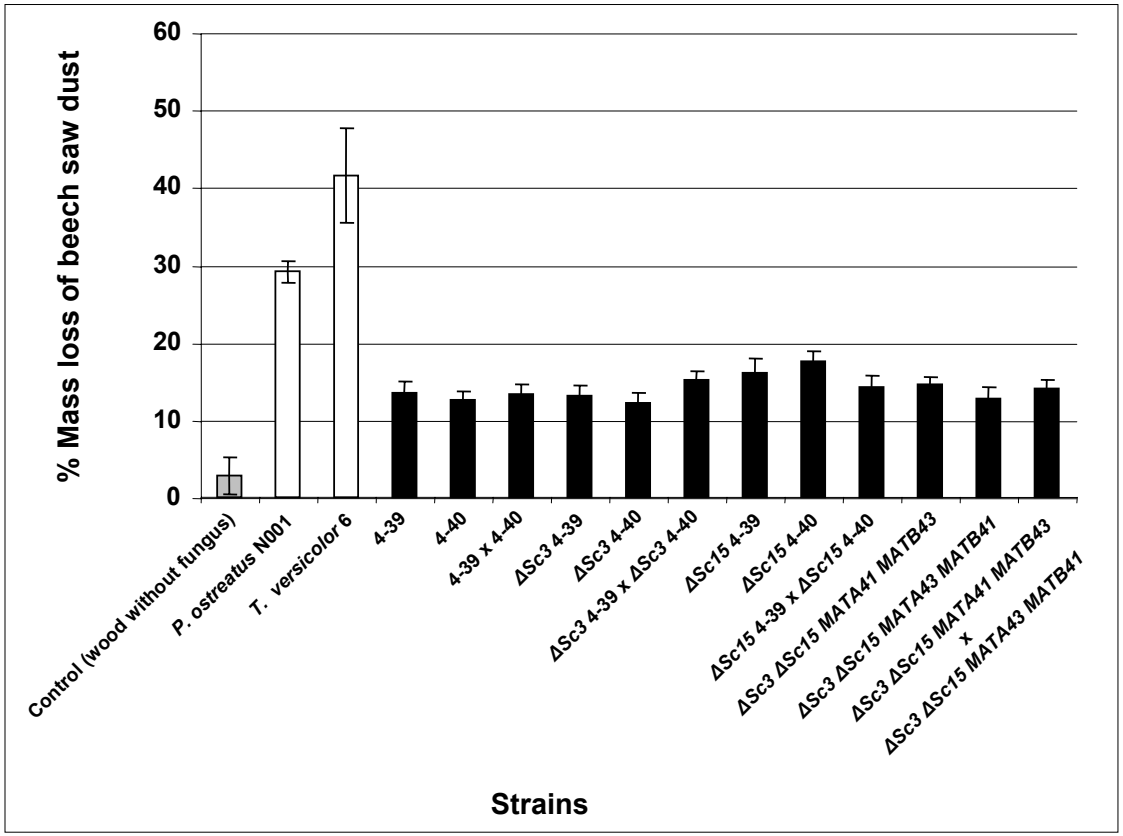

Fig. 16 Decay of beech saw dust by *S. commune* wild type strains, hydrophobin and SC15 mutants after 4 months of incubation as determined from mass losses. Strains were grown in a flask on 50 ml *S. commune* minimal medium at 25°C in continuous light containing 6 g saw dus t. Values shown were averages of 5 replicates.

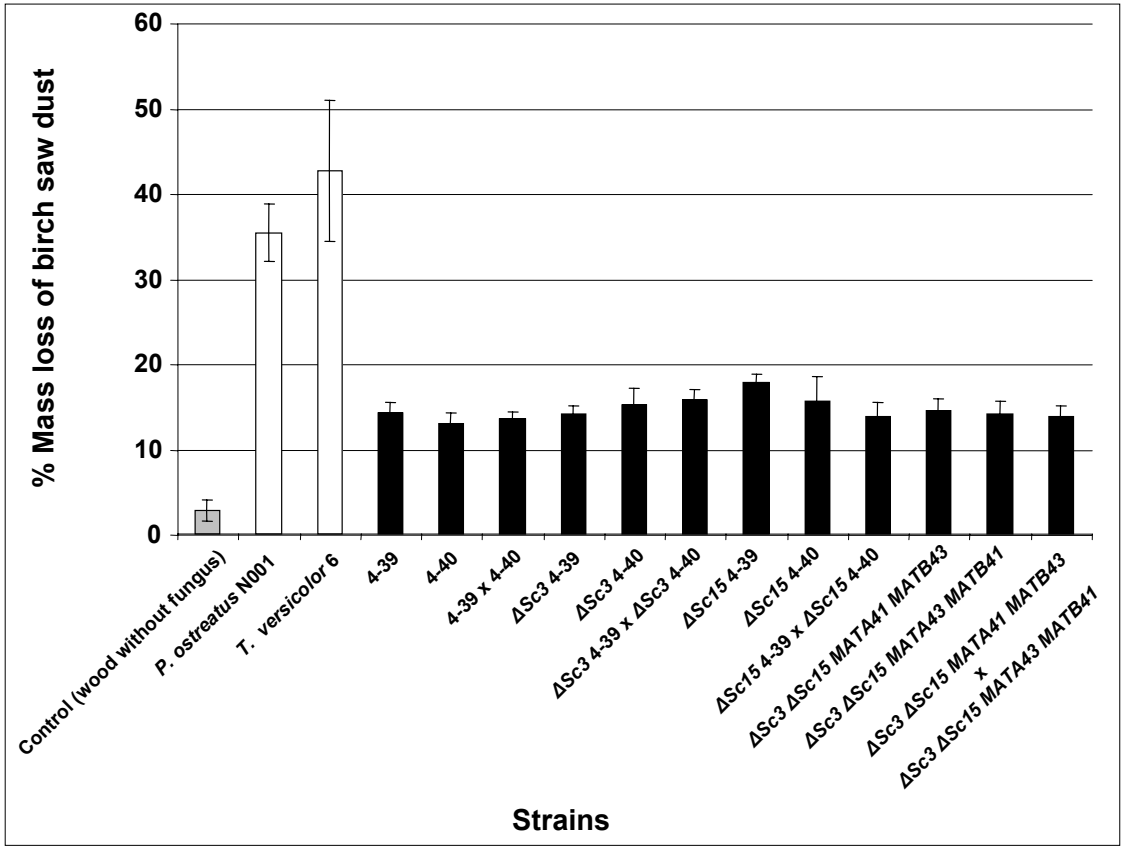

Fig. 17 Decay of birch saw dust by *S. commune* wild type strains, hydrophobin and SC15 mutants after 4 months of incubation as determined from mass losses. Strains were grown in a flask on 50 ml *S. commune* minimal medium at 25^{o}C in continuous light containing 6 g saw dus t. Values shown were averages of 5 replicates.

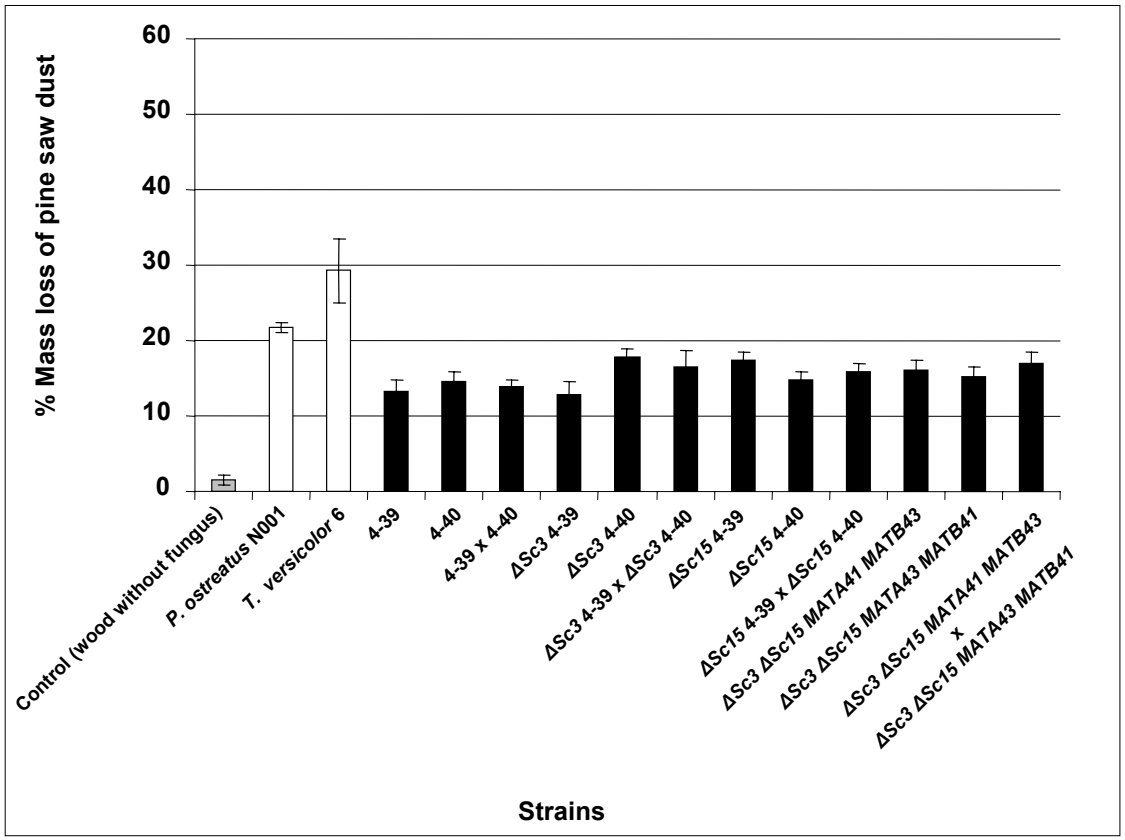

Fig. 18 Decay of pine saw dust by *S. commune* wild type strains, hydrophobin and SC15 mutants after 4 months of incubation. Strains were grown in a flask on 50 ml *S. commune* minimal medium at 25^{o}C in continuous light containing 6 g saw dust. Values shown were averages of 5 replicate.

Table 1 Statistical data (calculated by using the Duncan multiple range test) indicating significant differences ($p \leq 0.05$) in the mass loss of different types of saw dust (beech, birch and pine) caused by different fungal strains during 4 months of incubation.

Strains	*	Beech saw dust 4 months incubation different with	Birch saw dust 4 months incubation different with	Pine saw dust 4 months incubation different with
4-39	d	**a**, *b, c*, k	**a**, *b, c*	**a**, *b, c*, h, i, j, m, n, o
4-40	e	**a**, *b, c*, j, k	**a**, *b, c*, j	**a**, *b, c*, h, i, j, o
4-39 x 4-40	f	**a**, *b, c*, k	**a**, *b, c*, j	**a**, *b, c,* h, i, j, l, m, n, o
***ΔSc3* 4-39**	g	**a**, *b, c*, k	**a**, *b, c*	**a**, *b, c*, h, i, j, l, m, n, o
***ΔSc3* 4-40**	h	**a**, *b, c*, k	**a**, *b, c*	**a**, *b, c*, d, e, f, g, k, l
***ΔSc3* 4-39 x *ΔSc3* 4-40**	i	**a**, *b, c*	**a**, *b, c*	**a**, *b, c*, d, f, g
***ΔSc15* 4-39**	j	**a**, *b, c*	**a**, *b, c*, e, f, o, n	**a**, *b, c*, d, e, f, g, k
***ΔSc15* 4-40**	k	**a**, *b, c*, d, h, e, f, i, j, m, n, o	**a**, *b, c*	**a**, *b, c*, h, j
***ΔSc15* 4-39 x *ΔSc15* 4-40**	l	**a**, *b, c*, k	**a**, *b, c*	**a**, *b, c*, g, h
ΔSc3 ΔSc15MATA41 MATB43	m	**a**, *b, c*, j, k	**a**, *b, c*	**a**, *b, c*, d, g
ΔSc3 ΔSc15MATA43 MATB41	n	**a**, *b, c*, k	**a**, *b, c*, j	**a**, *b, c,* d, g
ΔSc3 ΔSc15MATA41 MATB43* x *ΔSc3 ΔSc15MATA43 MATB41	o	**a**, *b, c*, k	**a**, *b, c*, j	**a**, *b, c*, d, e, f, g
Control wood	**a**	*b*,*c*,d,e,f,g,h,i,j,k,l,m,n,o	*b*,*c*,d,e,f,g,h,i,j,k,l,m,n,o	*b*,*c*,d,e,f,g,h,i,j,k,l,m,n,o
***P. ostreatus* N001**	*b*	**a**,*c*,d,e,f,g,h,i,j,k,l,m,n,o	**a**,*c*,d,e,f,g,h,i,j,k,l,m,n,o	**a**,*c*,d,e,f,g,h,i,j,k,l,m,n,o
***T. versicolor* 6**	*c*	**a**,*b*,*c*,d.e,f,g,h,i,j,k,l,m,n,o	**a**,*b*,d,e,f,g,h,i,j,k,l,m,n,o	**a**,*b*,d,e,f,g,h,i,j,k,l,m,n,o

(*) For an easy understanding, fungal strains were marked by alphabetical letters.

Table 2 Comparison of percentage decay levels of beech, birch, pine wood blocks and saw dust by *S. commune* wild type, hydrophobin and SC15 mutants.

Strains	Beech wood blocks		Birch wood blocks		Pine wood blocks		Beech saw dust	Birch saw dust	Pine saw dust
	4 months incubation	5 months incubation	4 months incubation	5 months incubation	4 months incubation	5 months incubation	4 months incubation	4 months incubation	4 months incubation
4-39	4.2 ± 1.1	5.2 ± 1.7	2.7 ± 0.2	3.3 ± 0.1	3.0 ± 0.9	2.8 ± 0.1	10.8 ± 1.1	11.3 ± 1.5	11.7 ± 1.2
4-40	2.4 ± 0.5	2.5 ± 0.7	4.3 ± 1.6	3.2 ± 0.1	1.2 ± 0.03	2.3 ± 0.1	9.7 ± 0.9	10.2 ± 0.6	13.0 ± 1.1
4-39 x 4-40	2.0 ± 0.2	2.4 ± 0.3	2.4 ± 0.1	2.9 ± 0.1	7.0 ± 5.3	3.3 ± 2.2	10.6 ± 1.0	10.7 ± 1.0	12.3 ± 0.7
***ΔSc3* 4-39**	3.2 ± 0.6	3.0 ± 0.2	2.7 ± 0.1	3.4 ± 0.1	1.2 ± 0.05	2.4 ± 0.1	10.4 ± 0.9	11.3 ± 1.1	11.3 ± 1.3
***ΔSc3* 4-40**	2.7 ± 0.4	3.2 ± 0.4	3.6 ± 0.3	3.8 ± 0.6	1.4 ± 0.04	2.6 ± 0.1	9.4 ± 1.0	12.4 ± 0.8	16.2 ± 1.1
***ΔSc3* 4-39 x *ΔSc3* 4-40**	2.2 ± 0.3	4.0 ± 0.7	2.4 ± 0.1	2.9 ± 0.1	0.6 ± 0.03	2.4 ± 0.1	12.3 ± 1.0	12.9 ± 3.0	15.0 ± 1.8
***ΔSc15* 4-39**	3.6 ± 0.8	4.6 ± 0.8	3.1 ± 0.4	4.1 ± 0.3	0.4 ± 0.02	2.2 ± 0.1	13.4 ± 1.4	14.9 ± 2.3	15.8 ± 1.1
***ΔSc15* 4-40**	2.9 ± 0.3	3.5 ± 0.4	4.2 ± 1.0	3.4 ± 0.6	0.3 ± 0.02	1.9 ± 0.1	14.8 ± 1.1	12.8 ± 1.2	13.2 ± 0.9
***ΔSc15* 4-39 x *ΔSc15* 4-39**	2.4 ± 0.4	3.2 ± 0.6	2.2 ± 0.1	2.8 ± 0.04	0.1 ± 0.01	2.1 ± 0.1	11.5 ± 1.1	11.0 ± 1.7	14.2 ± 1.1
Δ*Sc3*Δ*Sc15* MATA41 MATB43	0.9 ± 0.04	2.1 ± 0.2	2.7 ± 0.2	3.4 ± 0.2	0.2 ± 0.01	2.1 ± 0.1	11.8 ± 0.7	11.6 ± 1.2	14.5 ± 1.1
Δ*Sc3*Δ*Sc15* MATA43 MATB41	2.2 ± 0.2	2.7 ± 0.3	3.9 ± 0.5	4.8 ± 0.5	0.9 ± 0.02	2.3 ± 0.1	10.0 ± 1.2	11.3 ± 1.3	13.7 ± 1.2
Δ*Sc3*Δ*Sc15* MATA41 MATB43 x Δ*Sc3*Δ*Sc15* MATA43 MATB41	2.1 ± 0.1	1.9 ± 0.2	1.6 ± 0.04	2.6 ± 0.1	0.6 ± 0.03	1.8 ± 0.1	11.3 ± 0.9	11.0 ± 1.0	15.3 ± 1.3
***P. ostreatus* N001**	18.1 ± 5.7	20.4 ± 7.8	26.8 ± 6.1	27.2 ± 6.2	30 ± 5.6	32.8 ± 10.5	26.3 ± 1.2	32.5 ± 0.8	20.2 ± 0.6
***T. versicolor* 6**	32.1 ± 22	52.2 ± 31	34.5 ± 2.5	40.6 ± 2.1	18.3 ± 6.8	34.5 ± 15	38.7 ± 5.7	39.8 ± 0.9	27.6 ± 3.9

Values were percentages obtained from (mass loss of fungal treated sample – mass loss of the control).

3.5 Discussion

In this study, *Schizophyllum commune* wild type strains and coisogenic hydrophobin and SC15 mutants were tested for their ability to decay wood. Hydrophobins are known to help the fungus in the formation of aerial hyphae and in the process of attachment of the fungus to hydrophobic materials (Lugones et al. 2004, Wessels et al. 1991, Wösten et al. 1993, 1994, 1999, Wessels 1997). Previous studies showed that, in the absence of the Sc3 hydrophobin, protein SC15 helps in the attachment to surfaces (Lugones et al. 2004). Only one PhD study by de Jong (2006) was performed previously to check the role of hydrophobins in the wood decay process and de Jong (2006) concluded that SC3 and SC15 proteins function in aerial hyphae formation but not in the substrate colonization during growth on wood. This conclusion is supported by the experiments presented in this chapter. No wood decay tests were performed in the study by de Jong (2006) to determine possible mass losses caused by various *S. commune* strains. Such tests are presented for the first time for hydrophobin mutants in this thesis.

In this study, *S. commune* single and double mutants of the SC3 hydrophobin as well as of the SC3 supporting protein SC15 were used to test their wood decay ability on three different types of wood saw dusts: beech, birch and pine. *S. commune* strains showed little decay effects when grown on beech wood blocks. Decay levels after 4 and 5 months of incubation were between 2 to 5% (percentage of mass loss of fungal treated sample after subtracting the mass loss of the control, see Table 2). These results were similar to the observations made for *S. commune* strains by Schmidt and Liese (1980). In the experiments of these authors, *S. commune* strains caused no or only little decay of beech wood blocks of about 0.4 to 4.1% after incubation for 5 months. In another beech block test performed by Leithoff and Peek (2001), the *S. commune* strain MUCL 31016 caused a minor mass loss of up to 1.3%.

In the birch wood block test performed in this PhD study, decay levels caused by different *S. commune* strains after 4 and 5 months of incubation varied from 1.5 to 4.2% (percentage of mass loss of fungal treated sample after subtracting the mass loss of the control, see Table 2). There was only one previous study of *S. commune* on

birch wood blocks mentioned in the literature by Erikson (1990) in which there was no mass loss (0%) observed after 12 weeks of incubation.

In this PhD study, the decay levels of pine wood blocks caused by *S. commune* strains were in between 0.1 to 7% (percentage of mass loss of fungal treated sample after subtracting the mass loss of the control, see Table 2) similar to the observations from previous studies by Schmidt and Liese (1980) who reported mass losses by *S. commune* on pine wood of 0 to 2.9%. Other studies performed by Schirp et al. (2003), Hegarty et al. (1987), Nilsson and Daniel (1983) revealed also only minor mass losses of 0, 0.6 and 4%, respectively, by *S. commune* on pine wood blocks.

Our data however suggest that the decay rate by *S. commune* increased when saw dust is used. Decay rates under such conditions were higher between 5 to 7% (Table 2). This could be due to a better aeration in the saw dust than in the wood blocks as well as due to a large wood surface area. However, one has to also consider in these series of experiments that BSM medium was added to the saw dust. It is possible that with the extra supplied nutrients, *S. commune* becomes stronger and its metabolism better regulated so that wood might be more aggressively attacked. Nutritional regulation of enzymes acting in wood decay in basidiomycetes is not uncommon. For example, supply of sufficient nitrogen can cause induction of decay enzymes such the laccases [e.g. see the review of Rühl et al. (2007) for further information].

There were no previous reported studies on decay tests of *S. commune* with saw dust of beech, birch or pine. In this study, *S. commune* strains caused decolorization of pine saw dust which might be an indication of a white rot activity (Fig. 15). In contrast to the *S. commune* strains, the white rots *P. ostreatus* and *T. versicolor* however did not decolorize the pine saw dust. The decolorization of pine wood therefore could be due to substrate specificity and thereby by specific enzyme or metabolite activities performed by the *S. commune* strains which may not be offered by *P. ostreatus* N001 and *T. versicolor* 6. All the *S. commune* strains and *P. ostreatus* N001 did not decolorize either beech or birch saw dust. *T. versicolor* in contrast decolorized beech but not birch or pine. The above data suggest that the secretion and

type of enzymes or metabolites by these fungi could be specific to the substrate used or that the different substrates react differently with them.

Hydrophobin mutants as well as SC15 mutants of *S. commune* were able to decolorize and degrade the pine saw dust at a similar rate than the wild type strains. Also, all *S. commune* strains were able to affect the strength of wood. Maximum strength loss caused by *S. commune* strains in pine wood blocks was 35% (Fig. 9) which is in agreement with the previous studies by Schirp et al. (2003) in which these authors observed 32% loss in strength of pine wood.

In conclusion from this study, *S. commune* wild type strains and hydrophobin and SC15 single as well double mutants, monokaryons as well as dikaryons, were able to grow on beech, birch and pine wood and could cause a mild decay at least when wood was supplied in form of saw dust. All *S. commune* strains used in this study were able to cause loss in strength of compact beech, birch and pine wood blocks.

3.6 References

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Chapter 4

FTIR spectroscopy of a *S. commune* monokaryotic wild type and a coisogenic *Sc3* hydrophobin mutant

4. FTIR spectroscopy of a *S. commune* monokaryotic wild type and a coisogenic *Sc3* hydrophobin mutant

4.1 Abstract

ATR-FTIR spectroscopy was used to study a *S. commune* wild type monokaryon and a SC3 hydrophobin mutant grown in standing liquid cultures. The spectra recorded at different growth ages revealed both qualitative and quantitative differences. A clear difference in the amine peak of the FTIR spectra associated with the proteins was observed from day 5 to day 10. This difference could result from the secretion of the SC3 hydrophobin which is usually the dominating secreted protein during the formation of aerial hyphae in the wild type strain. Quantification of the peak areas by an integration method detected higher values of protein associated peaks in the wild type strain than in the hydrophobin mutant at different ages of the cultures. In contrast, higher peak area values of the polysaccharide associated peak at wave number region (1190 to 900 cm^{-1}) were noted in the SC3 hydrophobin mutant.

4.2 Introduction

4.2.1 FTIR Spectroscopy

Fourier transform infrared (FTIR) spectroscopy has been successfully used as a tool for rapid identification of various bacteria, few fungi and algae at species as well as strain level (Choo-Smith et al. 2001, Mariey et al. 2001, Naumann et al. 1991, Ngo-Thi et al. 2003). The technique is generally used to determine the chemical composition of organic compounds in samples. In combination with artificial neural networks, application of FTIR spectroscopy offers a potential method for reliable identification of micro-organisms and biodiagnagnostics (Udelhoven et al. 2000). The principle of the technique is based on the absorption of the infrared radiation by molecular bonds such as C-H, O-H, C=O, N-H, C-C, etc. Characteristic transmittance or reflection patterns can be observed due to the bending, stretching or twisting of the molecular bonds caused by the energy absorption of the sample, thus an infrared spectrum represents a fingerprint which is a characteristic of any chemical substance. Wave number (ν), the reciprocal of wave length, is generally used as a physical unit of FT-IR spectroscopy (Tseng et al. 1996, Chiriboga et al. 1998, Kümmerle et al. 1998, Günzler and Gremlich 2002, Martín et al. 2005, Rosén and Persson 2006). The overall molecular composition is different for different organisms and hence the FTIR spectra will also be different (Maquelin et al. 2003). The polysaccharides, proteins, lipids and other compounds containing distinct molecular bonds or functional groups in the biological samples absorb the energy and give absorbance at the specific wave number region of the spectra due to characteristic molecular vibrations (Wolkers et al. 2004, Naumann et al. 2005, Holme and Jelle 2006). So, this technique offers a chemically based discrimination of intact microbial cells and produces complex classic biochemical fingerprint spectra that are highly reproducible and distinct for different microorganisms (Udelhoven et al. 2000, Lasch et al. 2003, Irudayaraj et al. 2002).

4.2.2 IR Spectral regions for microorganisms

Five different regions of the mid-infrared spectra are described for microbiological samples. I. the fatty acid region (3,050 to 2,800 cm^{-1}), II. the amide region (1,750 to 1,500 cm^{-1}) where protein and peptide bands dominate, III. the mixed region (1,500 to 1,200 cm^{-1}) containing vibrations of fatty acids, proteins and polysaccharides, IV. the

polysaccharide region (1,200 to 900 cm^{-1}) and V. the species specific region (900 to 700 cm^{-1}) that is characteristic at species level (Kümmerle et al. 1998, Udelhoven et al. 2000). Multivariate data analysis techniques such as factor analysis, cluster analysis, or artificial neural network analysis are the common strategies to study the hidden information in the microbial spectral data to differentiate, identify and classify microorganisms and tissue specific substructures without prior information (Yu and Irudayaraj 2005, Lasch et al. 2002).

FTIR spectroscopy is also a powerful tool for studying the secondary structure of proteins and subtle changes in the protein structure (Troullier et al. 2000). The ATR-FTIR (attenuated total reflection Fourier transform infrared) spectroscopy mode has an advantage that the infra-red beam penetrates a short distance called penetration depth which is about 3 μm into the sample and makes the measurement of solids, liquids and powders easy and quick and thus became a very powerful method to determine the structure of biological materials, in particular the components of biological membranes like proteins that cannot be studied by x-ray crystallography and NMR (Acha et al. 2000, Vigano et al. 2000, Galichet et al. 2001, Holme and Jelle 2006). ATR occurs when a beam of radiation enters from a denser medium into a less dense medium. It has also several other advantages that it requires very low amount (10 μg) of material and does not require the knowledge of protein concentration. The differences in the concentration can be eliminated by baseline correction and normalization (Faix 1991), not disturbed by turbid samples, such as large membrane fragments or precipitates, allows the determination of the secondary structures for membrane proteins (Goormaghtigh et al. 1990, Paris et al. 2005).

4.2.3 FTIR studies on bacteria, yeast and ascomycetes

Differentiation of bacteria such as *Salmonella* (*enteritidis* and *typhimurium*), *Escherichia coli* (serotype O26, O27 and O157: H7), *Yersinia enterocolitis* and *Shigella boydii* was achieved down to the strain level by FTIR micro spectrometry (Yu and Irudayaraj 2005). Identification and discrimination at the species and strain level up to 90% was described for *Lactobacillus* isolates (Curk et al. 1994). Discrimination of four bacterial isogenic strains of *Pseudomonas aeruginosa* was possible by ATR-FTIR spectroscopy in conjunction with statistical methods

(Sockalingum et al. 1997, Bouhedja et al. 1997). Identification of 9 strains of *Saccharomyces cervisiae* was achieved by FTIR microspectroscopy (Wenning et al. 2002). More recently, the intra-specific characterization of the airborne filamentous fungi *Aspergillus* and *Penicillium* was demonstrated on the genus, species and strain level (Fischer et al. 2006). The strains with mutations of biochemical characters such as slime production can have significantly different FTIR spectra due to major changes in cellular compositions (Kümmerl et al. 1998). Structural modifications in the cell wall of the mutated yeast strain AGLSC have been studied by FTIR spectroscopy (Galichet et al. 2001). Characterization and identification of moulds grown on different media is possible by using ATR-FTIR spectroscopy (Holme and Jelle 2006). The ATR technique has also been used to study the structure, orientation and tertiary structures in peptides and membrane proteins during the last years (Vigano et al. 2000). Screening large numbers of plants for a broad range of cell wall phenotypes has also been in practice by using FTIR spectroscopy (McCann et al. 2001).

4.2.4 FTIR studies on basidiomycetes

FTIR studies concerning the basidiomycete fungi have also been reported (Naumann et al., 2005, Holme and Jelle 2006), but they are very few. Genetically defined variation through mutation approaches offers best opportunity to identify a broad range of structural alterations in cell walls leading to the improved understanding of fungal growth. Our study is the first report describing FTIR studies of a fungal hydrophobin mutant. During mycelial growth, hydrophobins are the most abundant secreted proteins in cultures of *Schizophyllum commune* (Wessels 1999, Wösten et al. 1999, Wösten 2000). Disruption of *Sc3* hydrophobin gene has affected the cell wall composition in *S. commune* (van Wetter et al. 2000). The amount of mucilage [water soluble (1-3) β -glucan with single residues attached by (1-6) β-linkages)] secreted into the medium has increased dramatically in the *Sc3* knock out mutant (van Wetter et al. 2000). Molecules of the cell wall may provide mechanical strength, porosity and can control cell adhesion. The functions may not be only mechanical, but could also be biological (McCann et al. 2001).

In this study, we applied a quantitative integration method to a set of ATR-FTIR spectra recorded on fungal mycelium grown in liquid culture in order to later apply the technique to study the fungal growth on the surface and inside the wood (see chapter 5 of this thesis, Naumann et al. 2005). We wish to address the following questions, Is it possible to discriminate the wild type and its coisogenic SC3 hydrophobin mutant of *S. commune* with regard to protein and polysaccharide also with respect to the different growth age of cultures? Can FTIR spectra be used to identify the differences in the age of the fungal strains?

4.3 Materials and methods

4.3.1 Fungal strains

Schizophyllum commune wildtype monokaryotic strain 4-39 (*MATA41 MATB41*, CBS 341.81) and the coisogenic hydrophobin mutant 72-3 (Δ *Sc3 MATA41 MATB41*) characterized by a disrupted *Sc3* gene (Wessels et al. 1991, van Wetter et al. 1996) were kindly provided by Prof. Wösten, Utrecht, The Netherlands. *Trametes versicolor* 6 originated from the institute's collection is used as a general control.

4.3.2 Growth conditions

Strains were grown at 28 ^{o}C in dark and cultivated on BSM [Basidiomycete standard medium; 5g glucose monohydrate, 0.65g L-asparagine 1g KH_2PO_4, 0.5g $MgSO_4$ x $7H_2O$, 0.5g KCl, 0.5g yeast extract, 10g agar, 50 ml stock solution I (0.2g $FeSO_4$ x $7H_20$ per liter), 50ml stock solution II (0.16g $Mn(CH_3COO)_2$ x $4H_2O$, 0.04g $Zn(NO_3)_2$ x $4H_2O$, 1g $Ca(NO_3)_2$ x $4H_2O$, 0.06g $CuSO_4$ x $5H_2O$) per 1 liter H_2O, pH 4.5; Hüttermann and Volger 1973] liquid medium as standing cultures in 500 ml Erlenmeyer flasks containing 100 ml of the culture medium, inoculated with 10 ml homogenized mycelial macerate. Three replicates were grown for each harvest from each strain.

4.3.3 Culture harvest and mycelia grinding

Harvest of complete cultures was done on day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45 and 60. Mycelium was separated from the culture medium with a nylon mesh (0.3 mm pore size) in a funnel connected to vacuum. The scrapped mycelium

was collected into an Eppendorf tube and freeze dried in a lyophilisator. The dry weights of the mycelium were noted to determine growth curves (Fig. 1). Mycelium was grinded (about 1 hr per each sample) with a ball mill using tungsten carbide beads of 3 mm size.

4.3.4 ATR - FTIR Spectroscopy

All the powdered samples were left overnight at the temperature of 20 ^{o}C with the FTIR spectrometer to avoid temperature and moisture shifts during the measurements. About 1 mg of the mycelial powder was used for measuring the spectra. FTIR spectra of the mycelium were recorded with the FTIR spectrometer Equinox 55 (Bucker, Germany) combined with an attenuated total reflection (the infrared beam is attenuated and totally reflected) unit (Dura SamplIR). Conditions: 32 scans at 4 cm $^{-1}$ resolution. The mycelial powder was pressed onto the crystal where the infrared beam is attenuated at the interface between the sample and the crystal. Three measurements were made for three replicates of each harvest day from each strain.

4.3.5 Data analysis

FTIR spectra coming from the same day for a strain (i.e. in total 3 values) were baseline corrected, vector normalized and averaged. Spectral data were evaluated using the OPUS version 5.0 software (Bruker, Germany). Baseline correction was done by the rubber band method using 64 baseline points. Quantification of peak areas associated with proteins and polysaccharides was performed by integration method A described in the OPUS version 5.0 software, Bruker, Germany (Fig. 2). For integration of the peaks, the 3 replicate spectra from each growth day of each strain were vector normalized, baseline corrected and integrated.

4.3.6 Statistical analysis

Statistics was performed by using SPSS software release 9.0.0, standard version (SPSS Inc.). The individual peak area values from each replicate were used for t-test ($P<0.05$). In case of more than two data sets, data were compared using Duncan multiple range test up to 95% confidence level ($p\leq0.05$).

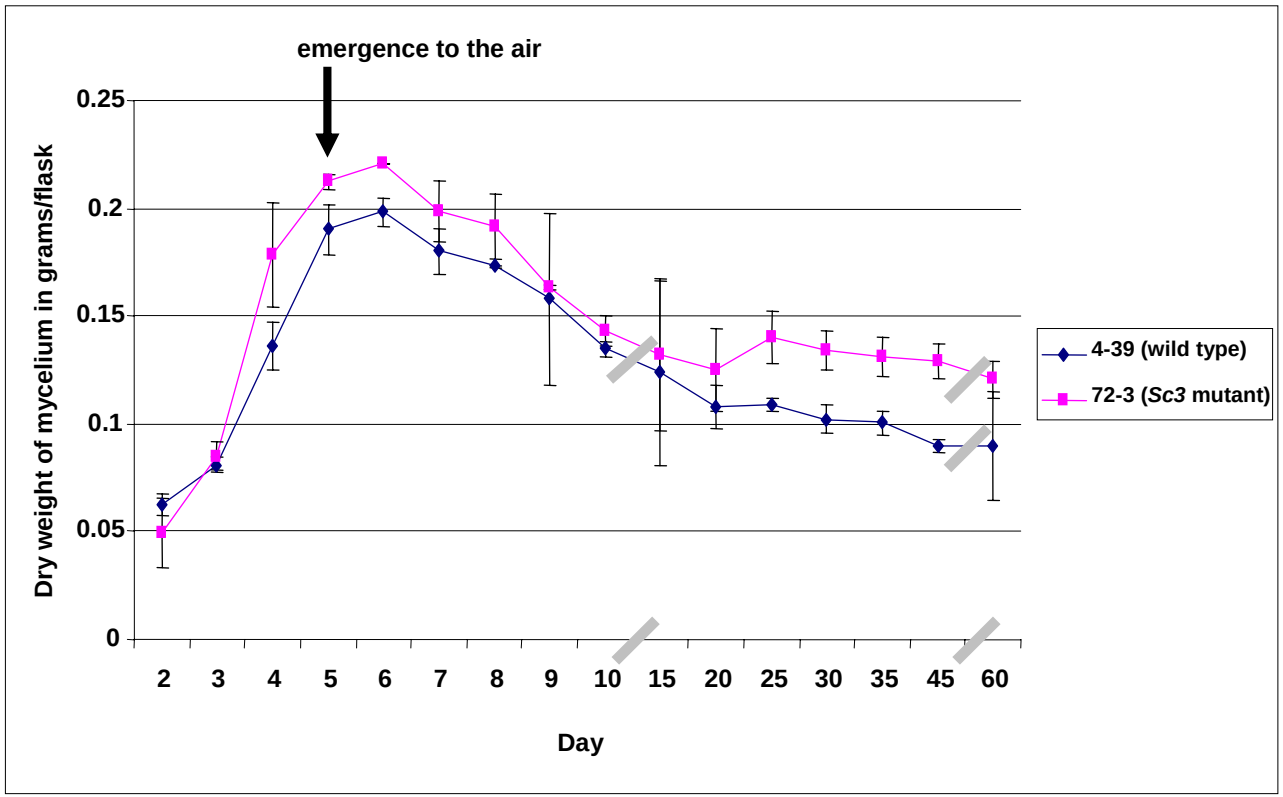


Fig. 1 Biomass production of *S. commune* monokaryon 4-39 and its coisogenic *Sc3* hydrophobin knockout mutant 72-3 over the time grown in standing BSM liquid medium at 28°C in dark condition. Each three 100ml cultures per day were analysed and average values and standard deviations calculated. The arrow indicates the day of emerge of mycelium into the air in the cultures of the monokaryon 4-39. Cultures of mutant 72-3 did not form aerial mycelium.

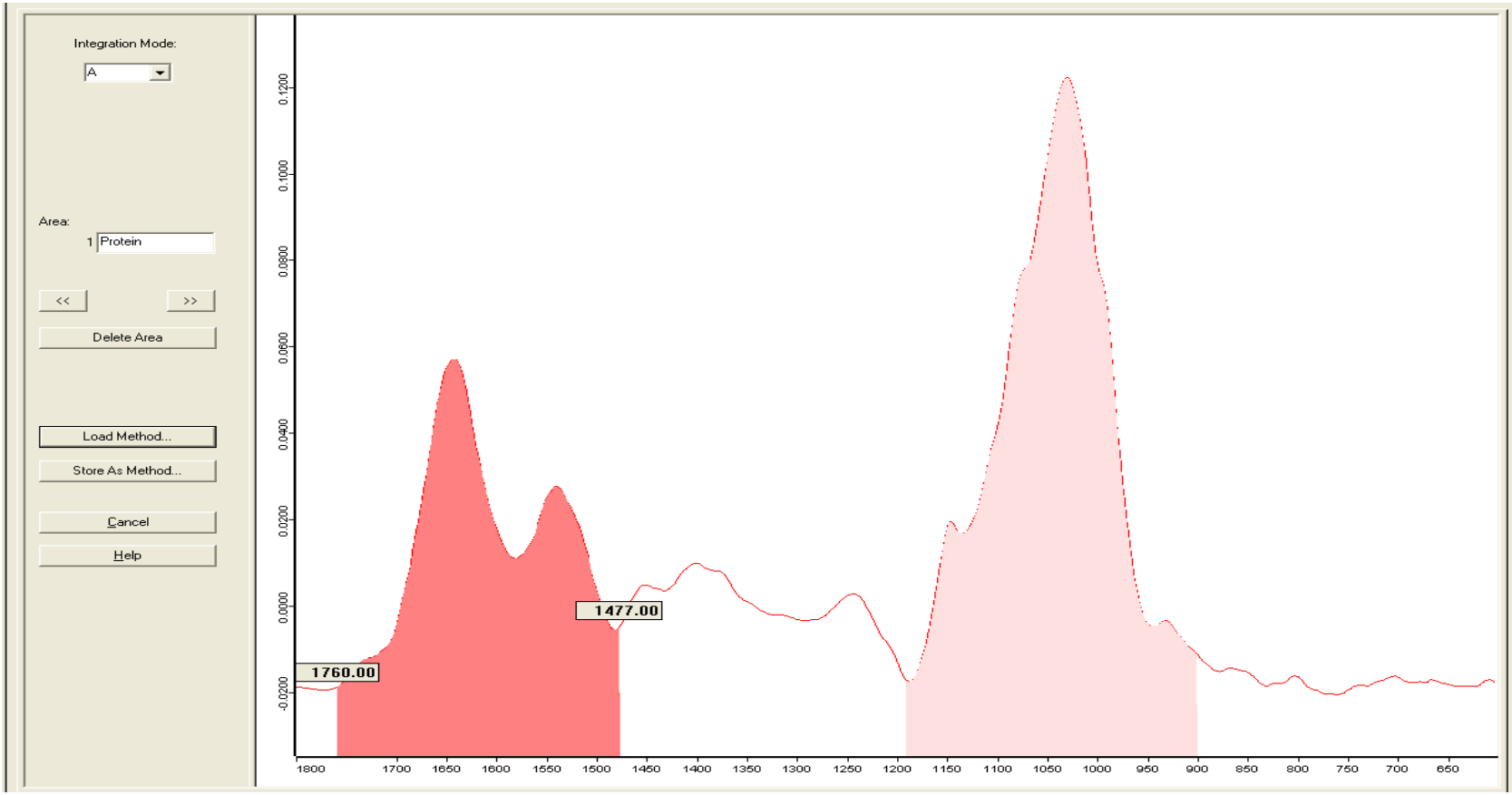


Fig. 2 Interactive set up method A for the integration of the peaks from the FTIR spectral regions associated with proteins (1760-1477 cm^{-1}) and polysaccharides (1190-900 cm^{-1}, values not marked in the this figure due to limitation of the software to display more than two values).

4.4 Results

The overall growth curve over the time of *S. commune* monokaryon 4-39 and the co-isogenic *Sc3* hydrophobin mutant followed the same pattern with exponential phase from day 3 to 5 of incubation, a growth peak at day 6 and a rapid decline of biomass afterwards (Fig. 1). The FTIR spectra of the *S. commune* wild type and hydrophobin mutant from the different age groups are shown in Fig. 3 and 4. Important differences were observed in FTIR absorption spectra between the wild type and the hydrophobin mutant from day 5 to day 10 particularly in the amide region of the spectra i.e., 1760 to 1477 cm^{-1} where protein and peptide bands dominate (Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005, Fig. 5e to 5j). These differences could result from the secretion of *Sc3* hydrophobin which is usually dominating during the formation of aerial hyphae in the wild type strain.

The differences in the appearance or disappearance of the peaks with bands numbered 1 to 6 in Fig. 3 and 4 indicates the possibility of this method to identify the changes occurring in the fungal cell walls in different age points. Comparison of the spectra of wild type and hydrophobin mutant showed both qualitative and quantitative differences at different growing ages (Fig. 5, Table 1 and 2). Integration of the peak areas associated with proteins (spectral region 1760 to 1477 cm^{-1}) showed higher values of wild type strain compared to the *Sc3* hydrophobin mutant strain (Table 1). Higher differences among peak areas were observed on day 9 and 10. In contrast to the protein peak area values, the mutant showed higher peak areas of the polysaccharide region (spectral region 1190 to 900 cm^{-1}) than the wild type strain over the whole growth period (Table 2).

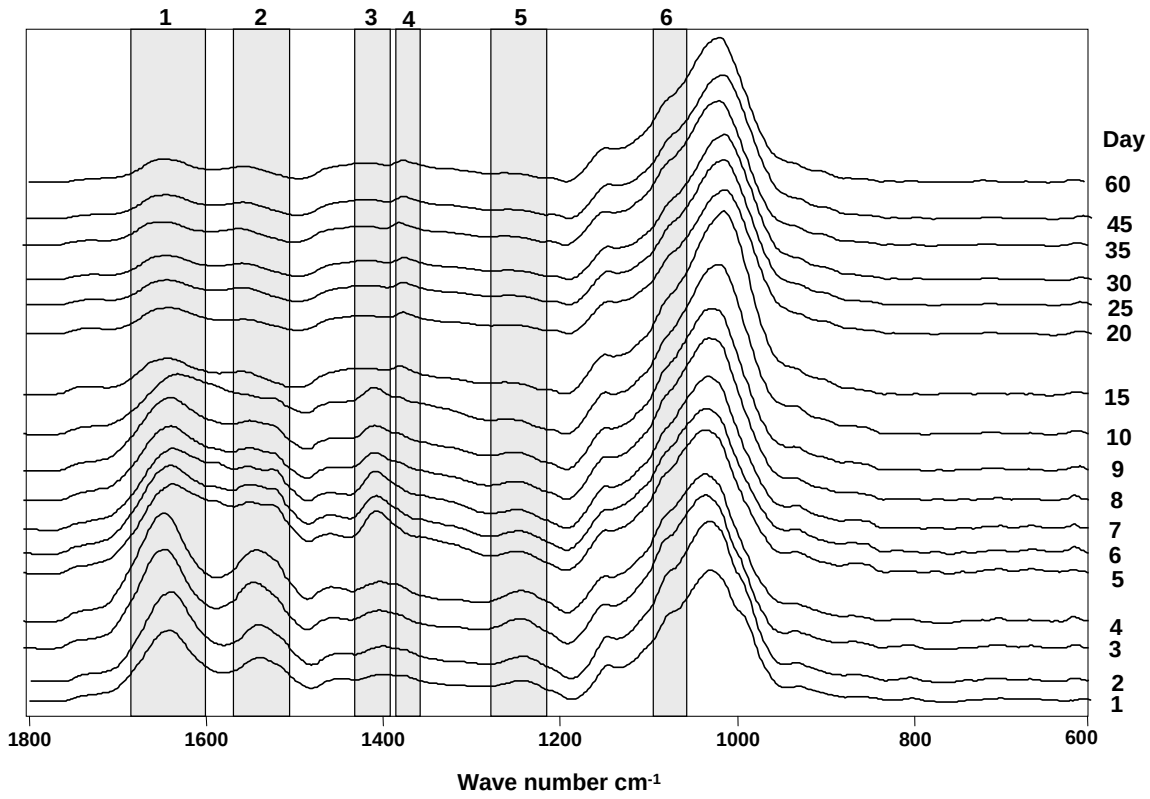


Fig. 3 ATR-FTIR spectra (baseline corrected and vector normalized) of the *S. commune* wild type 4-39 at different age growth. Bands marking regions1 to 6 indicate the differences in the peak intensities, appearance or disappearance of peaks in the FTIR spectral regions (1. 1680-1600 cm $^{-1}$ for amide I, 2. 1580-1500 cm $^{-1}$ for amide II, 3. 1432-1365 cm $^{-1}$ & 4. 1391-1361 cm $^{-1}$ for mixed region of fatty acids, proteins and polysaccharides, 5. 1272-1210 cm $^{-1}$ for amide III and 6. 1090-1060 cm^{-1} for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000,Yu & Irudayaraj 2005)].

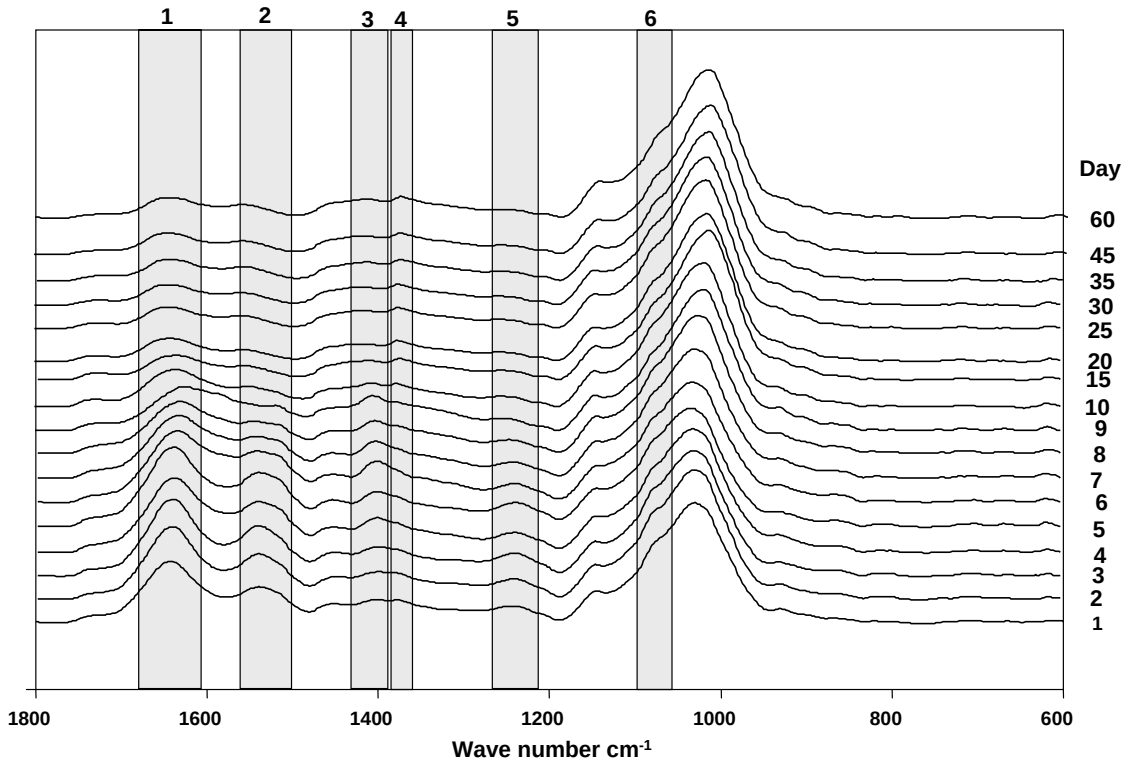
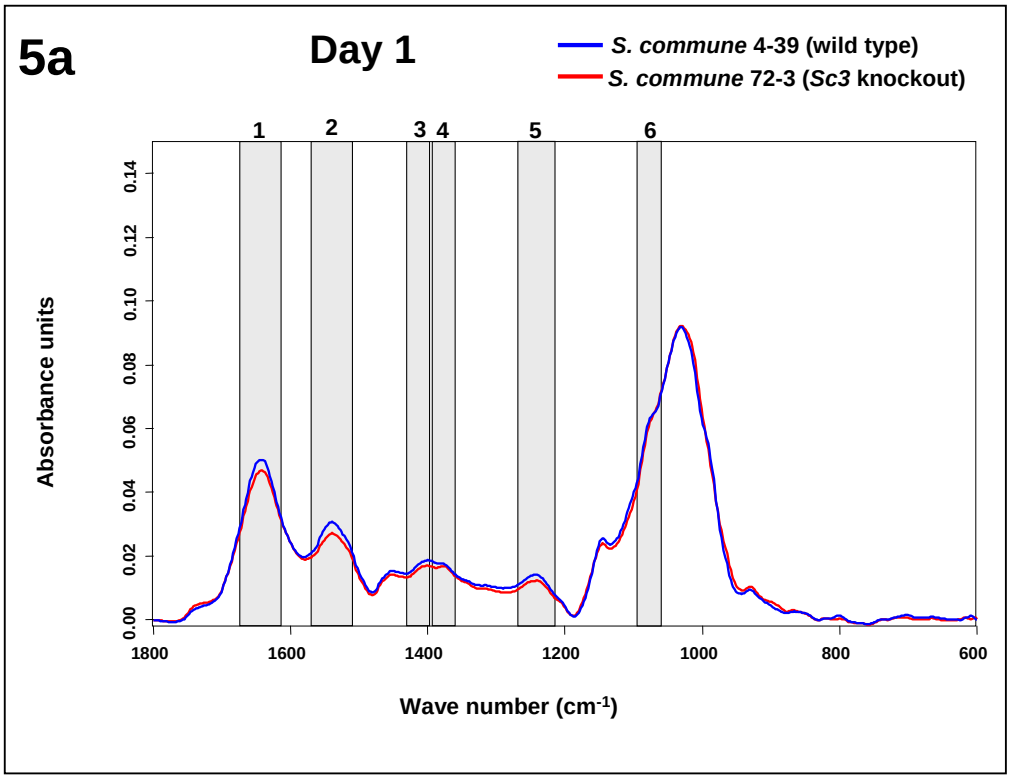
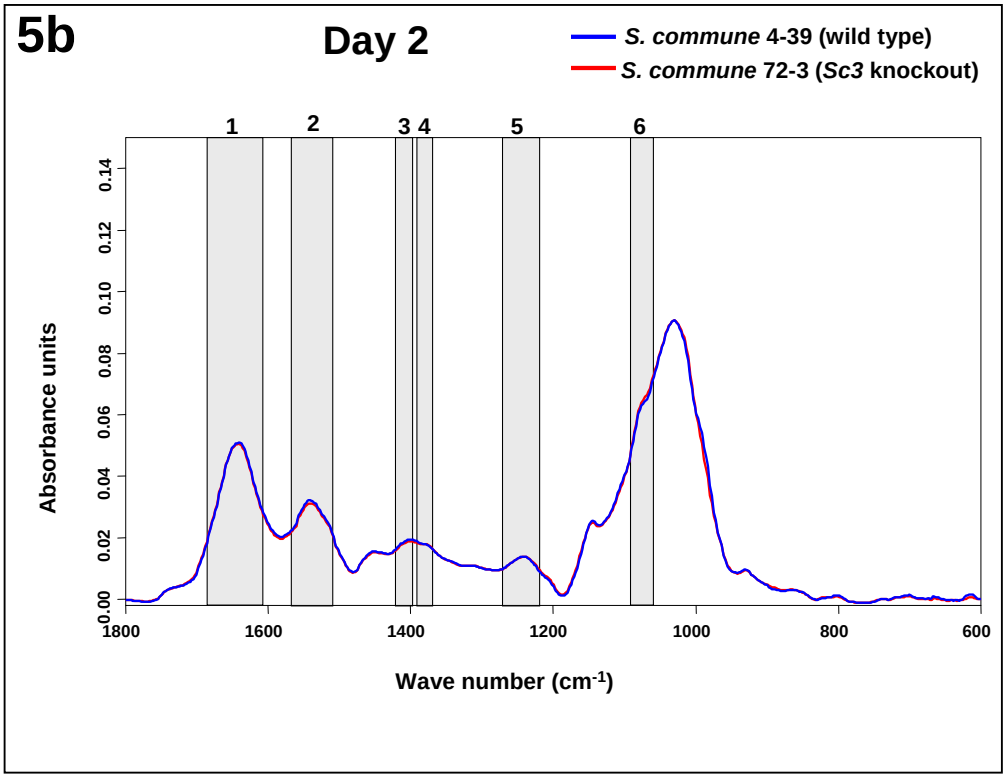
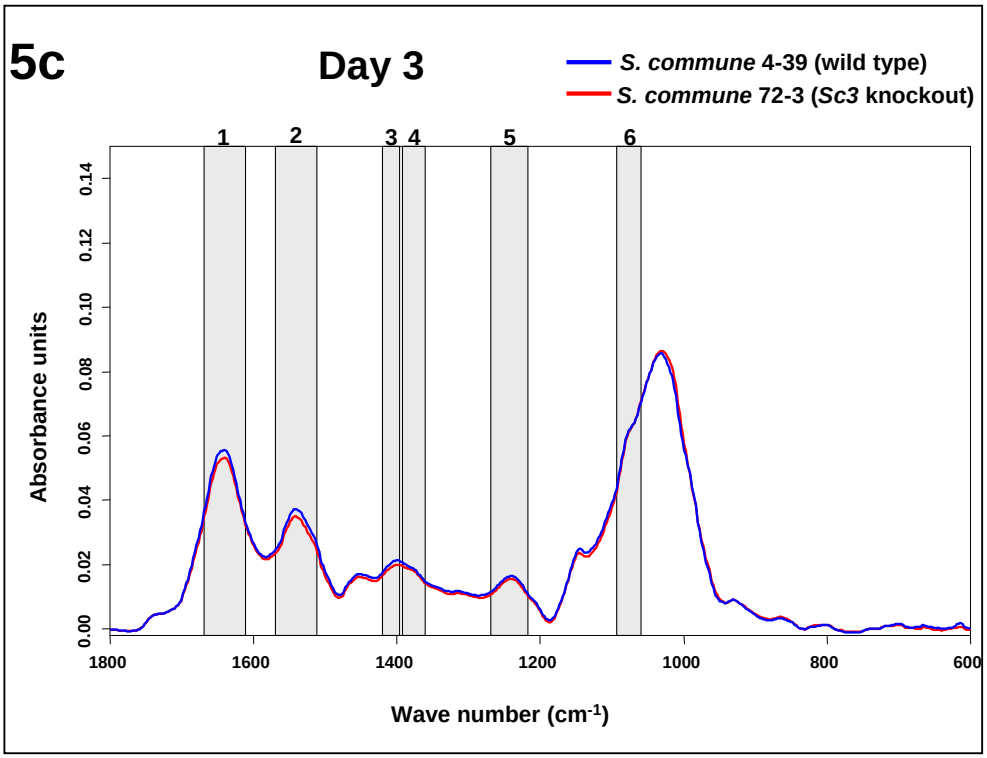
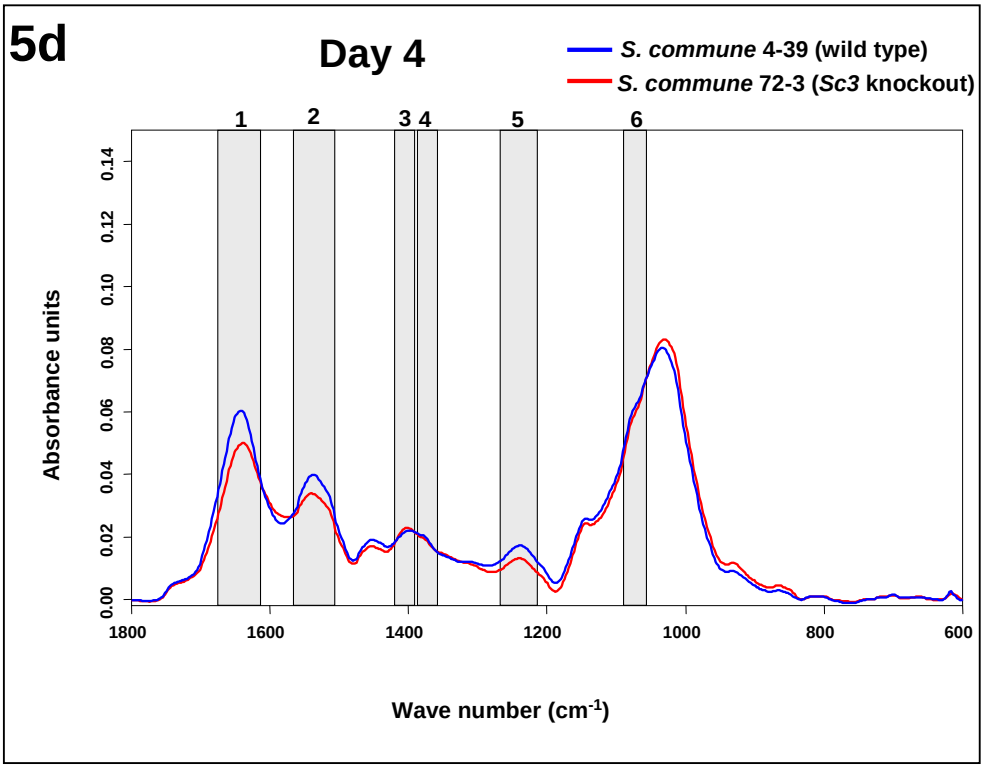


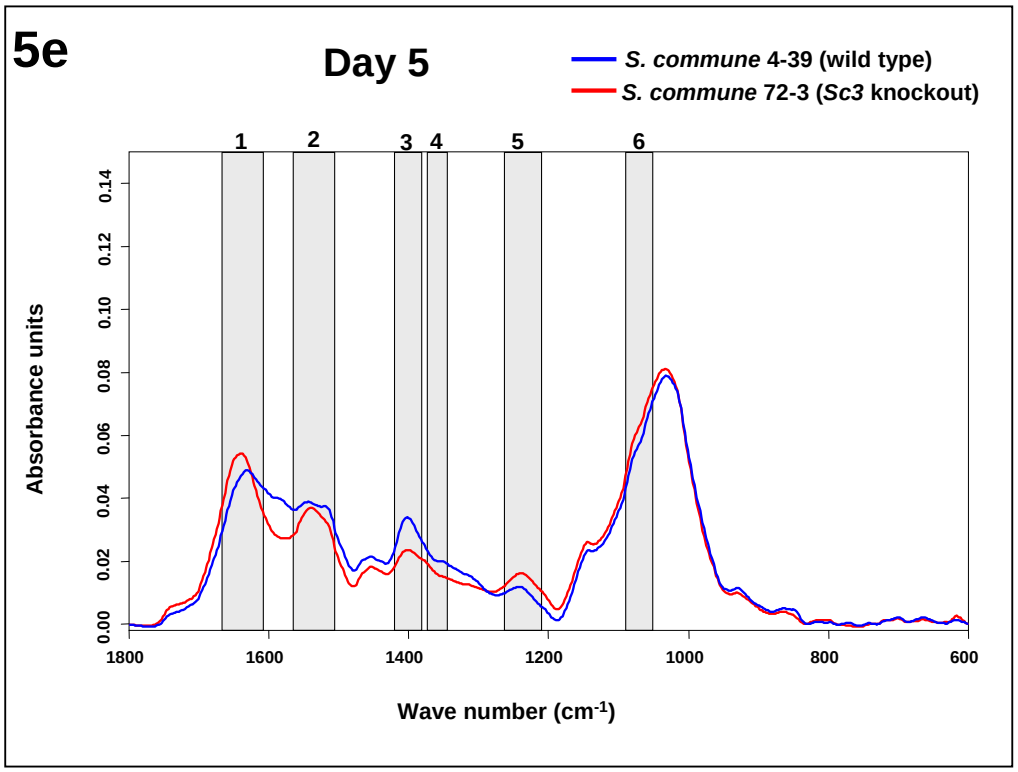
Fig. 4 ATR-FTIR spectra (baseline corrected and vector normalized) of the *S. commune Sc3* mutant 72-3 (*ΔSc3* 4-39) at different age growth. Bands 1 to 6 indicate the differences in the peak intensities, appearance or disappearance of peaks in the FTIR spectral regions (1. 1680-1600 cm $^{-1}$ for amide I, 2. 1580-1500 cm $^{-1}$ for amide II, 3. 1432-1365 cm $^{-1}$ & 4. 1391-1361 cm $^{-1}$ for mixed region of fatty acids, proteins and polysaccharides, 5. 1272-1210 cm $^{-1}$ for amide III and 6. 1090-1060 cm $^{-1}$ for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005)].

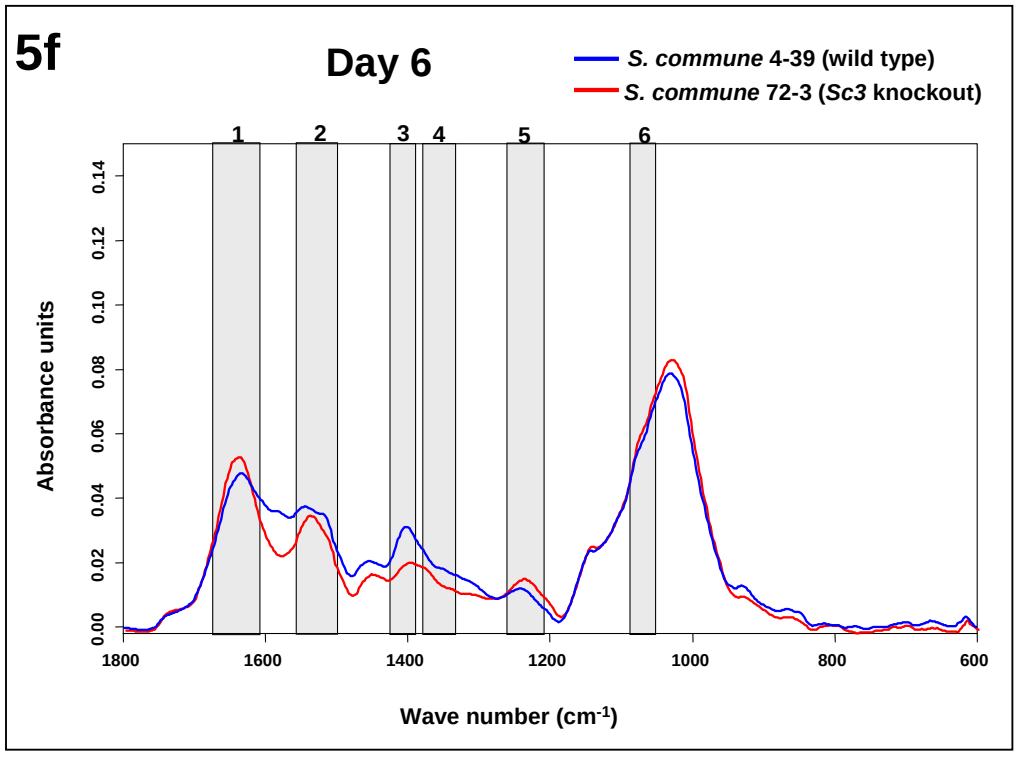

5a
Day 1
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

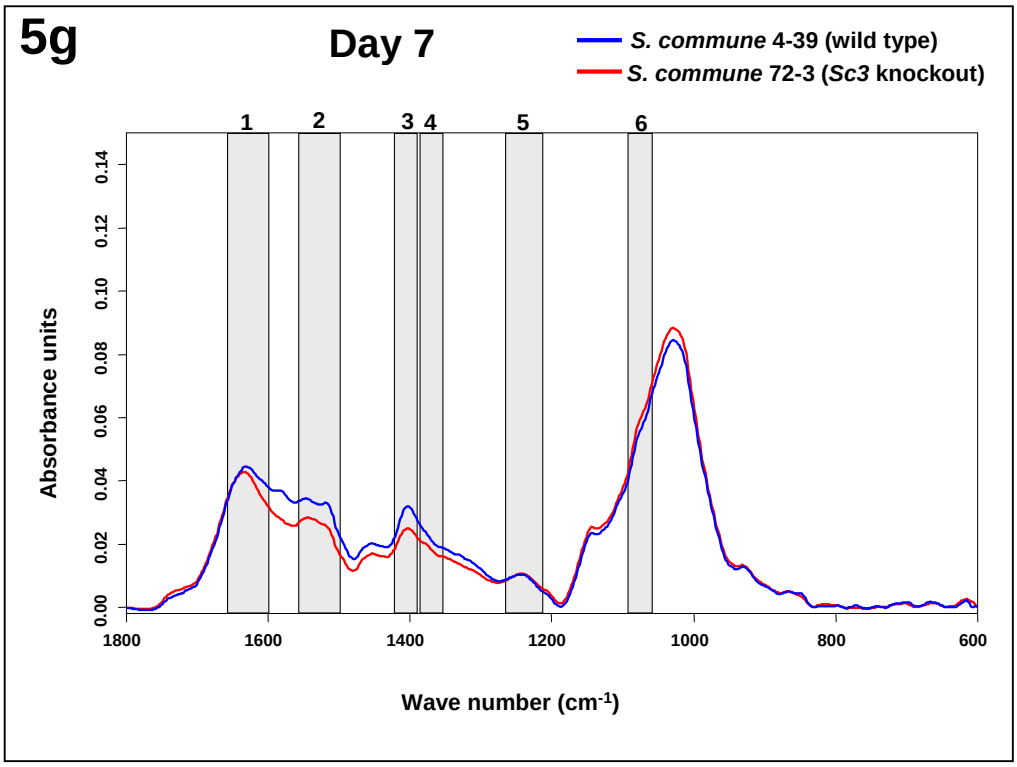

5b
Day 2
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

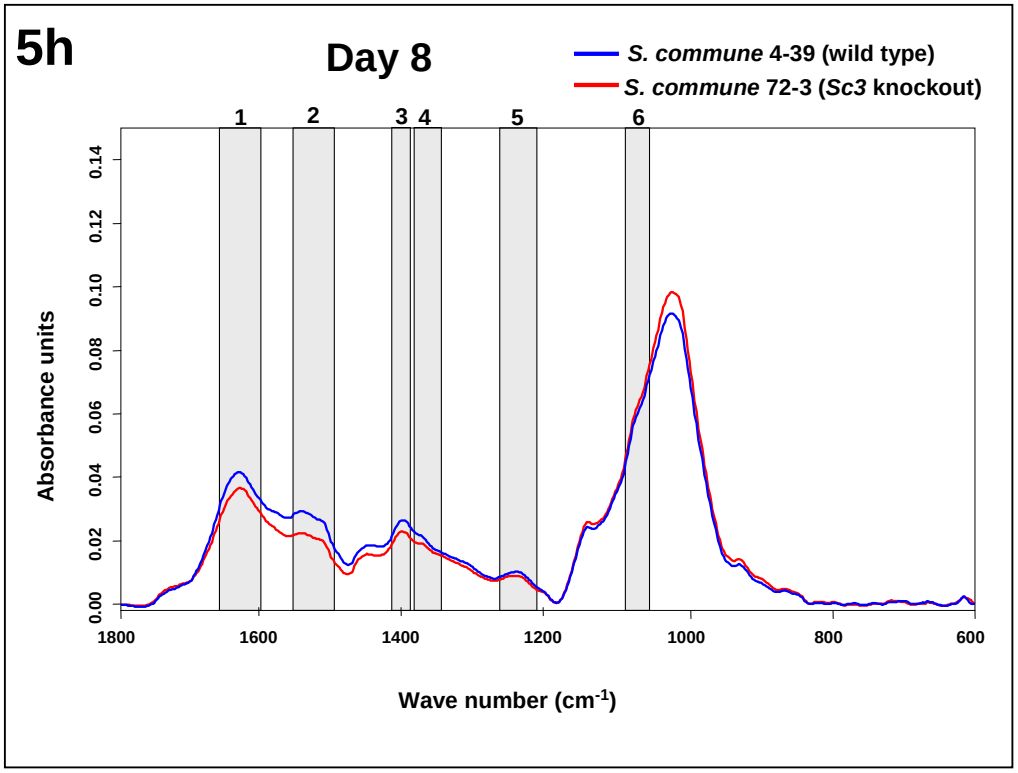

5c
Day 3
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

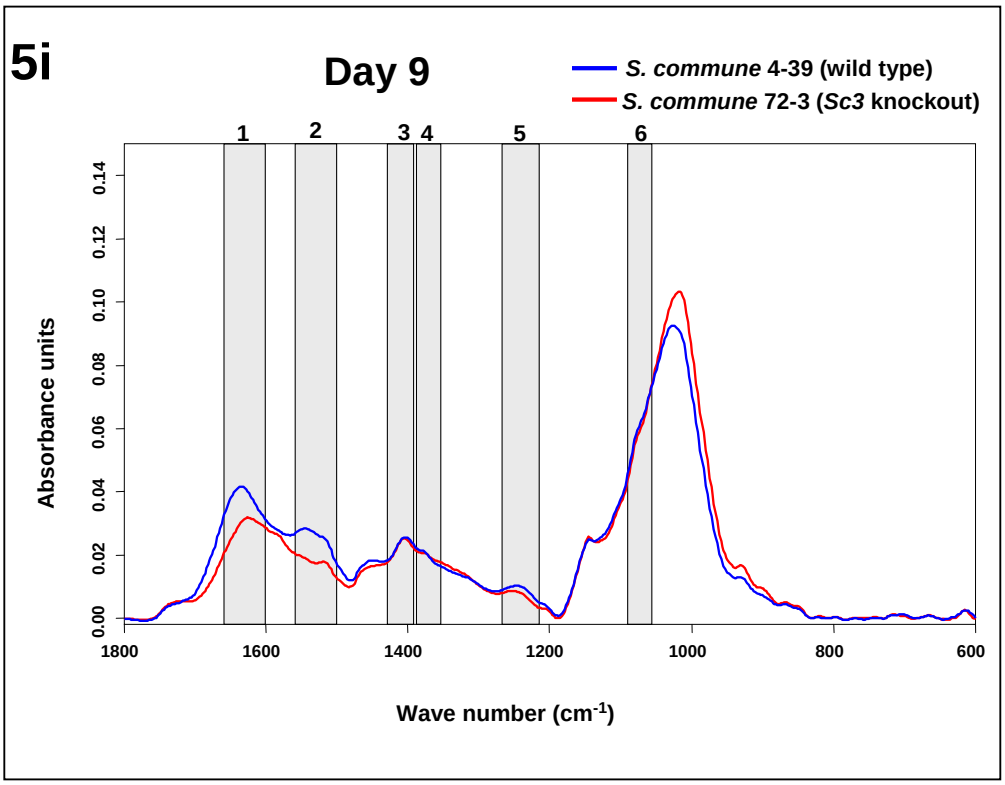

5d
Day 4
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

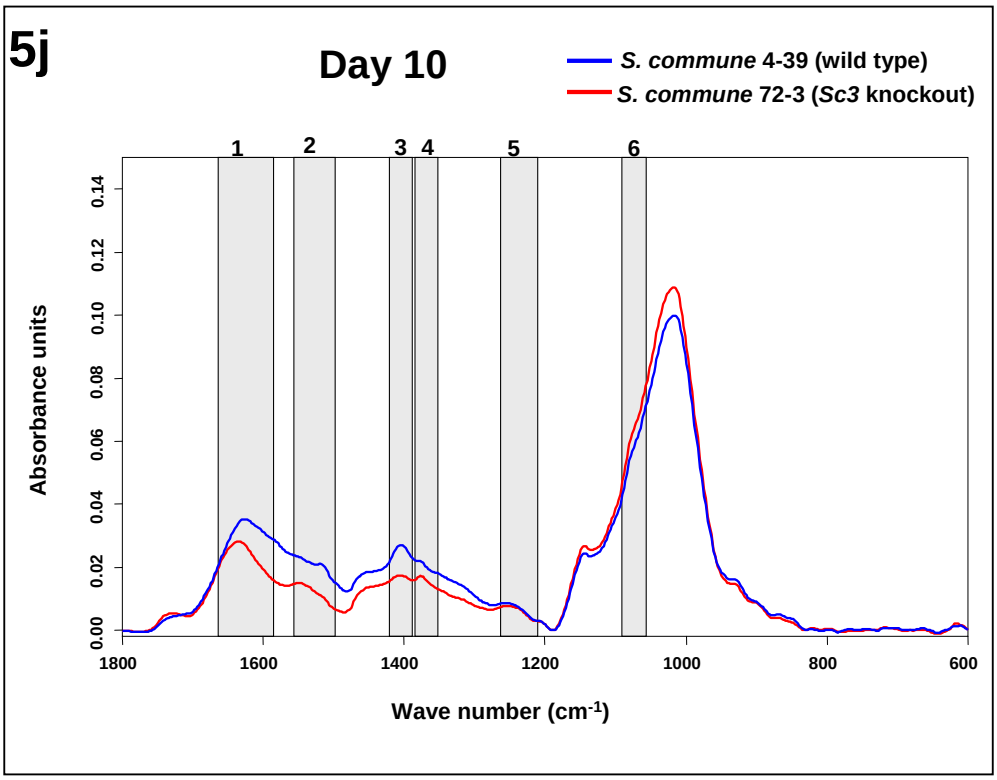

5e
Day 5
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

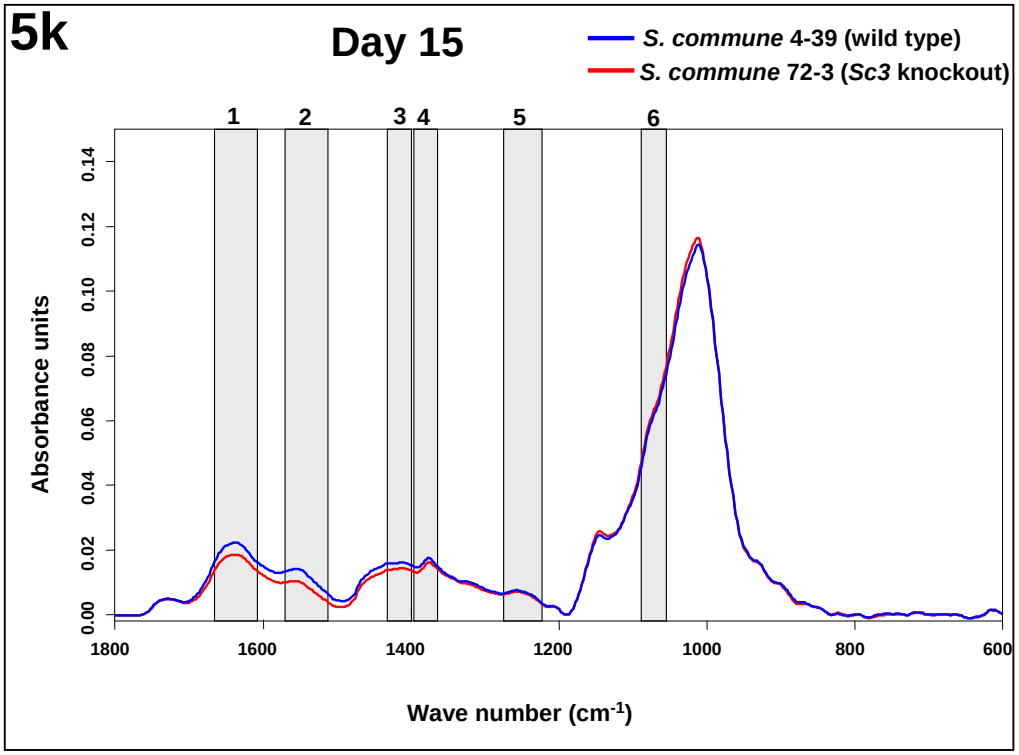

5f
Day 6
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

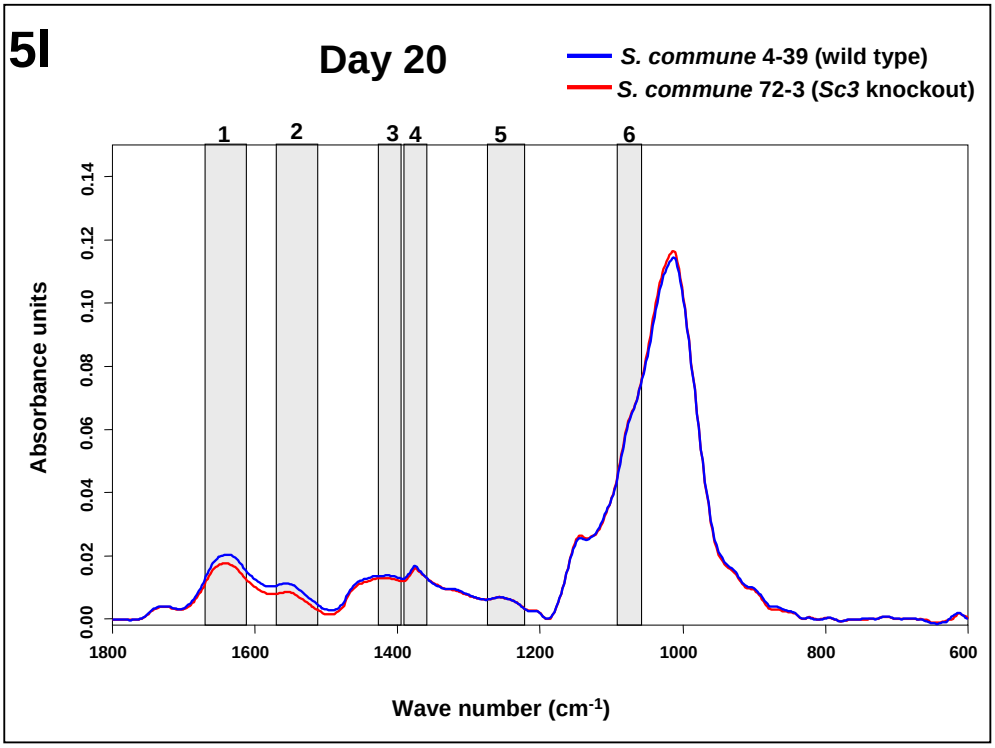

5g
Day 7
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

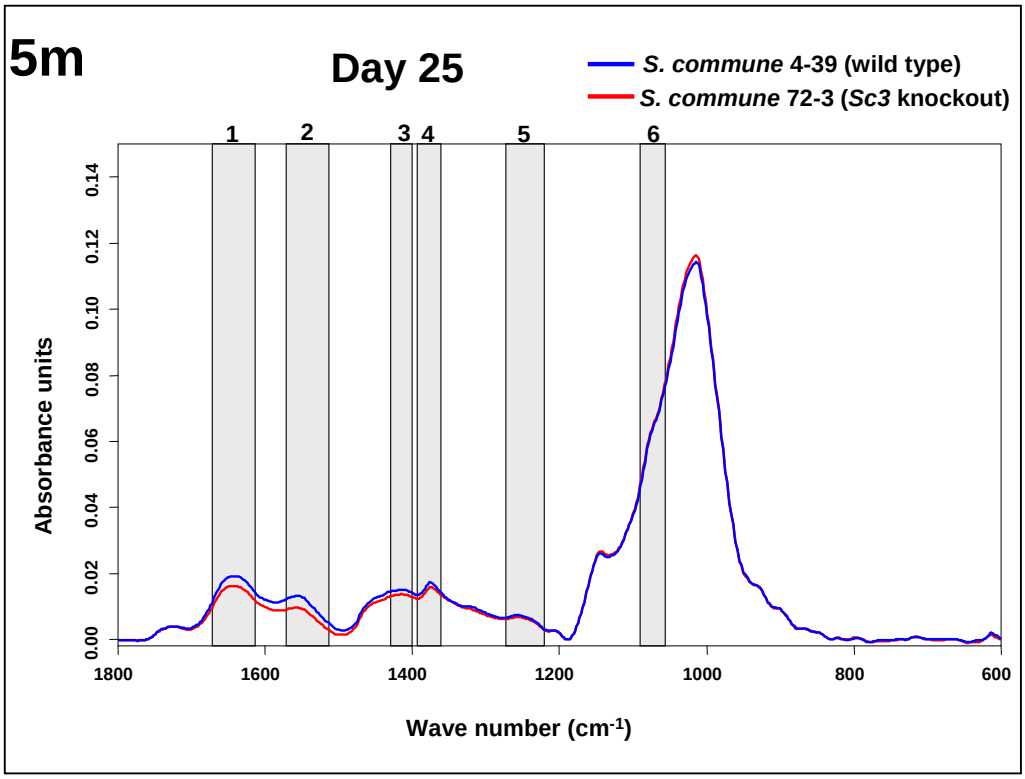

5h
Day 8
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

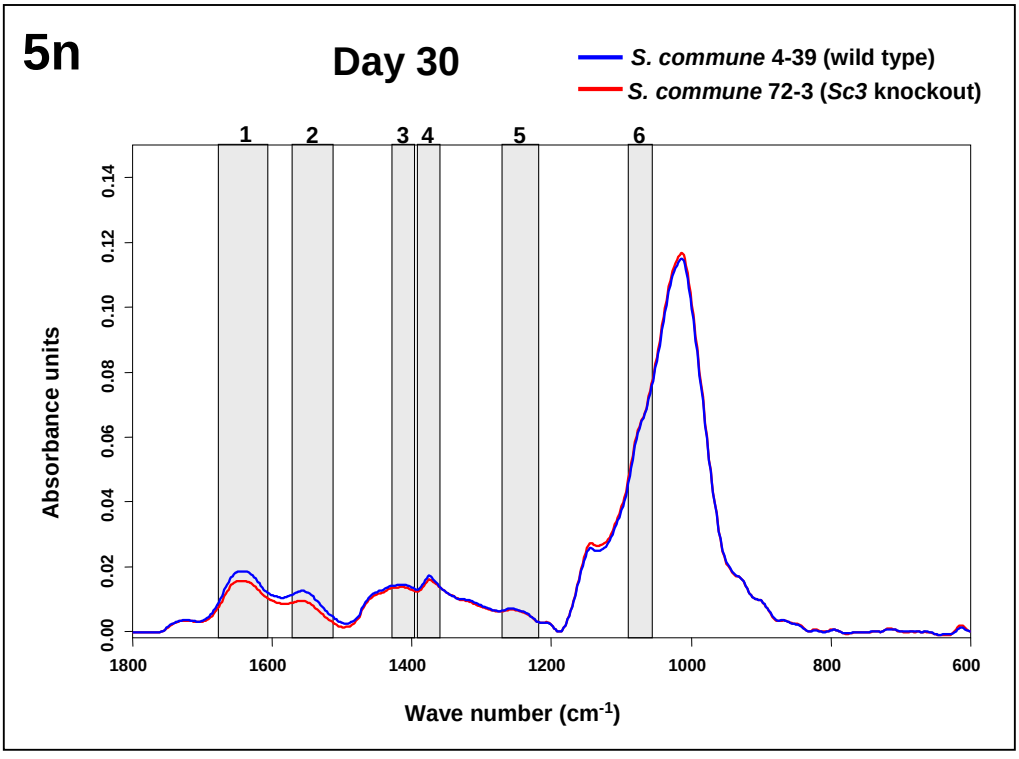

5i
Day 9
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

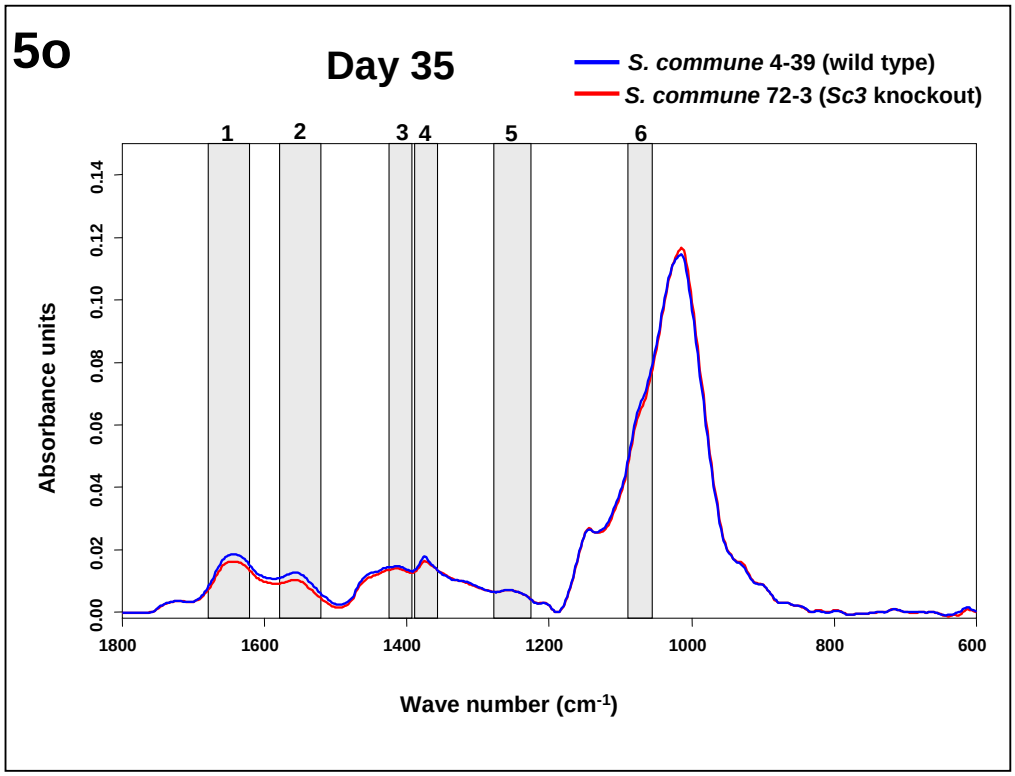

5j
Day 10
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

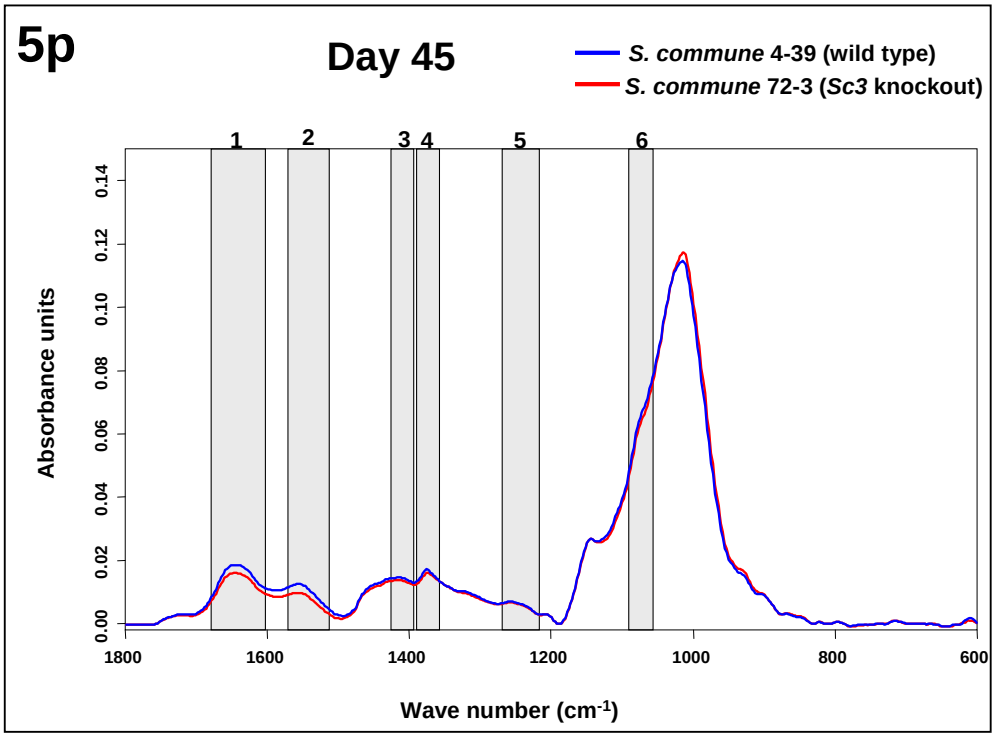

5k
Day 15
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)


5l
Day 20
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)


5m
Day 25
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)


5n
Day 30
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)


5o
Day 35
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)


5p
Day 45
S. commune 4-39 (wild type)
S. commune 72-3 (Sc3 knockout)
1
2
3
4
5
6
Absorbance units
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1800
1600
1400
1200
1000
800
600
Wave number (cm-1)

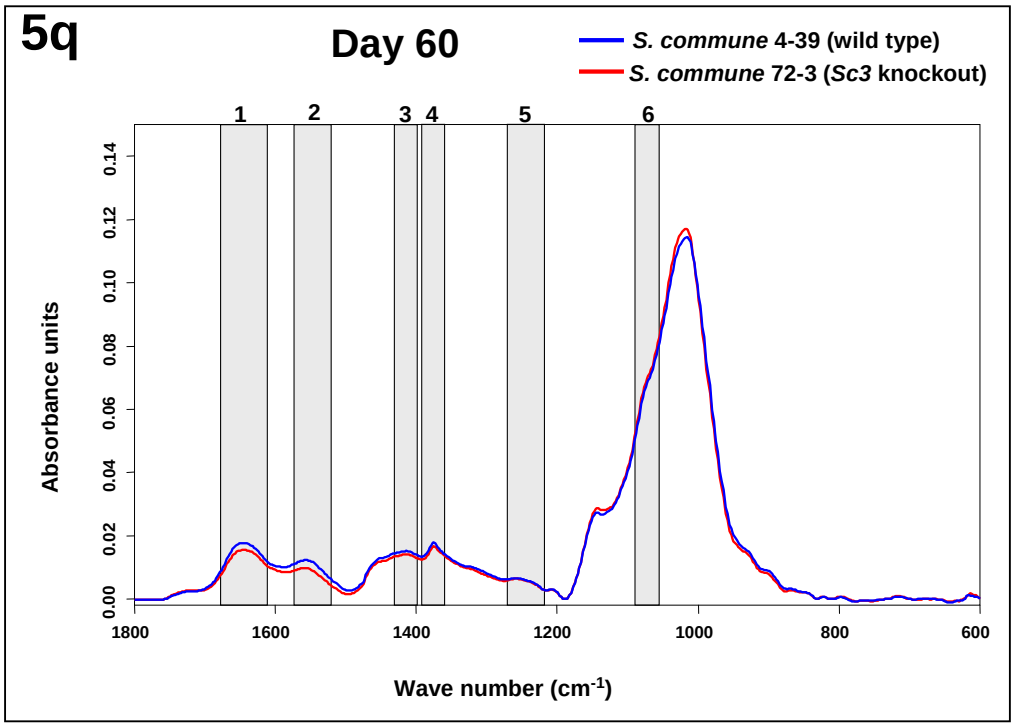


Fig. 5 Comparison of the ATR-FTIR spectra (vector normalized and base line corrected) of the *S. commune* 4-39 (wild type) and *Sc3* knockout mutant 72-3 (*ΔSc3* 4-39) grown in standing liquid structures at 28 C in dark from day 1 to day 60 of cultivation. Bands 1 to 6 indicate 1. 1680-1600 cm $^{-1}$ for amide I, 2. 1580-1500 cm $^{-1}$ for amide II, 3. 1432-1365 cm $^{-1}$ & 4. 1391-1361 cm $^{-1}$ for mixed region of fatty acids, protein and polysaccharides, 5. 1272-1210 cm $^{-1}$ for amide III and 6. 1090-1060 cm $^{-1}$ for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005)]. A summary of all spectra is shown as an overview in Fig. 8 in the appendix to this chapter.

Table 1 Comparison of peak area values of the protein part of the FTIR spectra (spectral region 1760 to 1477 cm $^{-1}$) from the mycelium of wild type and the *Sc3* hydrophobin mutant of *Schizophyllum commune* as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral region representing the peaks associated with amide I and amide II bands of the protein were used for integration.

Day of growth	***S. commune* wild type**	***S. commune Sc3* mutant**	**Difference of protein associated peak area**	**More area of protein associated peak in**	**Statistically significant difference**
1	6.7682	6.2219	0.5463	wild type	No
2	7.7218	6.8736	0.8482	wild type	Yes
3	8.1511	7.1684	0.9827	wild type	Yes
4	7.4917	7.1060	0.3857	mutant	No
5	6.5179	6.5840	0.0661	mutant	No
6	7.0272	6.1450	0.8822	wild type	No
7	7.1420	6.3430	0.799	Wild type	No
8	6.2741	5.2765	0.9976	wild type	Yes
9	6.1794	4.7005	1.4789	wild type	Yes
10	5.3012	3.7134	1.5878	wild type	Yes
15	3.0455	2.4262	0.6193	wild type	Yes
20	2.5535	2.1030	0.4505	wild type	Yes
25	2.5745	2.0640	0.5105	wild type	Yes
30	2.4478	1.9886	0.4592	wild type	Yes
35	2.4428	2.1039	0.3389	wild type	Yes
45	2.4229	2.0096	0.4133	wild type	Yes
60	2.3309	1.9674	0.3635	wild type	Yes

Table 2 Comparison of peak area values of the polysaccharide part of the FTIR spectra (spectral region1190 to 900 cm $^{-1}$) from the wild type and the *Sc3* hydrophobin mutant of *S. commune* as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral region 1190 to 900 cm $^{-1}$ was used for integration.

Day of growth	***S. commune* wild type**	***S. commune Sc3* mutant**	**Difference of polysaccharide peak area**	**More area of polysaccharide associated peak in**	**Statistically significant difference**
1	10.7192	10.8821	0.1629	Sc3 mutant	No
2	10.3598	10.6327	0.2729	Sc3 mutant	Yes
3	9.9530	10.4370	0.484	Sc3 mutant	Yes
4	10.4092	10.4916	0.0824	Sc3 mutant	No
5	10.9773	11.0367	0.0594	Sc3 mutant	No
6	10.5648	11.0499	0.4851	Sc3 mutant	No
7	10.54	10.9732	0.4332	Sc3 mutant	No
8	11.2829	11.9982	0.7153	Sc3 mutant	Yes
9	11.4979	12.4231	0.9252	Sc3 mutant	Yes
10	12.1726	13.0464	0.8738	Sc3 mutant	Yes
15	13.3619	13.5976	0.2357	Sc3 mutant	No
20	13.6399	13.7679	0.128	Sc3 mutant	Yes
25	13.4985	13.6781	0.1796	Sc3 mutant	No
30	13.5807	13.8025	0.2218	Sc3 mutant	Yes
35	13.5699	13.5786	0.0087	Sc3 mutant	No
45	13.6864	13.7978	0.1114	Sc3 mutant	No
60	13.7716	13.8613	0.0897	Sc3 mutant	No

4.5 Discussion

This study was performed in order to check whether FTIR spectroscopy can be used to differentiate the mycelium of a *Schizophyllum commune* wild type and a coisogenic hydrophobin mutant strains and in order to determine how cultural age will influence this. Previously, we published from fungal infected wood that FTIR spectra recorded from the fungal mycelium and the wood can be distinguished (Naumann et al. 2005). Knowing the differences among the wild type and the hydrophobin mutant will be very useful in studying the effect of hydrophobin during the growth of fungus inside wood. Hydrophobins are secreted by the fungus at their apices which self-assemble and coat the fungal hyphae (Wessels 1997, Wösten 2001, Walser et al. 2003). The *Sc3* hydrophobin is the most abundant protein produced by the *S. commune* wild type strain during aerial hyphae formation (Wessels 1997, Wösten 2001). Previous studies by van Wetter et al. (1996, 2000) showed that the disruption of the *Sc3* hydrophobin gene resulted in the hindrance of the aerial growth and the production of large amounts of mucilage into the liquid cultures. Our results support these previous studies by van Wetter et al. (2000). We observed in the FTIR spectra higher values of the peak areas of the polysaccharide associated peaks from the mycelium of the *Sc3* hydrophobin mutant than in the FTIR spectra of the corresponding wild type strain, particularly between day 5 to day 10 of incubation (Fig. 5e to 5j and Table 2) indicating that the hydrophobins affect the cell wall composition. Highest differences between the wild type and hydrophobin mutant of the protein and polysaccharide peak areas were observed in the cultures of day 9 and 10 (Table 1 and 2). Further to this, the spectra of both the wild type and the hydrophobin mutant showed changes in the intensity/appearance or disappearance of the peaks with the increasing culture age, which can indicate the possibility of the production of different proteins or polysaccharides or other metabolites specific to the fungal strains (Fig. 3 and 4).

4.6 References

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4.7 Appendix

T. versicolor 6 was used as a general control in the experiment and the details are presented as attachments to chapter 4. Although the growth peak of *T. versicolor* 6 occurred on day 7 of cultivation one day later than those in the *S. commune* cultures, the overall growth curve of *T. versicolor* 6 resembled that of the *S. commune* strains with a fast increase of biomass production, a one day peak of highest biomass and a rapid decline in biomass. The dry biomass of *T. versicolor* 6 was generally higher during the exponential growth stage than that of *S. commune* strains (Fig. 6). In general, as calculated by the integration method A, the mycelial protein content was significantly lower over the time in the cultures of *T. versicolor* 6 when compared with *S. commune* strains (Fig. 9, Table 3 and 4) and the polysaccharide contents were higher than in *S. commune* strains (Table 5 and 6). On the whole, the FTIR method worked for differentiating the fungi at species as well as strain level with respect to the protein and polysaccharides levels.

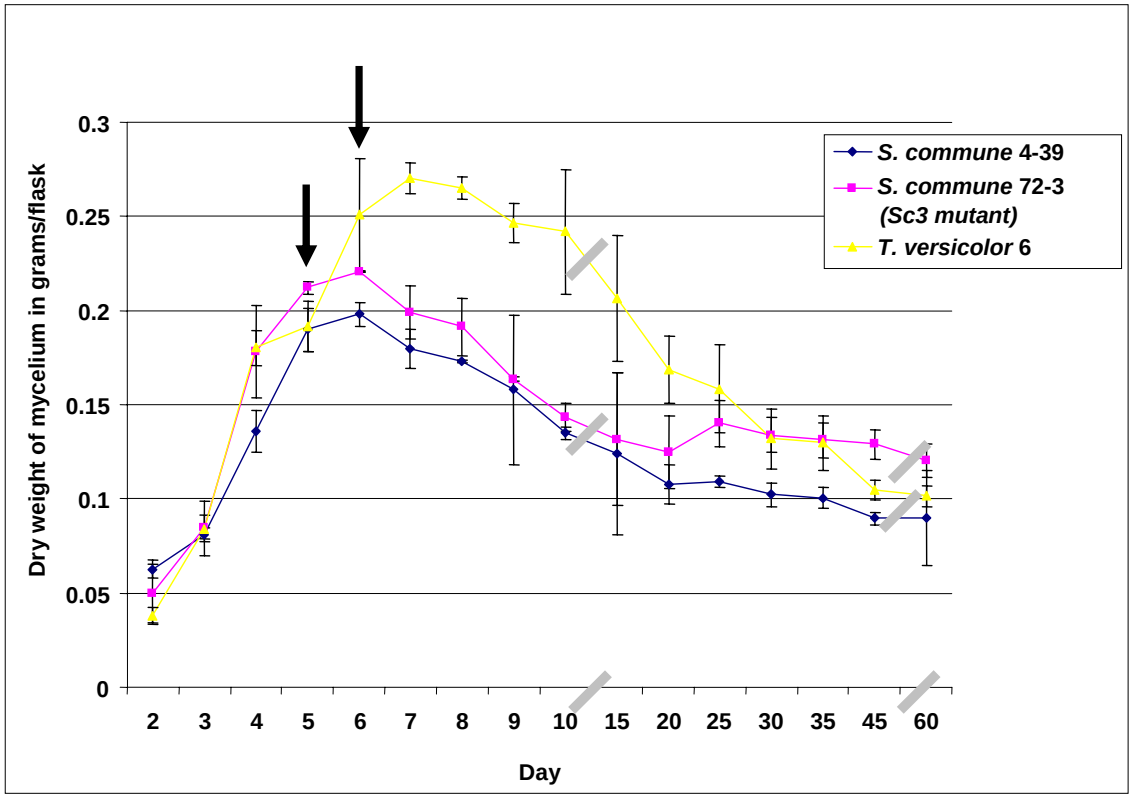


Fig. 6 Biomass production of *T. versicolor* 6, *S. commune* monokaryon 4-39 and its coisogenic *Sc3* hydrophobin knockout mutant 72-3 over the time grown at 28°C in dark condition. Each three 100 ml cultures per day were analysed and average values and standard deviations calculated. The arrows indicate the day of emergence of mycelium into the air in the cultures of the *S. commune* 4-39 and *T. versicolor* 6.

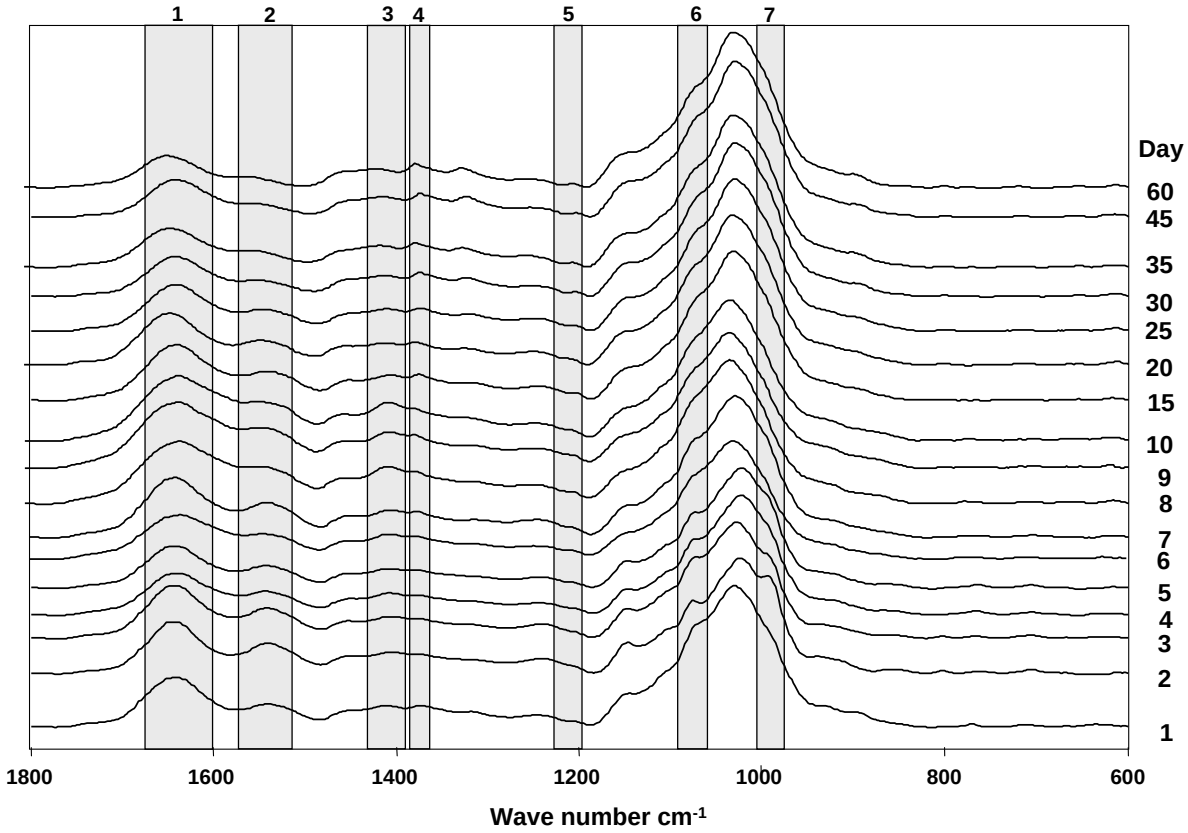


Fig. 7 ATR-FTIR spectra (baseline corrected and vector normalized) of the *T. versicolor* 6 at different growth ages. Bands 1 to 6 indicate the differences in the peak intensities, appearance or disappearance of peaks in the FTIR spectral regions (1. 1680-1600 cm $^{-1}$ for amide I, 2. 1580-1500 cm $^{-1}$ for amide II, 3. 1432-1365 cm $^{-1}$ & 4. 1391-1361 cm $^{-1}$ for mixed region of fatty acids, proteins and polysaccharides, 5. 1272-1210 cm $^{-1}$ for amide III and 6. 1090-1060 and 7. 1003-980 cm $^{-1}$ for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005)].

Table 3 Comparison of peak area values of the protein part of the FTIR spectra (spectral region 1760 to 1477 cm $^{-1}$) from the mycelium of *T. versicolor* 6, *S. commune* wild type 4-39 and the *Sc3* hydrophobin mutant *ΔSc3* 4-39 as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral region representing the peaks associated with amide I and amide II bands of the protein were used for integration.

Protein peak			
Day growth	*T. versicolor* 6	*S. commune* wild type	*S. commune* *Sc3* mutant
1	3.8783	6.7682	6.2219
2	4.4864	7.7218	6.8736
3	4.4639	8.1511	7.1684
4	4.5696	7.4917	7.1060
5	5.2304	6.5179	6.5840
6	5.5889	7.0272	6.1450
7	6.2691	7.1420	6.3430
8	6.0020	6.2741	5.2765
9	5.2722	6.1794	4.7005
10	4.2327	5.3012	3.7134
15	4.1290	3.0455	2.4262
20	3.5556	2.5535	2.1030
25	3.5120	2.5745	2.0640
30	3.2462	2.4478	1.9886
35	2.8771	2.4428	2.1039
45	2.4815	2.4229	2.0096
60	5.3345	2.3309	1.9674

Table 4 Statistical data (calculated by using the Duncan multiple range test) indicating the significant differences ($p \leq 0.05$) in the area of protein part of the FTIR spectra (spectral region 1760 to 1477 cm^{-1}) from the mycelium of *T. versicolor* 6, *S. commune* wild type 4-39 and the *Sc3* hydrophobin mutant *ΔSc3* 4-39 as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral regions representing the peaks associated with amide I and amide II bands of the protein were used for integration.
For an easy understanding, the fungal strains were indicated by the alphabetical letters.

Protein peak	a	b	c
Day growth	***T. versicolor* 6**	***S. commune* wild type**	***S. commune* *Sc3* mutant**
1	ND	ND	ND
2	b, c	a, c	a, b
3	b, c	a, c	a, b
4	b, c	a	a
5	b, c	a, c	a, b
6	b	a	ND
7	ND	ND	ND
8	c	c	a, b
9	ND	c	b
10	b, c	a, c	a, b
15	b, c	a, c	a, b
20	b, c	a	a
25	b, c	a, c	a, b
30	b, c	a, c	a, b
35	b, c	a, c	a, b
45	c	c	a, c
60	b, c	a, c	a, b

ND indicates no significant difference with other two strains.

Table 5 Comparison of peak area values of the polysaccharide part of the FTIR spectra (spectral region1190 to 900 cm $^{-1}$) from *T. versicolor* 6, *S. commune* wild type 4-39 and the *Sc3* hydrophobin mutant *ΔSc3* 4-39 as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral region 1190 to 900 cm $^{-1}$ was used for integration.

Polysaccharide peak			
Day growth	***T. versicolor* 6**	***S. commune*** **wild type**	***S. commune*** ***Sc3* mutant**
1	12.4236	10.7192	10.8821
2	11.8828	10.3598	10.6327
3	11.8980	9.9530	10.4370
4	11.7442	10.4092	10.4916
5	11.6066	10.9773	11.0367
6	11.6355	10.5648	11.0499
7	11.4772	10.54	10.9732
8	11.5715	11.2829	11.9982
9	12.0341	11.4979	12.4231
10	12.3120	12.1726	13.0464
15	12.9174	13.3619	13.5976
20	13.2148	13.6399	13.7679
25	13.1967	13.4985	13.6781
30	13.4114	13.5807	13.8025
35	13.7999	13.5699	13.5786
45	14.0490	13.6864	13.7978
60	11.5515	13.7716	13.8613

Table 6 Statistical data (calculated by using Duncan multiple range test) indicating the significant differences ($p \leq 0.05$) in the polysaccharide part of the FTIR spectra (spectral region1190 to 900 cm^{-1}) from the mycelium of *T. versicolor* 6, *S. commune* wild type 4-39 and the *Sc3* hydrophobin mutant *ΔSc3* 4-39 as calculated by the integration method A. The spectra used were vector normalized and base line corrected. Spectral region 1190 to 900 cm^{-1} was used for integration.

Polysaccharide peak	a	b	c
Day growth	***T. versicolor* 6**	***S. commune* wild type**	***S. commune* *Sc3* mutant**
1	b, c	a	a
2	b, c	a	a
3	b, c	a, c	a, b
4	b, c	a	a
5	b, c	a	a
6	b	a	ND
7	b, c	a	a
8	c	c	a, b
9	ND	ND	ND
10	ND	c	b
15	b, c	a	a
20	b, c	a	a
25	ND	ND	ND
30	b, c	a, c	a, b
35	ND	ND	ND
45	b, c	a	a
60	b, c	a	a

For an easy understanding, the fungal strains were indicated by the alphabetical letters.
ND indicates no significant difference with other two strains.

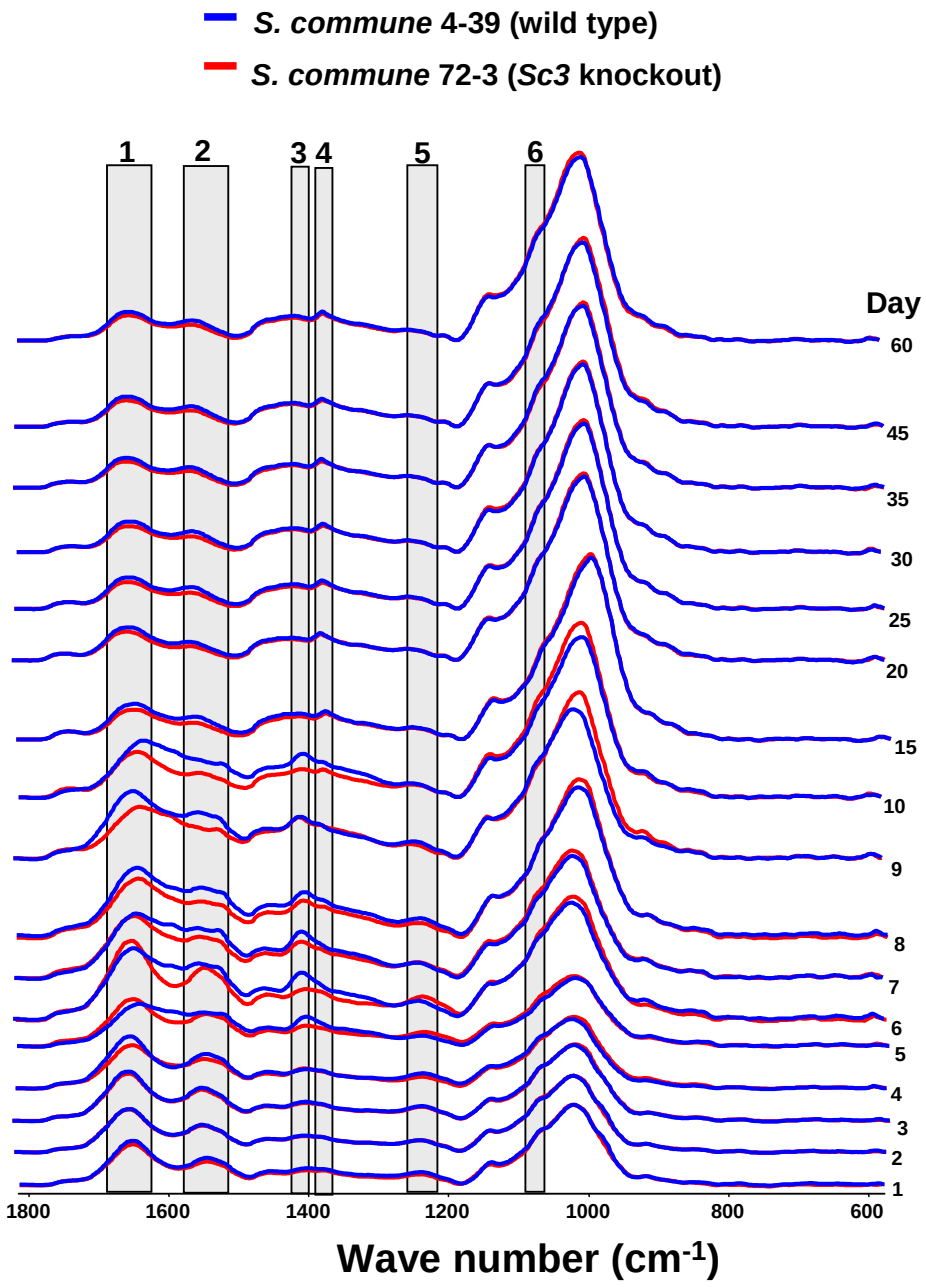
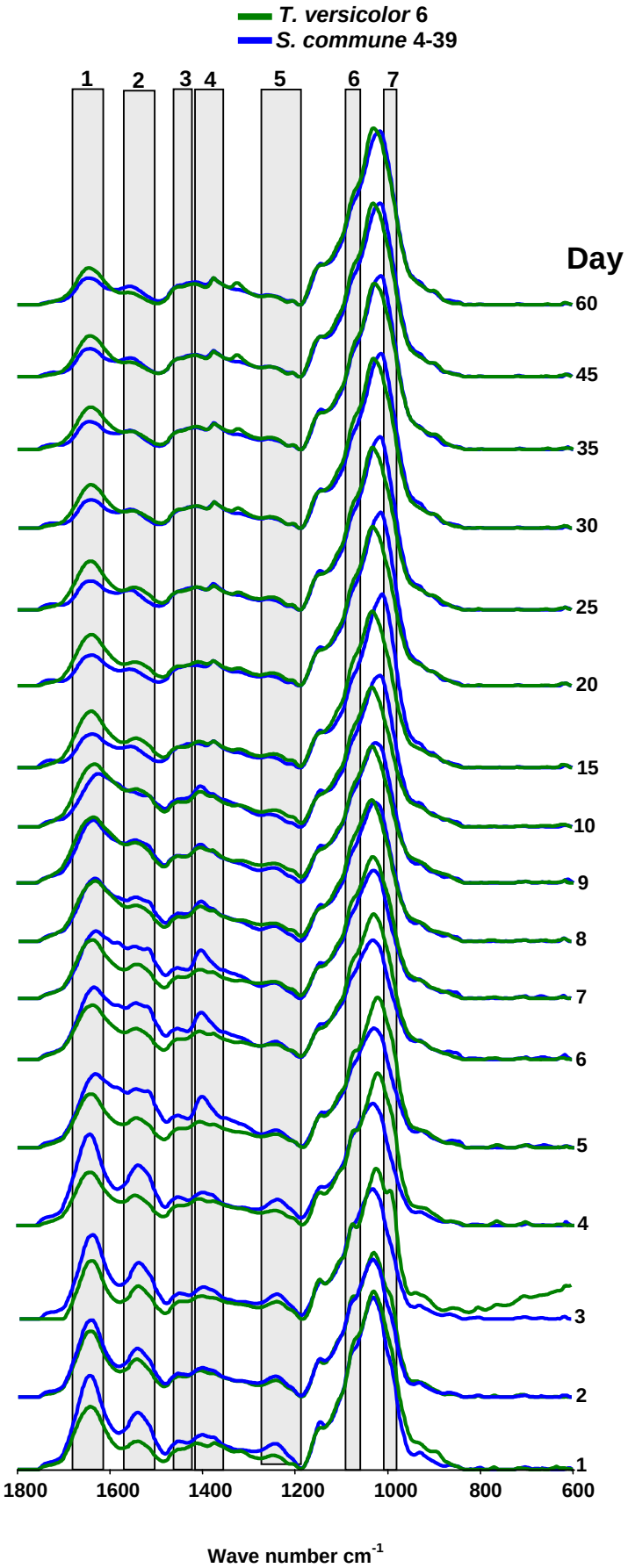


Fig. 8 Comparison of the ATR-FTIR spectra (vector normalized and base line corrected) of the *S. commune* 4-39 (wild type) and *Sc3* knockout mutant 72-3 (*ΔSc3* 4-39) grown in standing liquid structures from day 1 to day 60. Bands 1 to 6 indicate the differences in the peak intensities, appearance or disappearance of peaks in the FTIR spectral regions over the growth time (1. 1680-1600 cm $^{-1}$ for amide I, 2. 1580-1500 cm $^{-1}$ for amide II, 3. 1432-1365 cm $^{-1}$ and 4. 1391-1361 cm $^{-1}$ for mixed region of fatty acids, proteins and polysaccharides, 5. 1272-1210 cm $^{-1}$ for amide III and 6. 1090-1060 cm $^{-1}$ for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005)].


T. versicolor 6
S. commune 4-39
1
2
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Day
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6
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4
3
2
1
1800
1600
1400
1200
1000
800
600
Wave number cm-1

Fig. 9 Comparison of the ATR-FTIR spectra (vector normalized and base line corrected) of *T. versicolor* 6 and *S. commune* 4-39 (wild type) grown in standing liquid BSM cultures from day 1 to day 60. Bands 1 to 7 mark regions of major differences in the peak intensities, appearance or disappearance of peaks in the FTIR spectral regions over the growth time (1. 1680-1600 cm^{-1} for amide I, 2. 1580-1500 cm^{-1} for amide II, 3. 1432-1365 cm^{-1} and 4. 1391-1361 cm^{-1} for mixed region of fatty acids, proteins and polysaccharides, 5. 1272-1210 cm^{-1} for amide III and 6. 1090-1060 cm^{-1} and 7. 1003-980 cm^{-1} for polysaccharides) [(Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005)].

Chapter 5

FTIR microscopy of *S. commune* in wood

5. FTIR microscopy of *S. commune* in wood

5.1 Abstract

FTIR microscopy has been used for the qualitative and quantitative analysis of protein and polysaccharide content in the mycelium of *S. commune* wild type strains and SC3 hydrophobin mutants, while growing on the surface and inside of beech wood. Classification by cluster analysis differentiated wood fibers, the empty lumen of wood vessels, and the fungal strains from the surface of the beech wood. Within the wood, all the *S. commune* strains clustered together but they were discriminated from *P. ostreatus*. Peak area values of the vector-normalized, baseline-corrected and integrated FTIR spectra of the mycelium of different strains were used for quantitative analysis of proteins and polysaccharides.

5.2 Introduction

FTIR microscopy has recently been used for detecting fungi within wood (Naumann et al. 2005, 2007). Analysis of fungi concentrated so far on distinguishing *Trametes versicolor* and *Schizophyllum commune*. These fungi were clearly differentiated from each other and mycelium within a species was differentiated according to whether it grew on the surface or within the wood (Naumann et al. 2005). In a study on food yeasts, it had been shown that strains with mutations of biochemical characters such as slime production can have significantly different FTIR spectra due to major changes in cellular compositions (Kümmerle et al. 1998). In chapter 4 it has been reported that a *S. commune* wild type strain and a coisogenic *ΔSc3* hydrophobin mutant differ in slime production as well as in protein content and that this differences can be detected by FTIR analysis.

FTIR is also a useful technique for studying changes in the wood caused by fungal attack. Pandey & Pitman (2003) and Pandey & Nagveni (2007) studied the relative changes in the intensities of peak areas and height values of lignin and carbohydrate associated bands from the spectra of brown-rot and white-rot decayed wood. Recently, the technique has been used for cell wall analysis of beech and poplar wood (Naumann & Polle 2006). There were however no previous FTIR studies on specific differences in polysaccharide and protein production occurring between mycelia of different fungal strains when the fungi are growing on and within the wood.

The objective of the study described in this chapter was therefore to analyze the protein and polysaccharide contents of mycelia from *S. commune* strains growing on and within wood. Monokaryons and dikaryons were considered being either wild type or knock-out mutants of the *Sc3* hydrophobin gene(s).

5.3 Materials and methods

5.3.1 Fungal strains

The co-isogenic *S. commune* monokaryons 4-39 (*MATA41 MATB41,* CBS 341.81), and 4-40 (*MATA43 MATB43,* CBS 340.81), and the corresponding *ΔSc3* hydrophobin mutants 72-3 (*ΔSc3 MATA41 MATB41*), and *ΔSc3 4-40* (*ΔSc3 MATA43 MATB43*) were kindly provided by Prof. Wösten, Utrecht, The Netherlands. Dikaryotic

S. commune strains 4-39 x 4-40 (*MATA41 MATB41* x *MATA43 MATB43*) and *ΔSc3* 4-39 x *ΔSc3* 4-40 (*MATA41 MATB41* x *MATA43 MATB43*) were produced by mating the corresponding monokaryons. *P. ostreatus* N001 (dikaryon) kindly provided by Prof. Ramirez, Universida Publica de Navarra, Pamplona, Spain, was used as a control strain for growth on and within wood.

5.3.2 Growth conditions

All strains were grown at 28°C in dark condition. *S. commune* strains were cultivated on *S. commune* minimal medium (20 g glucose, 1.5 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ x $7H_2O$, 1 g yeast extract, 0.12 mg thiamine-HCl, 0.1 mg pyridoxine HCl, 0.005 mg biotin, 0.2 mg $CuSO_4$ x $5H_2O$, 0.08 mg $MnCl_2$ x $4H_2O$, 0.4 mg cobaltous chloride hexahydrate, 1.2 mg calcium nitrate tetrahydrate per 1 liter H_2O; Dons et al. 1979), and *P. ostreatus* on SMY medium (10 g of sucrose, 10 g of malt extract, 4 g of yeast extract, per 1 liter H_2O; pH 5.6; Peñas et al. 2002).

5.3.3 Wood block test

Beech wood was cut into 3 x 1 x 0.5 cm^3 (longitudinal to the axis x radial to the axis x tangential to the axis) blocks, quality selected and used for the study. The wood was dried in an oven at 100°C for two days. Initial weights of the dried wood blocks were determined. Each three wood blocks were used in 18 cm Petri dishes. Per each fungal strain, 3 different Petri dishes were used. To avoid the direct contact of the wood blocks with the agar, sterile steel grids were used beneath the wood blocks. Wood blocks were transferred onto the steel grids when the mycelium of the growing fungi covered half of the Petri dishes. Mycelium was inoculated in the middle of the plates by placing a block of agar with mycelium that was cut with a cork borer (1cm Ø) from the edge of the Petri dish pre-cultures grown on 1% agar *S. commune* medium. Petri dishes were sealed with Parafilm (PECHINEY, Chicago, USA) and incubated at 28°C both for initial mycelial growth as well as for further incubation, once the wood blocks were placed onto the grids. To check decay by the strains, harvests were performed after 8, 12, 16, 20 and 24 weeks of incubation. Plates without fungi but with wood blocks were used as negative controls. After 24 weeks of incubation, wood blocks were harvested. One wood block from each Petri dish was stored at -20°C, until further use for FTIR microscopy. One wood block from each Petri dish was used

for light microscopy. The mycelium attaching to the other wood block/Petri dish was separated by using a scalpel. The wood blocks were then dried at 100 ^{o}C for 3 days and weighted to note the final (dried) weight. Mass loss of wood was calculated as (initial wt – final wt)/initial wt x 100.

5.3.4 Light Microscopy

Sections of 30 µm thickness were prepared by a microtome and placed onto a glass slide, stained for 5 min with 20% lactophenol blue, washed with water, treated with 0.1 M sodium acetate and 100 µM MBTH (3-methyl-2-benzothiozolinone hydrazone hydrochloride monohydrate) for 5 min and then a drop of glycerine was placed onto the section which was then covered with a cover-slip. Samples were analysed with a Zeiss Axiophot photomicroscope (Zeiss, Göttingen, Germany) equipped with a soft imaging colour view II Mega pixel digital camera (Soft Imaging System, Münster, Germany) that was linked to a computer equipped with analySIS® software programme (Soft Imaging System, Münster, Germany).

5.3.5 Sample preparation for FTIR microscopy

For the measurement of spectra from the surface of the wood, mycelium from wood blocks stored at -20^{o}C was collected carefully with forceps onto a glass slide. To measure spectra of hyphae inside the wood blocks, sections were prepared transversely through the wood cells. Wood blocks were cut with a freeze microtome at a thickness of 30 µm and sections were air dried on glass slides under cover slips using weights (approx. 70g) of lead blocks.

5.3.6 FTIR microscopy

FTIR absorption spectra of wood sections were recorded with the FTIR spectrometer Equinox 55 combined with the IR microscope Hyperion 3000 (Bruker Optics, Ettlingen, Germany) including a single channel MCT detector and a 64 x 64 focal plane array 1detector (FPA). Wood sections were placed on a KBr window (2 mm). For single channel detector measurements, regions were defined by visual control with a knife edge aperture of 20 x 35 µm. Spectra of empty vessels, wood fibres, mycelia from the surface and inside wood were recorded with a 15 x Cassegrain objective and the single channel detector, resolution 4 cm^{-1}, 100 scans. FTIR images

of wood sections containing the mycelium were produced with a focal plane array detector with resolution 12 cm^{-1} and 100 scans.

5.3.7 Spectral data analysis

For each fungal strain, three wood blocks were used (each one from three different plates). One section from each wood block was analysed. Three different spectra per section from three different places were recorded each for wood fibres, vessel lumina and mycelium in vessels. In addition, three different spectra were recorded from mycelium detached with forceps from the surface of the wood. Spectral data were evaluated using OPUS version 5.0 software (Bruker, Germany). Cluster analysis was performed with original spectra subjected to first derivative, vector normalization of the region 1800 to 600 cm^{-1}. A dendrogram was constructed executing Ward's algorithm. For quantitative analysis, individual spectra were vector normalized, base line corrected (rubber band method with 64 base line points) and integrated (method A, described in the OPUS version 5.0 software, Bruker, Germany). Peak areas corresponding to the protein (region 1760 to 1477 cm^{-1}) and polysaccharide bands (1190 to 900 cm^{-1}) were used for integration. To check the distribution of mycelia inside the wood blocks, mycelial spectra were extracted from the Focal plane array (FPA) data set and cut to a range of 1250-922 cm^{-1} and correlated with the 4096 spectra of the imaging data set with the trace computation function of the OPUS software.

5.3.8 Statistical analysis

Statistics was performed by using SPSS software release 9.0.0, standard version (SPSS Inc., Chicago, USA). Data sets were compared using Tukey HSD multiple range test up to 95% confidence level ($p \leq 0.05$).

5.4 Results

5.4.1 Wood decay test and light microscopy

Wood decay tests were performed with beech wood and the *S. commune* monokaryotic strains 4-39 and 4-40, the dikaryon 4-39 x 4-40 and their co-isogenic *Sc3* hydrophobin mutants *ΔSc3* 4-39, *ΔSc3* 4-40 and *ΔSc3* 4-39 x *ΔSc3* 4-40,

respectively. Beech wood sections were analyzed under the microscope after 8 weeks of incubation with the fungal strains. Light microscopic analysis revealed the presence of hyphae inside the vessel lumina of the beech wood sections (Fig. 1). Clamps at the septa of hyphae that accumulated inside the vessel lumina were observed in case of the wild type *S. commune* dikaryon 4-39 x 4-40 and its hydrophobin mutant *ΔSc3* 4-39 x *ΔSc3* 4-40 (Fig. 1c and 1f). Note also the chlamydospore production by monokaryons and dikaryons in the vessels lumina (Fig. 1).

The mass loss of beech wood caused by *S. commune* strains calculated after 8, 12, 16, 20 and 24 weeks of incubation was between 0 and 2%. *P. ostreatus* used as general control decayed beech wood almost completely (93%) over the time (Table 1).

Table 1 Mass loss of beech wood by *S. commune* strains and *P. ostreatus* after 8, 12, 16, 20 and 24 weeks of incubation at 28°C in dark condition.

Strain	**Mass loss in % after incubation of**				
	8 weeks	**12 weeks**	**16 weeks**	**20 weeks**	**24 weeks**
4-39	1.20 ± 0.56	1.18 ± 1.0	1.22 ± 2.11	1.46 ± 0.46	0.89 ± 0.24
***ΔSc3* 4-39**	0.02 ± 0.78	1.49 ± 0.78	0.66 ± 0.43	2.12 ± 2.72	0.53 ± 1.38
4-40	1.58 ± 2.37	1.46 ± 0.89	2.19 ± 1.64	2.20 ± 0.75	0.8 ± 0.51
***ΔSc3* 4-40**	0.96 ± 1.09	3.31 ± 4.89	0.69 ± 0.01	1.61 ± 3.97	0.49 ± 0.7
4-39 x 4-40	0.97 ± 1.51	1.94 ± 2.43	2.24 ± 1.49	1.62 ± 0.61	0.48 ± 2.55
***ΔSc3* 4-40 x *ΔSc3* 4-40**	0.39 ± 0.95	1.18 ± 2.09	1.86 ± 0.01	0.30 ± 0.06	0.72 ± 1.69
***P. ostreatus* N001**	54.38± 3.95	74.2 ± 7.2	81 ± 0.86	86.12 ± 1.63	93.95 ± 2.29
Control	0.41± 0.49	0.04 ± 0.78	2.17 ± 0.83	0.32 ± 0.11	0.39 ± 0.09

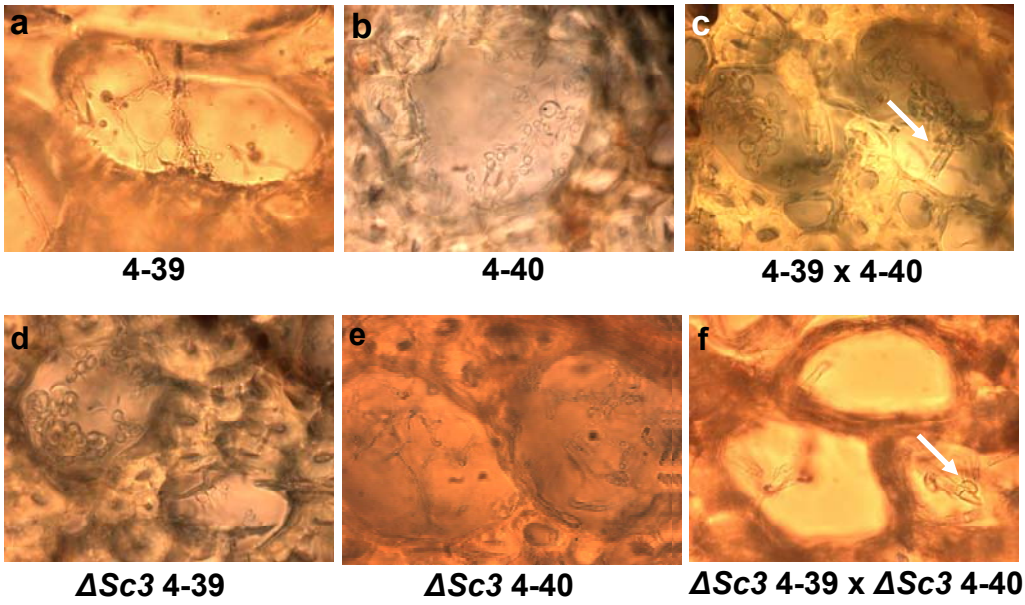


Fig. 1 Light microscopy pictures of *S. commune* wild type monokaryons 4-39 (a), 4-40 (b), the dikaryon 4-39 x 4-40 (c) and the corresponding *Sc3* hydrophobin mutants *ΔSc3* 4-39 (d), *ΔSc3* 4-40 (e) and *ΔSc3* 4-40 x *ΔSc3* 4-40 (f) inside beech wood vessel lumina after 24 weeks of incubation at 28°C in constant dark. Clamp cells at hyphal septa of the dikaryons are indicated by arrows. Note that there is apparently chlamydospore production within the vessels by monokaryons and dikaryons

5.4.2 Discrimination of fungi and wood by FTIR analysis

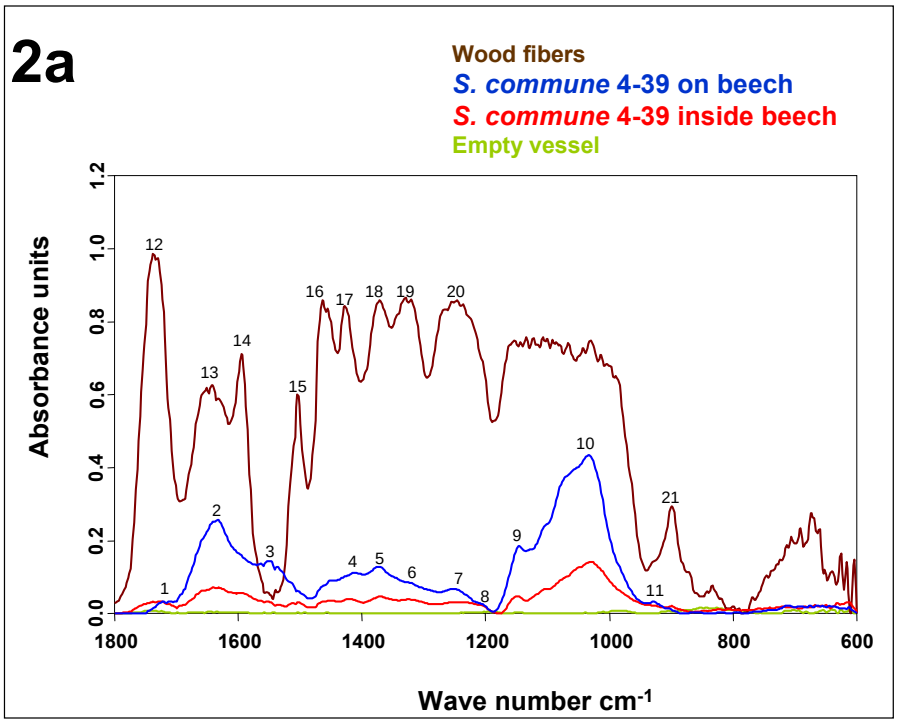
Beech wood blocks were infected with *S. commune* wild type strains and hydrophobin mutants or with *P. ostreatus* strain N001 and incubated for six months before determining FTIR spectra of wood fibers, empty vessels lumina and mycelia on and within wood. The spectra from the wood fibers, empty vessels, mycelia on the surface of beech and mycelia inside beech could in all cases be visually distinguished (Fig. 2). Several peaks were observed in the spectra of the mycelia and the wood fibers typical for fungal mycelia and beech wood, respectively. These were assigned according to Mochaček-Grošev et al. (2001), Naumann et al. (2005) and Pandey and Pitman (2003) (see Fig. 2a and Table 2).

Table 2 Peak assignments of the absorption bands in the FTIR spectra of fungal mycelium and beech wood (Fig. 2a).

Type of material	Peak number	Peak position	Peak assignment
Fungal mycelium	1	1735-1720 cm^{-1}	C=O stretching for esters (lipids)
	2	1650-1630 cm^{-1}	amide I (protein)
	3	1557-1542 cm^{-1}	amide II (protein)
	4	1424-1408 cm^{-1}	C-H bending
	5	1390-1360 cm^{-1}	amide III (protein)
	6	1326-1315 cm^{-1}	amide III (protein)
	7	1268-1240 cm^{-1}	
	8	1214-1200 cm^{-1}	C-C stretching, C-O stretching, C-H deformation (pyranose ring)
	9	1150-1136 cm^{-1}	
	10	1060-1030 cm^{-1}	
	11	934-925 cm^{-1}	α-anomer C-H deformation (glucan band)
Beech wood fibers	12	1738 cm^{-1}	unconjugated C=O (in xylans)
	13	1650 cm^{-1}	absorbed O-H, conjugated C-O
	14	1596 cm^{-1}	aromatic skeletal vibrations in lignin
	15	1505 cm^{-1}	aromatic skeletal vibrations in lignin
	16	1464 cm^{-1}	C-H deformation in lignin and carbohydrates
	17	1429 cm^{-1}	
	18	1370 cm^{-1}	C-H deformation in cellulose and hemicellulose
	19	1331/1321 cm^{-1}	C-H vibration in cellulose, C1-O vibration in syringyl derivatives
	20	1245 cm^{-1}	syringyl ring and C-O stretch in lignin and xylan
	21	899 cm^{-1}	C-H deformation in cellulose

Note: The spectra of the wood fibers in the wavelength region of 1200 to 1000 cm^{-1} were not well resolved in this study probably due to the relative high thickness (30 µm) of the samples. This thickness of wood sections was required since with a lower thickness of samples, there is the possibility that the mycelium could drop out from the wood vessels (Naumann et al. 2005).

Peak assignments were done according to Mochaček-Grošev et al. 2001, Naumann et al. 2005, and Pandey and Pitman 2003)


2a
Wood fibers
S. commune 4-39 on beech
S. commune 4-39 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
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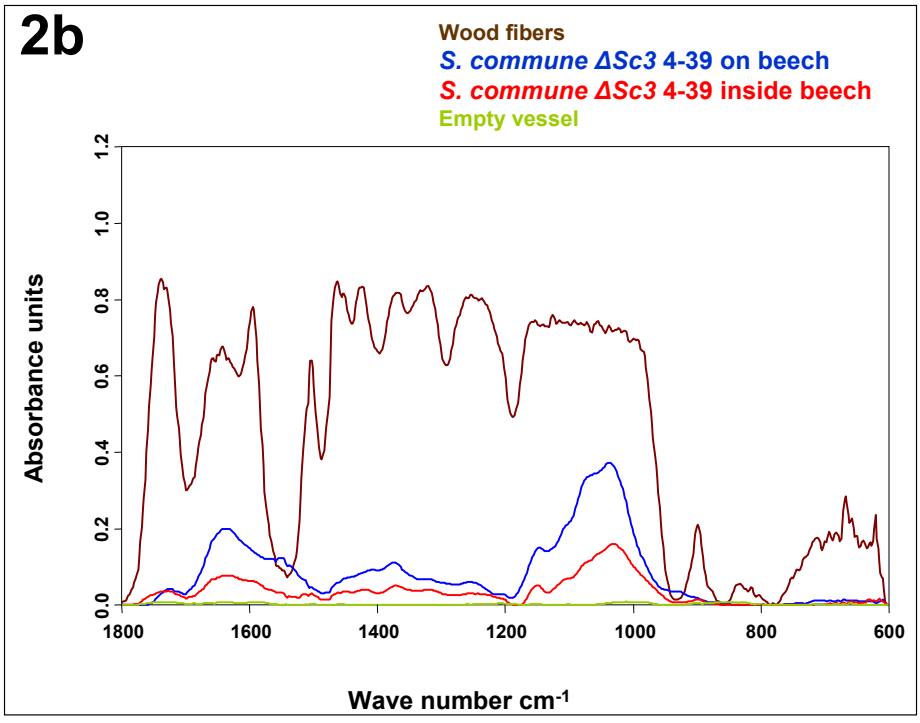
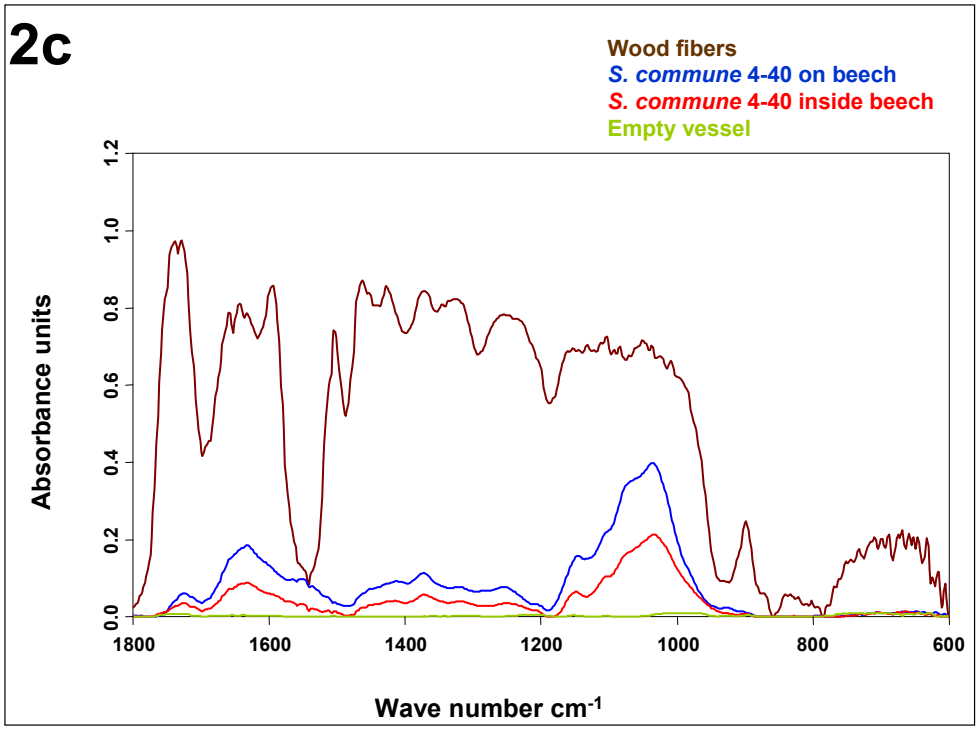
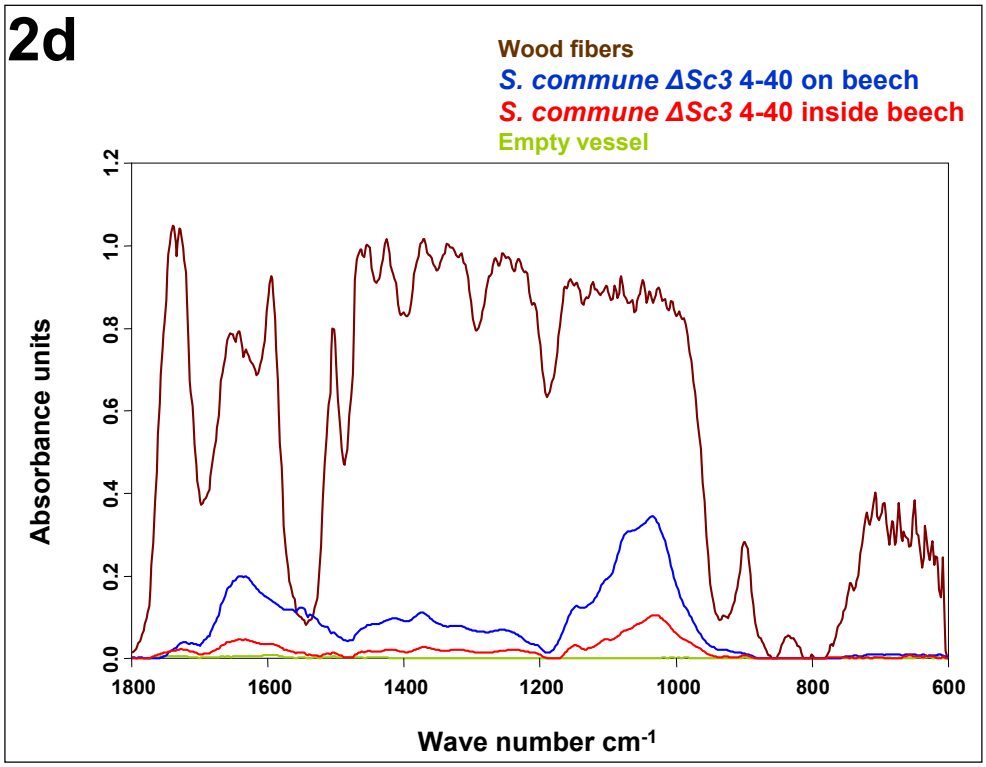
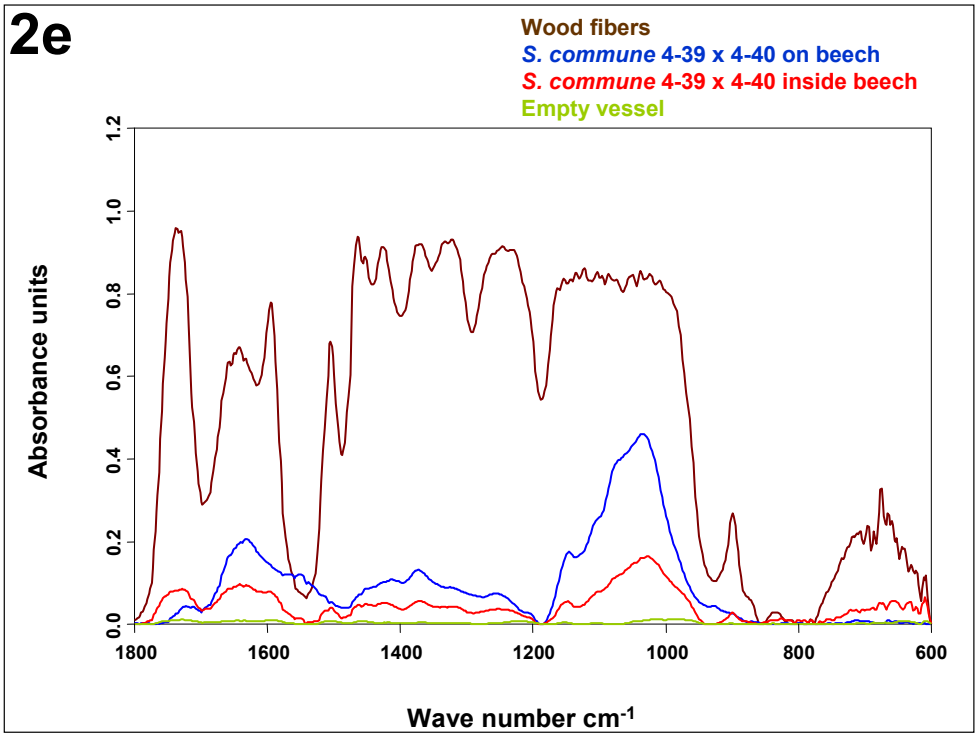
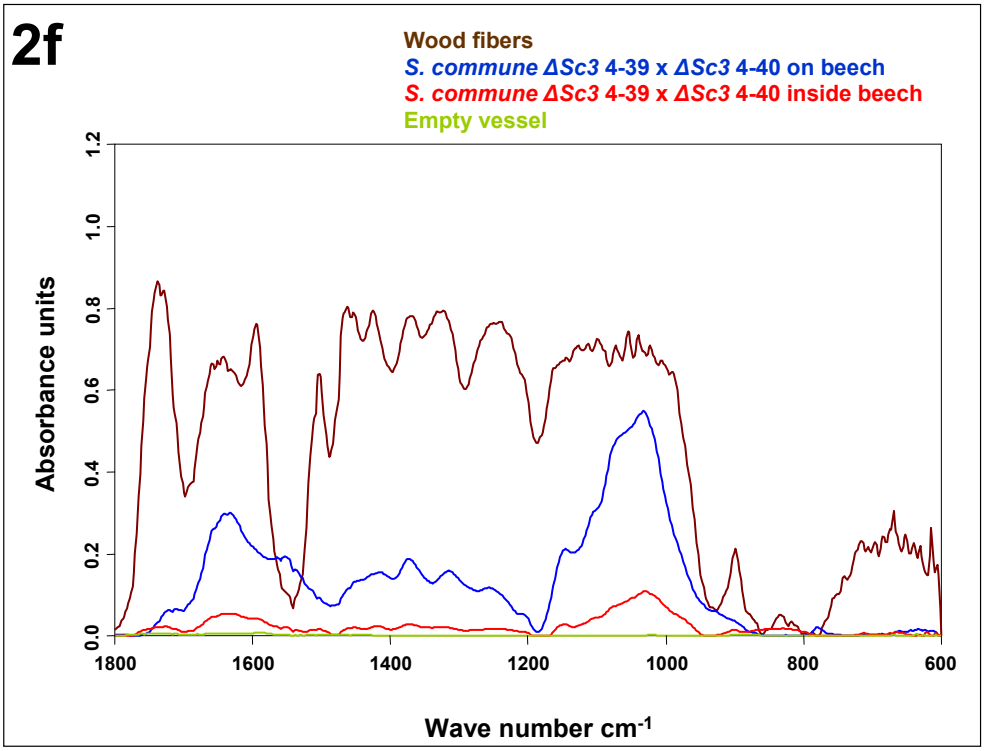
17
18
19
20
21


2b
Wood fibers
S. commune ΔSc3 4-39 on beech
S. commune ΔSc3 4-39 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1


2c
Wood fibers
S. commune 4-40 on beech
S. commune 4-40 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1


2d
Wood fibers
S. commune ΔSc3 4-40 on beech
S. commune ΔSc3 4-40 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1


2e
Wood fibers
S. commune 4-39 x 4-40 on beech
S. commune 4-39 x 4-40 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1


2f
Wood fibers
S. commune ΔSc3 4-39 x ΔSc3 4-40 on beech
S. commune ΔSc3 4-39 x ΔSc3 4-40 inside beech
Empty vessel
Absorbance units
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1800
1600
1400
1200
1000
800
600
Wave number cm-1

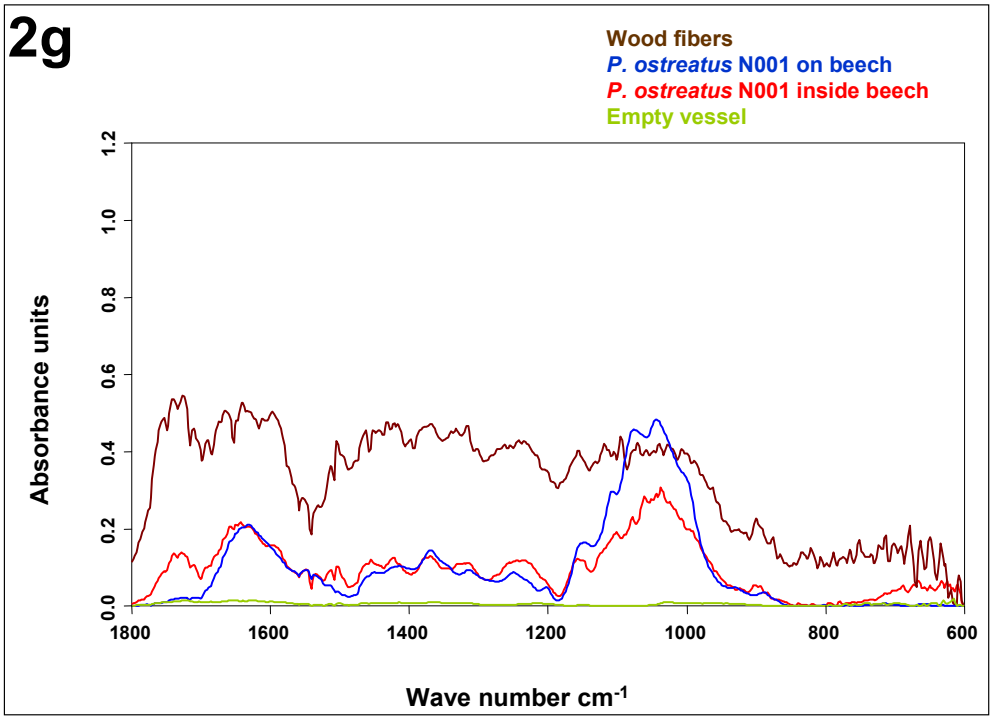


Fig. 2 FTIR spectra of beech wood fibers (brown), mycelium grown either on the beech wood surface (blue) or, inside the vessel lumina (red) and the empty wood vessel without mycelium (green). Measurements were performed after 24 weeks of fungal incubation with wood at 28°C under constant dark condition. The following strains were analyzed: 4-39 (2a), *ΔSc3* 4-39 (2b), 4-40 (2c), *ΔSc3* 4-40 (2d), 4-39 x 4-40 (2e), *ΔSc3* 4-39 x *ΔSc3* 4-40 (2f) and *P. ostreatus* N001 (2g). Each spectrum represents the average of 9 spectra, three from each section of three different wood blocks, each from different Petri dishes. The spectra were processed by baseline-correction with the rubber-band method using 64 baseline points as described in the OPUS version 5.0 software (Bruker, Germany).

Note: The *P. ostreatus* N001 inhabited wood (Fig. 2g) was very much destroyed and, hence, the spectra of the wood fibers showed a high noise and were not well resolved.

5.4.3 Discrimination of fungal strains

Cluster analysis resulted in a high heterogeneity between the spectra of wood and the fungal mycelium (Fig. 3). Two main clusters were observed, one from the wood and the other from the fungal mycelia. The spectra of wood fibers and the empty vessel lumina discriminated well and clustered separately from the spectra of the fungal mycelia. From the fungal cluster, two sub-clusters were observed. One sub-cluster is from the mycelium inside the wood which showed a very irregular pattern, irrespective of mono- and dikaryons and irrespective of wild type strains or

hydrophobin mutants. The other sub-cluster is from the mycelium growing on the wood surface, which again splitted into two small sub-clusters of monokaryons and dikaryons. These small sub-clusters distinguished further into individual strains with low heterogeneity. Overall from the cluster analysis, wood was clearly discriminated from fungal mycelia and fungi could be discriminated to the species as well as strain level on the surface of the wood. Inside the wood, *S. commune* and *P. ostreatus* were distinguished but not the individual *S. commune* strains.

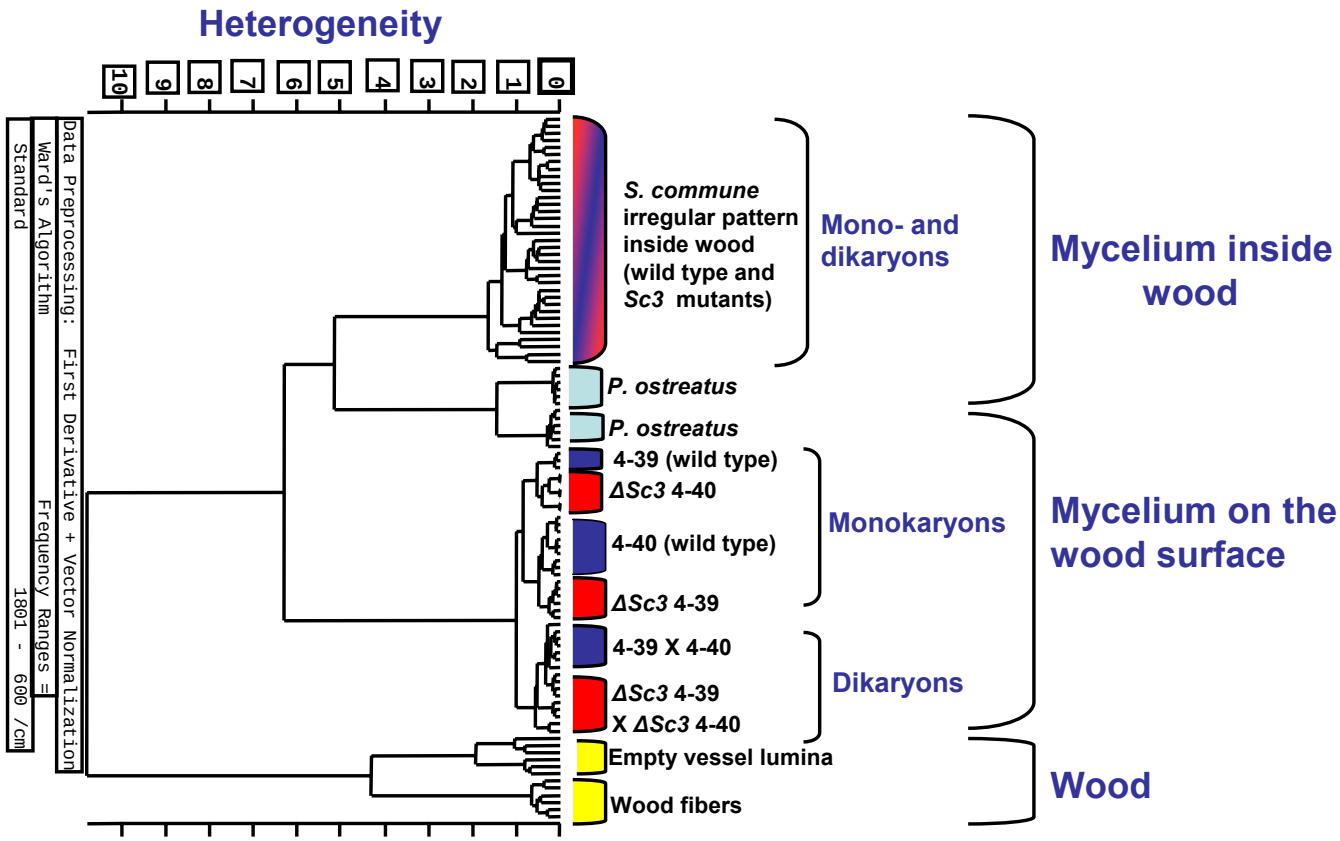


Fig. 3 Cluster analysis of FTIR spectra of mycelium of *S. commune* wild type and *Sc3* mutants inside and on the beech wood. Wood and fungal mycelium were discriminated. Mycelium on the wood surface and inside the wood clustered separately. Sub-clusters of strains were observed on the wood surface. For cluster analysis, original spectra were subjected to first derivative vector normalization in the frequency range of 1800 to 600 cm^{-1} using OPUS version 5.0 software. The dendrogram was constructed using Ward's algorithm described in the OPUS version 5.0 software, Bruker, Germany.

5.4 4 Distribution of *S. commune* mycelium within beech wood

To investigate the distribution of mycelium of the wild type and hydrophobin mutants inside the beech wood, the spectra from the FPA data set were subjected to trace computation by the OPUS version 5.0 software (Bruker Optics, Germany) and false color images were created (4C, 4D, 5C, 5D). The areas filled with mycelium inside the vessel lumina with high and low concentrations were clearly visible as indicated by the pink-red and yellow color in Fig. 4 and Fig. 5. These FPA spectral data of the mycelium inside the beech wood were further used for the quantification of mycelial protein and polysaccharide contents as described in the following.

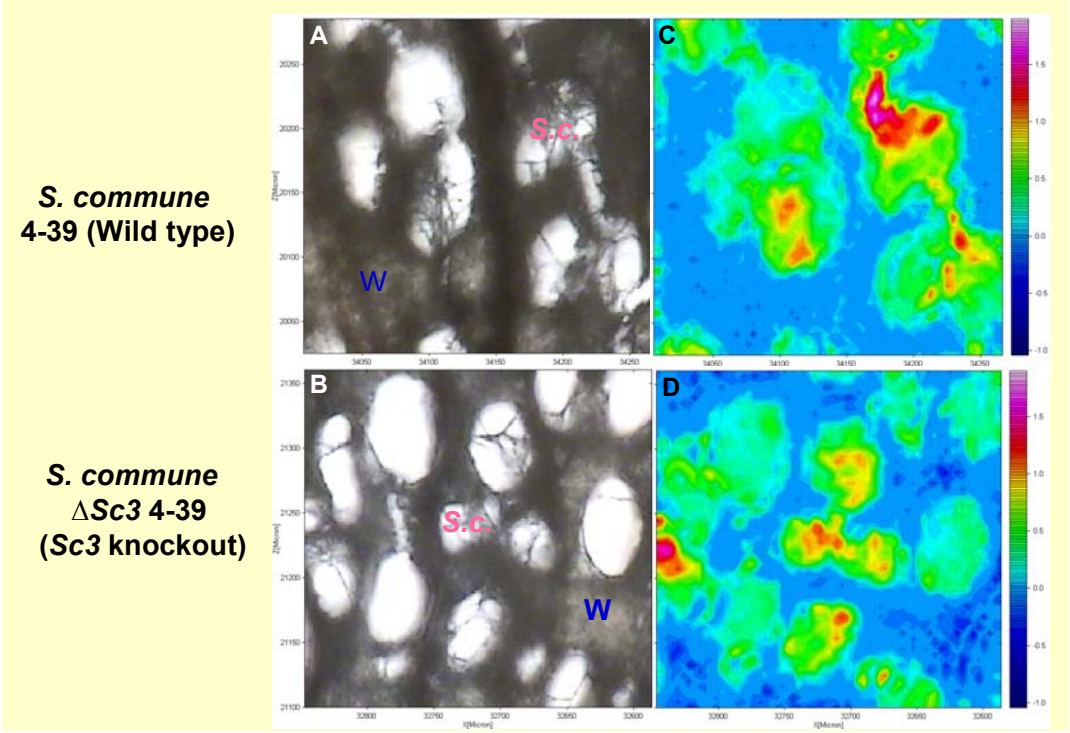


Fig. 4 Light microscopic view of beech wood cross section (A, B) presenting wood fibers (w) and vessel lumina with *S. commune* mycelium (*S. c.*). FTIR microscopic image of the same sections computed by correlation of the wave number region 1250-922 cm^{-1} with the FPA data set by the trace computation function (C, D). Dense *S. commune* mycelium is displayed pink-red and sparse mycelium in green. Blue indicates areas with no correlation with the fungal spectrum.

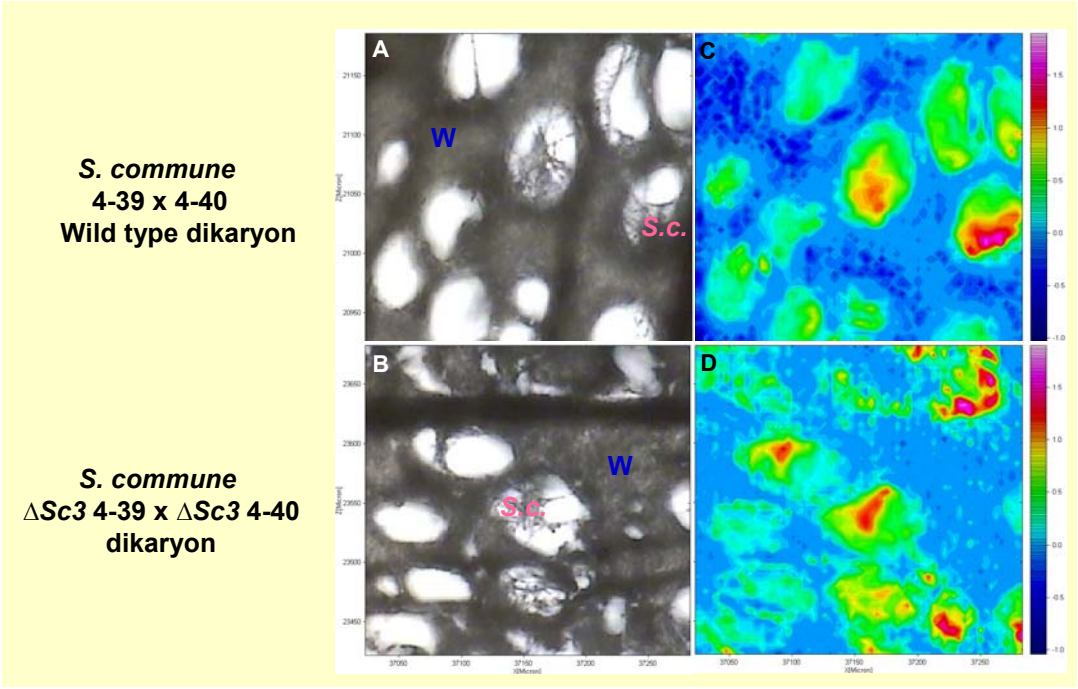


Fig. 5 (A, B) Light microscopic view of beech wood cross section presenting of wood fibers (w) and vessel lumina with *S. commune* (*S. c.*). (C, D) FTIR microscopic image of the same section computed by correlation of the by wave number region 1250-922 cm^{-1} with the FPA data set by the trace computation function. Dense *S. commune* mycelium is displayed pink-red and sparse mycelium in green. Blue indicates areas with no correlation with the fungal spectrum.

5.4.5 Quantification of protein and polysaccharide contents in the mycelium

The protein and polysaccharide content in the mycelium of *S. commune* strains growing on the surface of beech wood and inside the beech wood, respectively, have been calculated from the FTIR spectra by using the integration method as described in the OPUS version 5.0 software, Bruker, Germany. The area values of the protein and polysaccharide associated peaks of different strains are shown in Fig. 6 and 7, respectively.

On the surface of beech wood, the mycelial protein content of different strains of *S. commune* is not significantly different from each other (Fig. 6, Table 3), but the dikaryotic hydrophobin mutant strain *ΔSc3* 4-39 x *ΔSc3* 4-40 had a higher

polysaccharide content than wild type strain 4-39 and the hydrophobin mutant monokaryons *ΔSc3* 4-39 and *ΔSc3* 4-40 ($p<0.05$) (Fig. 7, Table 4). In contrast, compared to other strains, strain *ΔSc3* 4-40 showed a significantly lower carbohydrate content inside the wood.

When comparing mycelium from a given strain grown on the surface of wood and within wood, significant differences were seen in the protein content only for the monokaryotic hydrophobin mutant strain Δ*Sc3* 4-40, whilst two strains, the monokaryotic hydrophobin mutant Δ*Sc3* 4-40 and the dikaryotic mutant Δ*Sc3* 4-40 x Δ*Sc3* 4-40 showed significant differences in polysaccharide contents. On wood, these hydrophobin mutants produced by higher amounts of polysaccharides than within wood (Fig. 7).

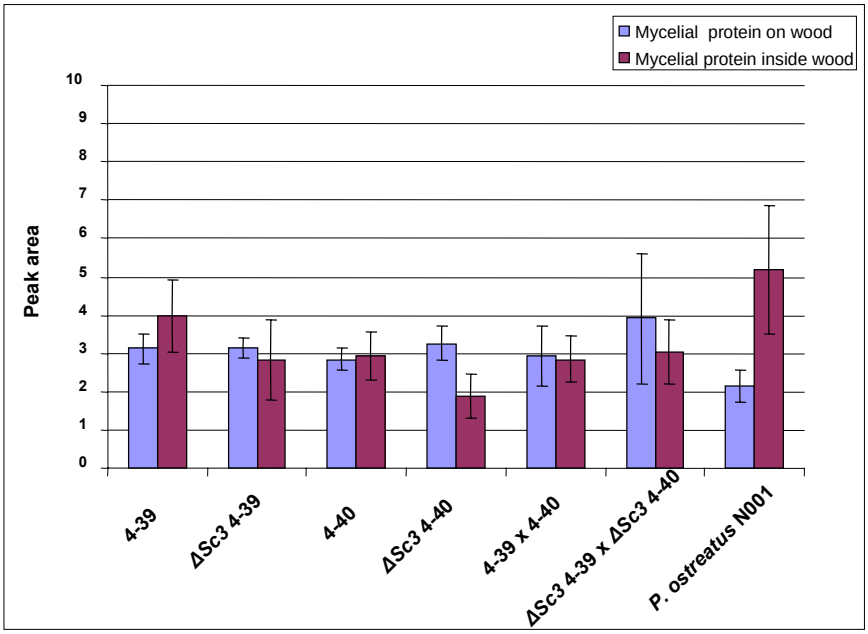


Fig. 6 Comparison of peak area values of the protein part of the FTIR spectra from wild type strains of *S. commune* and *Sc3* hydrophobin mutants while growing on the surface of beech wood and inside beech wood, respectively. Peak area values were calculated by the integration method A as described in the OPUS version 5.0 software, Bruker, Germany. The spectra used were vector-normalized, baseline-corrected and integrated. The spectral region 1760 to 1477 cm^{-1} which represents the peaks associated with amide I and amide II bands of the proteins (Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005) was used for integration.

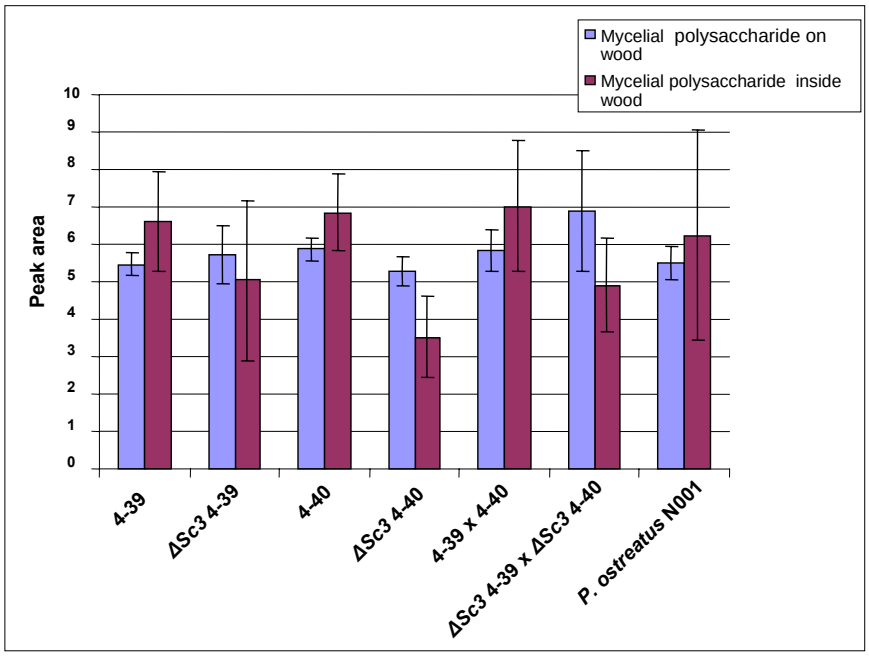


Fig. 7 Comparison of peak area values of the polysaccharide part of the FTIR spectra from wild type and *Sc3* hydrophobin mutants of *S. commune* while growing on surface of beech wood and inside beech wood, respectively. Peak area values were calculated by the integration method A as described in the OPUS version 5.0 software, Bruker, Germany. The spectra used were vector-normalized, baseline-corrected and integrated. The spectral region 1190 to 900 cm^{-1} which represents the peak associated with polysaccharides (Kümmerle et al. 1998, Jung 2000, Salzer et al. 2000, Yu & Irudayaraj 2005) was used for integration.

Table 3 Statistical data (calculated by using Tukey multiple comparisons, SPSS software release 9.0.0, standard version SPSS Inc., Chicago, USA) indicating the significant differences in between the fungal strains ($p \leq 0.05$) in the area of the protein (spectral region 1760 to 1477 cm^{-1}) and the polysaccharide part (spectral region 1190 to 900 cm^{-1}) of the FTIR spectra measured from the mycelium of *S. commune* wild type and the SC3 hydrophobin mutants and *P. ostreatus* when growing on beech wood surface and inside the wood, respectively.

Difference in between fungal strains					
Strain	*	**Protein**		**Polysaccharide**	
		On beech wood different from	Inside beech wood different from	On beech wood different from	Inside beech wood different from
4-39	a	ND	d	ND	d
ΔSc3 4-39	b	ND	g	f	ND
4-40	c	ND	g	ND	d
ΔSc3 4-40	d	ND	a, g	f	a, e
4-39 x 4-40	e	ND	g	ND	d
ΔSc3 4-39 x *ΔSc3* 4-40	f	g	g	a, b, d, g	ND
P. ostreatus N001	g	f	a, b, c, d, e, f	f	ND

* For an easy understanding, the fungal strains were indicated by the alphabetical letters.
ND indicates no significant difference with other strains.

Peak area values were calculated by the integration method A as described in the OPUS version 5.0 software, Bruker, Germany. The spectra used were vector-normalized and baseline-corrected. Each value represents the average of 9 replicate spectra.

Table 4 Statistical data (calculated by using Tukey multiple comparisons, SPSS software release 9.0.0, standard version, SPSS Inc., Chicago, USA) indicating the significant differences within each fungal strain ($p \leq 0.05$) in the area of the protein (spectral region 1760 to 1477 cm $^{-1}$) and the polysaccharide part (spectral region 1190 to 900 cm $^{-1}$) of the FTIR spectra measured from the mycelium of *S. commune* wild type and the SC3 hydrophobin mutants and *P. ostreatus* when growing on beech wood surface and inside the wood, respectively. The spectra used were vector normalized and base line corrected. Each value is represented from 9 replicate spectra.

Difference within fungal strains		
Strain	**Protein**	**Polysaccharide**
	on and inside beech wood	on and inside beech wood
4-39	ND	ND
ΔSc3 4-39	ND	ND
4-40	ND	ND
ΔSc3 4-40	S	S
4-39 x 4-40	ND	ND
ΔSc3 4-39 x *ΔSc3* 4-40	ND	S
P. ostreatus N001	S	ND

S indicates significant difference of the same strain growing on and inside beech wood, ND indicates no significant difference.
Peak area values were calculated by the integration method A as described in the OPUS version 5.0 software, Bruker, Germany. The spectra used were vector-normalized and baseline-corrected. Each value is represented from 9 replicate spectra.

5.5 Discussion

FTIR spectroscopy is a powerful tool to identify and classify microorganisms (Mariey et al. 2001). It is also in use to study chemical alterations in wood caused by different fungi (Anderson et al. 1991a, Anderson et al. 1991b, Owen & Thomas 1989, Pandey 1999). In chapter 4 of this thesis, the FTIR method has been successfully employed to study the protein and polysaccharide contents in the mycelium of a wild type and a SC3 hydrophobin mutant strain when grown in the liquid cultures. In this chapter, FTIR spectroscopy has been employed to study the combination of fungi and wood in order to further elucidate the possible role of hydrophobins in the wood colonization

and decay processes. By FTIR-spectra, fungal mycelium was easily distinguished from spectra of wood vessel's cell walls and lumina (Fig. 2). This finding allowed a more detailed analysis of fungal mycelia growing on and in wood. van Wetter et al. (2000) previously showed that hydrophobin mutants release higher amounts of glucans into the culture medium. The data in chapter 4 of this thesis suggested also that the polysaccharide content from the mycelium of the *Sc3* hydrophobin mutants is higher than that of the wild type strain, fully in agreement with the results of van Wetter et al. (2000). Here, FTIR microscopy has been applied to investigate the protein and polysaccharide contents from *S. commune* mycelium while growing on and within beech wood. By applying cluster analysis of whole spectra of the wild type strains 4-39, 4-40 and 4-39 x 4-40 and their hydrophobin mutants *ΔSc3* 4-39, *ΔSc3* 4-40 and *ΔSc3* 4-39 x *ΔSc3* 4-40 (Fig. 3), the *S. commune* strains were clearly discriminated while growing on the surface of beech wood. Further, between most of the strains there were however no significant differences in the protein or polysaccharide content in the mycelia but for the dikaryotic hydrophobin mutant *ΔSc3* 4-39 x *ΔSc3* 4-40 that showed a higher polysaccharide content. Furthermore, hydrophobin mutant strains *ΔSc3* 4-40 and *ΔSc3* 4-39 x *ΔSc3* 4-40 showed relatively higher polysaccharide contents on the surface of wood and lower polysaccharide contents inside the wood.

Also the differences by high polysaccharide content were obvious for two mutant strain compared to wild type strains, the overall results did not follow the expectations given by the results from the liquid cultures. Physiologically, growth in liquid artificial medium and growth in and on wood is likely a very different situation for the fungi. The liquid medium offers easily accessible nutrients in contrast to wood that likely needs the production of many degradative enzymes. This might be a reason for the observations presented here with solid wood, where the fungus never needs to grow within an unnatural liquid medium. However, another factor to be considered is the age of the cultures. FTIR-measurements on the wood samples were performed after 24 weeks of incubation. At this time, very likely aging processes will have happened that might eliminate previous differences in protein and polysaccharide contents of the fungi. Therefore, in the future FTIR-measurements of fungal

mycelium should be performed with young fungal mycelium shortly after infestation of wood, such as in week 1, 2, 3 and 4 of incubation.

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Chapter 6

Hydrophobins in wood technology

6. Hydrophobins in wood technology

6.1 Abstract

Hydrophobins are small amphiphatic proteins that self-assemble on surfaces into films, thereby rendering hydrophilic surfaces hydrophobic and vice versa. The surface activity of the SC3 hydrophobin from *Schizophyllum commune* has been tested on hydrophobic and hydrophilic materials. Hydrophobic Teflon and form-war film turned hydrophilic and hydrophilic glass turned hydrophobic on coating with SC3 hydrophobin. Further, the amphipathic nature of films of this protein was tested on solid beech wood. Beech wood turned hydrophilic or hydrophobic, depending on the concentration of SC3 hydrophobin applied. Lower concentrations (15, 25, 50 µg/ml) of SC3 resulted in hydrophobic behavior and higher concentrations (100 to 400 µg/ml) resulted in hydrophilic behavior of beech wood. Compared to the untreated control wood, ATR-FTIR spectra of the hydrophobin-treated beech wood showed an increase in the intensity of the peak associated with the amide I band of the protein part of the spectra in a concentration dependent manner, indicating the binding of the hydrophobin to the wood polymers.

6.2 Introduction

Hydrophobins are small proteins (±100 amino acids) produced by filamentous fungi. So far, they are known in basidiomycetes and ascomycetes (Hakanpaa et al. 2004, Paris et al. 2003, Scholtmeijer et al. 2001, Wösten 1994, Wösten 2000, Wösten 2001). Two classes of hydrophobins, namely class I and class II were described based on the hydropathy patterns and solubility characters of the protein. Both classes of hydrophobins are characterized by eight conserved cysteines (Wessels 1997, Wösten & de Vocht 2000, Whiteford & Spanu 2002, Walser et al. 2003). Intramolecular cross -linked cysteines are considered important for maintaining protein solubility and preventing premature self-association (de Vocht 2000). The SC3 hydrophobin produced by the vegetative mycelium of *Schizophyllum commune* is the best studied hydrophobin until now (Wösten 2001, de Vocht et al. 2002, Fan et al. 2006). It belongs to class I type hydrophobins because of its insoluble nature in aqueous solvents such as hot SDS solutions. It can only be dissolved by harsh treatments using organic solvents such as tri-fluoro acetic acid or formic acid. In contrast, class II hydrophobins are readily dissolved in solvents such as 60% ethanol or water (Torkkeli et al. 2002, Wessels 1997, Walser et al. 2003).

The treatment with the tri-fluoro acetic acid or formic acid process of hydrophobins oxidizes cysteine to cystic acid and thus breaks the disulphide bridges, thereby permanently breaking the hydrophobin structure. The process however was found to be effective in disrupting short range hydrophobic interactions leaving the hydrophobin monomers intact and biologically active, thus allowing them to reassemble into amphipathic polymers upon exposure to aeration (Kershaw & Talbot 1998).

Hydrophobins are surface active proteins (Wösten 2001). Their natural function is to adhere to surfaces and function as surfactants (Kurppa et al. 2007). They have a characteristic property to assemble into an amphipathic protein film when confronted to a hydrophilic-hydrophobic interface (Wessels 1997). In this way, they can change the polarity of a surface (Rillig 2005). They are thus able to change the biophysical properties of surfaces and can also attach molecules to surfaces that do not have an affinity in general (Scholtmeijer et al. 2001). Hydrophobins can work as coatings and

adhesive agents (Kisko et al. 2005). Their ability to self-assemble into amphipathic membranes, to modify both hydrophobic and hydrophilic surfaces as applied from aqueous solutions, to adsorb onto the surfaces differing from the other proteins (for example in contrast to Class I hydrophobins, BSA can be removed with hot SDS) make them interesting candidates for medical and technical applications (Hektor & Scholtmeijer 2005, Janssen et al. 2004, Scholtmeijer et al. 2001 & Stroud et al. 2003, Wessels 1997, Wessels 2000). Inspite of their remarkable biophysical properties, very little is know about their structural features (Torkkeli et al. 2002).

SC3 from *S. commune* as the best described hydrophobin is known to be the most surface active protein (Wösten 1999 & Wösten 2001). The activity of hydrophobins is at least similar to the traditional biosurfactants containg glycolipids, phospholipids, lipoproteins, neutral lipids, substituted fatty acids and lipopolysaccharides (Jenny et al. 1991, Wösten & de Vocht 2000). In contrast to the above mentioned surfactants, the surface activity of the hydrophobins is depending on amino acid sequences and not on a lipid molecule. Three states of SC3 have been spectroscopically identified namely the monomeric state, the α-helical and the β-sheet state. The monomeric state converts to the α-helical state upon binding to the surface which ends up with the β-sheet state upon time (de Vocht et al 2002). Simulations of the molecular dynamics of the SC3 hydrophobin showed that the loop between the third and fourth cysteines is responsible for both the binding and the transition to the α-helical state. The lack of determination of the three-dimensional structure of SC3 protein has limited the further understanding of its binding to surfaces (Fan et al. 2006).

In this chapter, the coating ability of the SC3 hydrophobin on different hydrophobic and hydrophilic materials and on solid wood was tested and its surface activity was discussed.

6.3 Materials and methods

6.3.1 Fungal strain

The *S. commune* monokaryotic strain 4-40 (*MATA43 MATB43,* CBS 340.81) was kindly provided by Prof. Wösten, Utrecht, The Netherlands.

6.3.2 Growth conditions

Strain 4-40 was grown at 25°C in continuous light on *S. commune* minimal medium (20 g glucose, 1.5 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ x $7H_2O$, 1 g yeast extract, 0.12 mg thiamine-HCl, 0.1 mg pyridoxine HCl, 0.005 mg biotin, 0.2 mg $CuSO_4$ x $5H_2O$, 0.08 mg $MnCl_2$ x $4H_2O$, 0.4 mg cobaltous chloride hexahydrate, 1.2 mg calcium nitrate tetrahydrate per 1 liter H_2O; Dons et al. 1979). Pre-cultures were prepared on 1% agar *S. commune* minimal medium. Petri dish cultures were incubated for 7 days. Blocks of agar (1 cm^3) were cut from the edge of the growing colony and used as inoculums for the pre-cultures in 500 ml Erlenmeyer flasks containing 100 ml liquid medium. Pre-cultures were incubated for 10 days. Main cultures were inoculated with 10 ml homogenized mycelial macerate from the pre-cultures in 100 ml liquid medium.

6.3.3 Culture harvest

Cultures were grown for 10 days. The mycelium was separated from the culture medium with a nylon mesh (0.3 mm pore size) in a funnel connected to vacuum. The scrapped mycelium was collected into a 50 ml Falcon tube (SARSTED AG & Co, Nümbrecht, Germany) and freeze dried in a lyophilisator. Lyophilized mycelium was finely grinded by using mortar and pestle in the presence of liquid nitrogen.

6.3.4 Purification of SC3 hydrophobin

SC3 hydrophobin was purified from the aerial hyphae according to Wösten et al. (1993). Non-covalently bound lipids were removed from the mycelium by extracting with chloroform/methanol (2:1 [v/v]) for 5 times. The resulting pellet was extracted twice with hot SDS (100°C), washed eight times with water and freeze-dried. The freeze-dried material was dissociated by sonicating 3 times for 30 seconds each in presence of TFA (Tri-fluoro acetic acid, 200 µl/mg of freeze-dried material) at 4°C. The suspension was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected into a fresh Falcon tube and dried. The dried pellet was re-suspended in 60% ethanol (1 ml/mg), 0.1 M Tris, pH 8.0, tightly closed and left overnight at room temperature. The suspension was centrifuged and the supernatant was dialyzed (dialysis tubing, size 2, Dia 18/32"-14.3 mm, Mol. wt. cut off 12-14 kD, Medicell International Ltd, London) against three changes of water for 60 min each time. The

solution was bubbled with compressed air (with a needle connected to the tube from air supplying device) and the foam was collected into a fresh Falcon tube and freeze-dried in a lyophilisator to get the powdered form of the SC3 protein. Protein was quantified by the Lowry method according to Peterson (1977). About 0.1 % (w/w) of SC3 hydrophobin was obtained through this process (0.5 mg of SC3 hydrophobin from 500 mg of mycelium). For subsequent usage, 4 mg of SC3 powder was solubilized in 10 ml of water.

6.3.5 Analytical procedures

1D-SDS-PAGE was performed according to Laemmli (1970). A 12 x 12 cm discontinuous 15 % acrylamide gel with 0.025 M Tris base, pH 8.3 was used as electrode buffer. The purified SC3 hydrophobin sample was mixed with sample buffer containing 0.06 M Tris-Cl, pH 6.8, 10 % glycerol and 0.025 % bromophenol blue. The samples were reduced with 2β-mercapto-ethanol to a 5 % total volume, cooked at 98°C for 4 min and then cooled to room temperature. A 20 µl sample with 1 µg/µl protein was loaded and run at 10-15°C with a constant current of 20 mA. Gels were fixed overnight in 30 % methanol with 12 % acetic acid, 0.05 % formaldehyde and stained with silver as described by Blum et al. (1987). The stained gel was scanned at 300 dpi resolution (Microtek, TMA, Germany).

6.3.6 Coating SC3 hydrophobin on Teflon, form-war film and glass

Teflon sheets of size 1 x 1 cm, 100 µm thickness (Plast Brand, PTFE Manschetten, Brand GMBH + Co, Werthein, Germany) were treated with 1 mg/0.5 ml of SC3 protein for 24 hrs and dried at room temperature for 5 hrs. To check the surface activity, the Teflon sheets were tested with 5 µl of colored water. Form-war films were prepared by immersing Parafilm (PECHINEY, Chicago, USA) of size 0.5 x 0.5 cm into 5 % polyvinyl formaldehyde in water free chloroform supplied by PLANO, Wetzlar, Germany and are treated with 1.5 mg/0.5 ml of SC3 protein for 24 hrs and dried at room temperature for 5 hrs. To check the surface activity of the formed films, form-war films were tested with 5 µl of colored water. Glass cover slips (size 22 x 22 mm, Menzel GmbH Co KG, Braunschweig, Germany) were treated with 30 µg/ml of SC3 protein for 24 hrs and dried at room temperature for 5 hrs and tested with 5 µl of colored water.

6.3.7 Coating beech wood with SC3 hydrophobin

Beech wood blocks (0.7 x 0.5 x 1 cm^3, longitudinal to the axis, radial to the axis x tangential to the axis) with the cross-sectioned fibers facing downwards were immersed in 1 ml solution containing SC3 hydrophobin in glass tubes (1.5 cm in Ø, 10 cm in length) which were tightly closed. 5, 10, 15, 25, 50, 100, 150, 200, 250 and 400 µg/ml concentrations of SC3 protein were used. Note that the solutions with 5 and 10 µg/ml were produced using a 20 µl pipette by pipetting 12.5, respectively 25 µl of a 4 mg/10 ml hydrophobins solution into water, in order to give a final volume of 1 ml hydrophobins solution. Higher concentrations were prepared by pipetting with either a 200 µl or an 1000 µl pipette appropriate volumes of the original hydrophobin solution of 4 mg/10 ml into water, in order to give a final volume of 1 ml with the required final concentration of hydrophobins. Wood blocks immersed in water without SC3 hydrophobin were used as controls. Three replicate wood blocks were used for each concentration and incubated at room temperature for 24 hrs without disturbing. After incubation, wood blocks were taken out onto a glass plate with forceps and oven dried at 70°C for 2 days. Three independent sets of experiments were performed, thereby using wood blocks from the same batch whereas the hydrophobin solutions were each time newly prepared from aliquots of the SC3 hydrophobin powder.

6.3.8 Water absorption measurements

After drying, the 0.7 x 0.5 x 1 cm^3-sized wood blocks were placed into a glass Petri dish, 5 µl of water was placed onto the tangential section of the wood blocks and the Petri dish was closed with the lid. The time for the absorption of the water by the wood was noted with a stop watch.

6.3.9 FTIR-spectroscopy

6.3.9.1 Purified hydrophobin

About 0.5 mg purified SC3 hydrophobin was used for the measurement. The hydrophobin powder was pressed onto the crystal with a concave press tip (specified for materials such as powders, pellets and irregular shaped solids as described by the manufacturer, Dura SamplIR) where the infrared beam in the FTIR-measurements is attenuated at the interface between the powder and the crystal. FTIR absorption

spectra of hydrophobin were recorded (conditions: 32 scans at 4 cm^{-1} resolution) with an FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an Attenuated Total Reflection (the infrared beam is attenuated and totally reflected) unit (Dura Sampl IR). Three replicate measurements were performed for one SC3 hydrophobin preparation. The spectra were vector-normalized, baseline-corrected (rubber band method, 64 points as described in the OPUS version 5.0 software, Bruker, Germany) and averaged.

6.3.9.2 Control Teflon and SC3 treated Teflon

Teflon sheets of size 1 x 1 cm, 100 μm thickness (Plast Brand, PTFE Manschetten, Brand GMBH + Co, Werthein, Germany) were used for FTIR measurement as described above. The Teflon sheets were pressed onto the crystal with a flat press tip (specified for materials such as films, wood blocks, paper, etc.) as described by the manufacturer, Dura SamplIR) where in the FTIR-measurements the infrared beam is attenuated at the interface between sample and the crystal. Each 3 replicate measurements were performed for 3 samples of control and of SC3-treated Teflon. Total 9 spectra each of control Teflon and SC3-treated Teflon were vector-normalized, baseline corrected (rubber band method, 64 points as described in the OPUS version 5.0 software, Bruker, Germany) and averaged.

6.3.9.3 Control beech wood and SC3 treated beech wood

Beech wood blocks that were used for the water absorption measurements were oven dried at 70°C for 2 days and kept in the FTIR-measurement room at 19°C for one day. FTIR absorption spectra of wood blocks were recorded with an FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an Attenuated Total Reflection as described above. The wood blocks were pressed onto the crystal with a flat press tip (specified for materials such as wood blocks, paper etc. as described by the manufacturer, Dura SamplIR) where in the FTIR-measurements the infrared beam is attenuated at the interface between the wood block and the crystal. At least 3 replicate measurements were made for each replicate wood block of each concentration of hydrophobin-treated wood blocks.

6.3.9.4 FTIR-spectral analysis

Spectral data were evaluated using OPUS version 5.0 software (Bruker, Germany). All FTIR spectra from purified SC3 hydrophobin, Teflon and wood blocks treated with the different hydrophobin concentrations were vector-normalized, baseline-corrected and averaged. Baseline-correction was made by the rubber band method using 64 base line points as described in the OPUS version 5.0 software, Bruker, Germany.

6.3.9.5 Wood vessel measurement

Beech wood sections of 25 µm thickness were prepared by a microtome and placed onto a glass slide. Samples were analysed with a Zeiss Axiophot photomicroscope (Zeiss, Göttingen, Germany) equipped with a soft imaging colour view II Mega pixel digital camera (Soft Imaging System, Münster, Germany) that was linked to a computer equipped with analySIS® software programme (Soft Imaging System, Münster, Germany). In total, each one section from three different wood blocks were analysed. Per section, three different areas of 1.13 x 0.84 μm^2 were considered. All vessels in this area were counted. In average, 110 ± 10 were present. Per observation area, the diameter of 30 vessels were determined as shown in Fig. 1, i.e. in total 270 vessels.

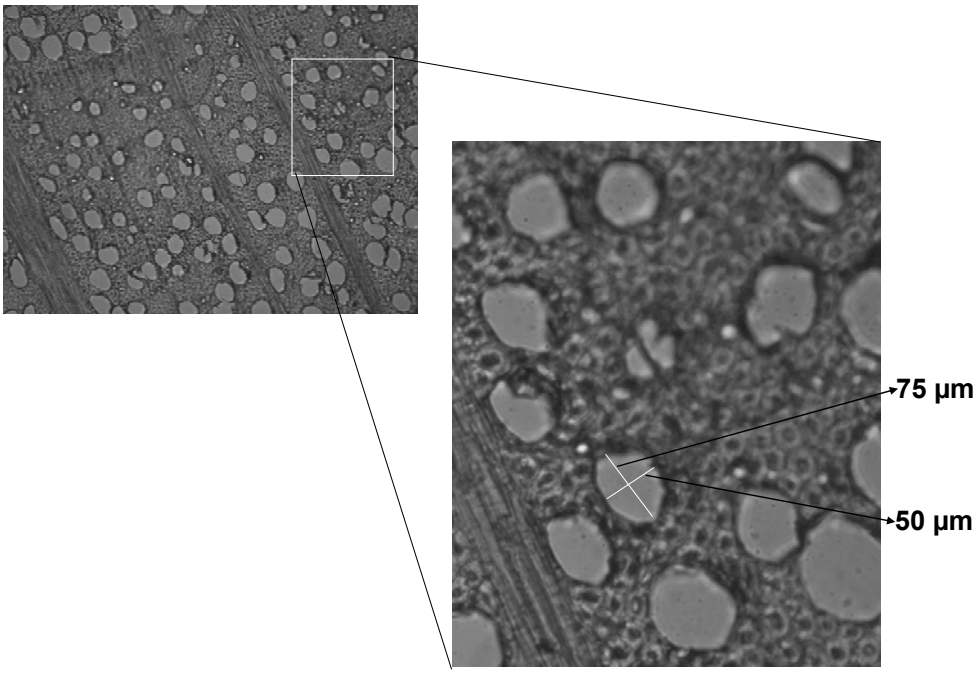

Fig. 1 An example of a wood cross-section showing the strategy of measurement of vessel diameter inside the beech wood. The measured data were used for the calculation of the total surface area of the vessels in wood blocks as given in the results section.

6.3.9.6 Statistical analysis

Statistics was performed by using SPSS software release 9.0.0, standard version (SPSS Inc., Chicago, USA). Data sets were compared using LSD test up to 95% confidence level ($p \leq 0.05$).

6.4 Results

6.4.1 Purified hydrophobin

About 0.1% (w/w) of SC3 hydrophobin (Fig. 2) from the mycelium of *S. commune* (0.5 mg of SC3 hydrophobin from 500 mg of mycelium) was obtained through the isolation process described in the methods section. The molecular weight and the purity of the protein was confirmed by SDS-PAGE (Fig. 3) showing an intense band at 24 kD being the size described previously for SC3 (Wösten et al. 1993, Lugones et al. 2004). FTIR analysis of SC3 hydrophobin revealed a number of absorption peaks that are formed due to amide vibrations of the peptide group of the protein (Fig. 4, Table 1, compare Jung 2000).

Fig. 2 Powdered form of purified SC3 hydrophobin from the mycelium of *S. commune* monokaryon 4-40 (*MATA*43*MATB*43, CBS 340.81).

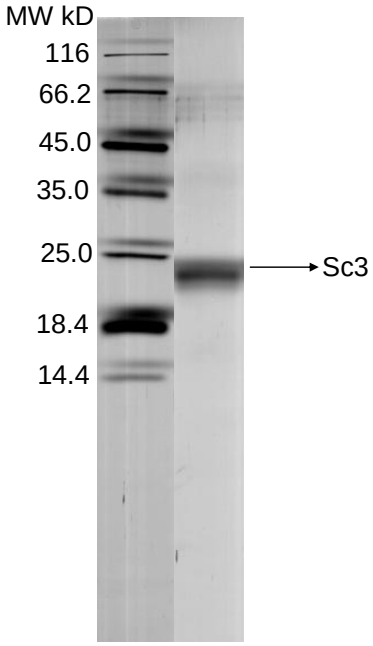

Fig. 3 Silver stained 1D-SDS-PAGE of purified SC3 hydrophobin from the mycelium of *S. commune* 4-40. Molecular weight markers are given at the left. 20 µg of protein was loaded on to the gel.

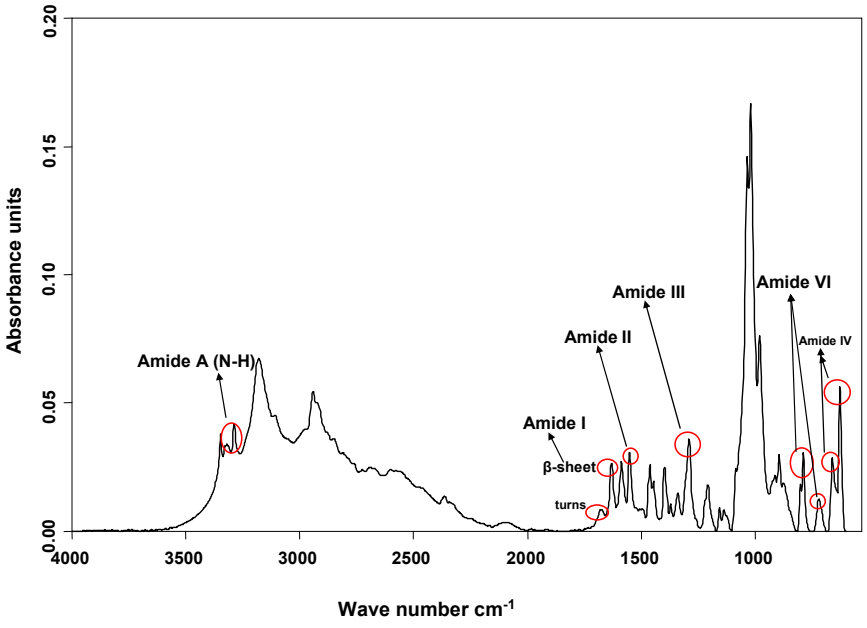

Fig. 4 ATR-FTIR spectrum of purified SC3 hydrophobin. The spectrum shown was vector-normalized, baseline-corrected and averaged from 3 replicate spectra.

Table 1 Amide vibrations of the peptide group **(Fig. 4)** in the absorption bands in the FTIR spectra of the SC3 hydrophobin

Peak position (cm^{-1})	Band	Assignment
3320 and 3389	Amide A	NH stretch, in resonance with amide II overtone
3171 and 2942	unknown	
1678 and 1633	Amide I, ß-sheet	Mainly CO stretching, slightly coupled with CN stretching, CCN deformation, NH bending
1587	unknown	
1549	Amide II	NH bending coupled with CN stretching
1461, 1392, 1339, 1293	unknown	
1293	Amide III	NH bending and CN stretching
1213, 1140, 1022	unknown	
789, 724	Amide VI	Out-of-plane NH bending
659, 629	Amide IV	OCN bending, coupled with other modes

Peaks were assigned according to Jung 2000.

6.4.2 SC3 coatings on hydrophobic and hydrophilic materials

Teflon sheets (size 1 x 1 cm, 100 μm thickness), form-war film (size 0.5 x 0.5 cm), and glass slides (size 22 x 22 mm) were treated with purified SC3 solutions as described in the methods section. The surface activity of hydrophobic and hydrophilic materials changed upon treatment with SC3 hydrophobin. Hydrophobic Teflon and form-war film turned hydrophilic (Fig. 5A, 5B, 5C, 5D) and hydrophilic glass turned hydrophobic (5E, 5F) on coating with SC3 hydrophobin. For FTIR-analysis of coated material, Teflon was chosen by its hardness which can resist pressure applied during the FTIR-measurement from the flat press tip (see methods). Whilst the glass samples would brake by such measurement and the very thin film form-war samples would not easily release from the parafilm for such measurements.

FTIR analysis of control Teflon and SC3 hydrophobin-treated Teflon showed differences in the spectra (Fig. 6). An intense peak associated with the amide I band of the peptides in the protein at 1653 cm^{-1} was observed in the hydrophobin-treated

Teflon. There were also four unknown peaks observed at 1468, 1376, 1022 and 1002 cm^{-1} in the hydrophobin-treated Teflon.

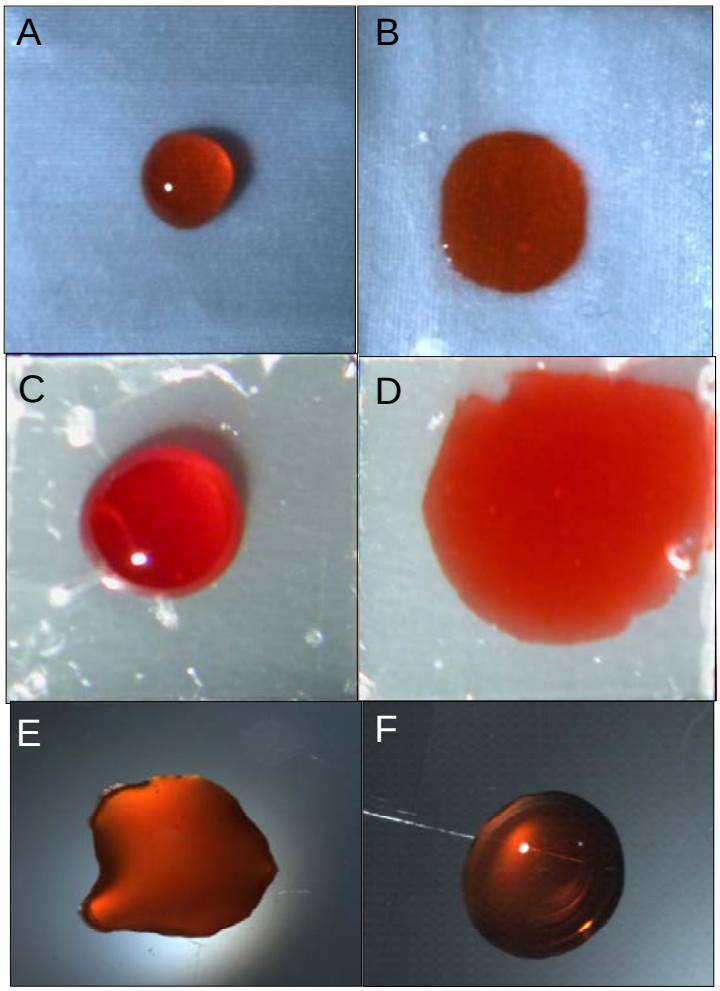

Fig. 5 Coating of SC3 hydrophobin on hydrophobic (Teflon, Form-war film) and hydrophilic (glass) materials. Control Teflon (A), SC3-coated Teflon (B), control form-war film (C), SC3-coated form-war film (D), control glass (E) & SC3-coated glass (F). 1 mg, 1.5 mg and 30 µg SC3 hydrophobin concentrations for coatings were randomly chosen in each 1 ml water was used for coating Teflon, form-war film and glass, respectively.

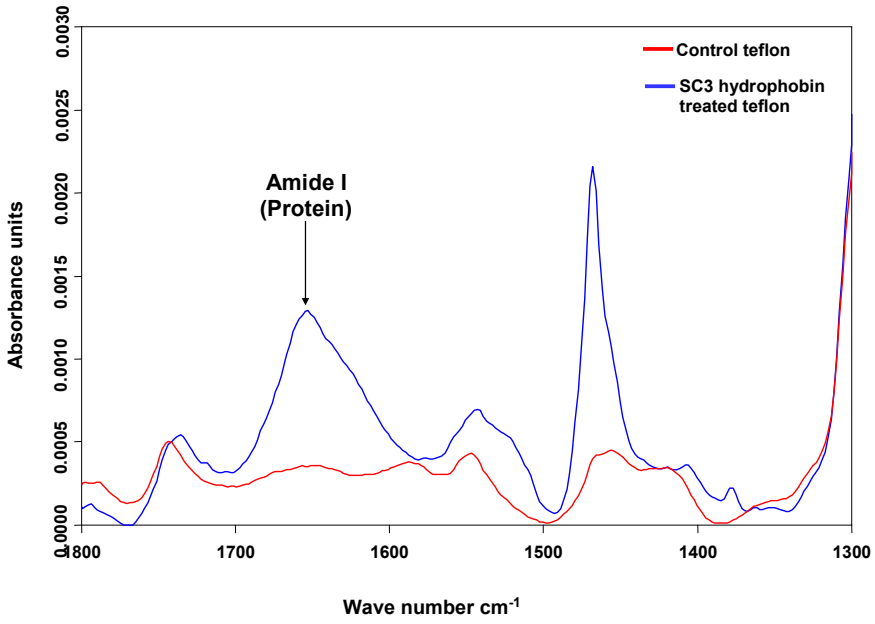

Fig. 6 ATR-FTIR spectra of control Teflon and SC3 hydrophobin treated Teflon. Spectra shown were vector normalized, baseline corrected and average of 9 replicate spectra of three different samples each of control Teflon and SC3 treated Teflon.

6.4.3 Hydrophobin activity on beech wood surface

Beech wood samples were treated with purified SC3 solutions as described in the methods section. The surface activity of beech wood blocks changed upon treatment with SC3 hydrophobin when compared with the control wood (wood treated only with water, Fig. 7 and 8). The change in surface activity was dependent on the hydrophobin concentration. Lower SC3 hydrophobin concentrations (between 15 and 100 µg total hydrophobin) resulted in lower hydrophilic behavior of the wood blocks and higher SC3 hydrophobin concentrations (150 to 400 µg total hydrophobin) resulted in higher hydrophilicity of the wood compared to the untreated control wood samples (Fig. 9). The differences in water repellency were significant in the range of 15 to 50 µg and in the range of 200 to 400 µg (see Table 2).

Since the wood vessels show a capillary action to transport water in the wood (Sperry 2003), it has to be assumed that the inner surface of the wood vessels acts hydrophilic. During the treatment with hydrophobins in watery solutions, it is likely that hydrophobin applied to the wood may be onto the surface of the vessels within the

wood. To estimate the minimum amount of hydrophobin required to bring a change in the nature of the wood, the overall wood vessel surface was calculated. For this, the total inner surface area of vessels was calculated by analyzing each 3 different measurement areas each 0.84 x 1.13 mm^2 per section of three different wood blocks contained on an average 110 ± 10 (Fig. 1). The surface area A of a wood vessel was calculated as:

$A = \Pi \times (d_1 + d_2)/2 \times h$

$\Pi = 22/7 = 3.141592$

d_1 = longest diameter of the vessel (e.g., 75 µm in Fig. 1)

d_2 = shortest diameter of the vessel (e.g., 50 µm in Fig. 1)

h = height of the wood block (assuming that the vessel goes through the whole block in a more or less even way).

On an average, 5794 vessels per whole wood block cross section of 10 x 5 mm^2 were present. A single vessel going through the whole wood block of 5 mm in length would have a surface of about 0.98 ± 0.31 mm^2. In total, 56.8858 cm^2 = 0.00568 m^2 vessel surface arise for the whole wood block. Wessels (1997) reported that 1.5 mg of SC3 hydrophobin is needed to cover a Teflon surface area of 1 m^2. In accordance, it should require 0.00855 mg (8.55 µg) to cover the 0.00568 m^2 vessel surface. However, the minimum amount of SC3 hydrophobin needed in laboratory experiments to bring a drastic change in the nature of wood from hydrophilic to hydrophobic was about 15 µg for 0.00568 m^2 that equals to 2.6 mg for 1 m^2 surface area of the wood vessel. This value is about 1.73 fold higher than the value given by Wessel (1997) for covering the same surface area of Teflon.

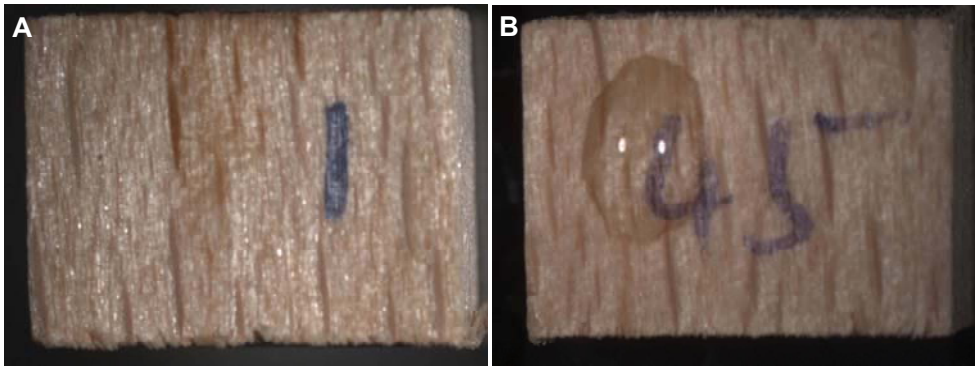

Fig. 7 Effect of treatment of beech wood with SC3 hydrophobin on water uptake of the wood. SC3-treated wood (A) and control wood (B). Photographs were taken 1 minute after placing 5

µl water on the wood blocks. 400 µg of SC3 hydrophobin in 1 ml water was used for the treatment of the sample.

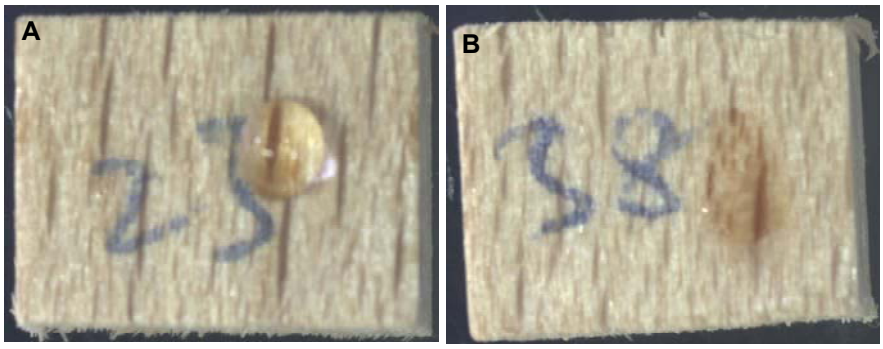

Fig. 8 Effect of treatment of beech wood with SC3 hydrophobin on water uptake of the wood. SC3-treated wood (A) and control wood (B). Photographs were taken 10 minute after placing 5 µl water on the wood blocks. 15 µg of SC3 hydrophobin in 1 ml water was used for the treatment of the sample.

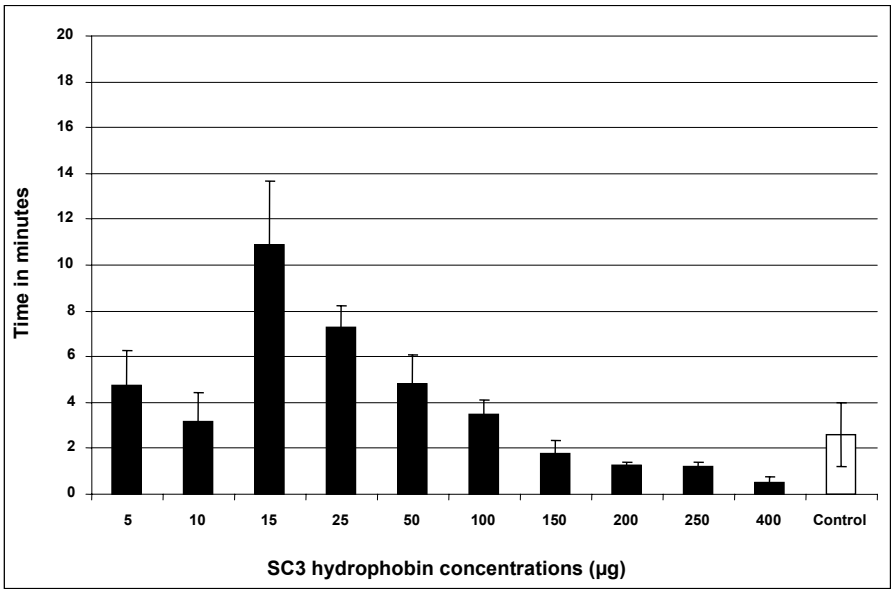

Fig. 9 Measurement of time required for the absorption of 5 µl water by the SC3 hydrophobin treated beech wood. Note, the jump in the results between the 10 µg and the 15 µg SC3 treatment might be due a fine error caused by a pipette change during the experiment (see material and methods). Values were averages of 9 values. The data presented in this figure were calculated from that of three individual sets of experiments. The results of the individual experiments are presented in Fig. 11, 12, 13 in the appendix.

Table 2 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of SC3-hydrophobin treated beech wood in comparison with the untreated control beech wood.

SC3 hydrophobin concentration (µg)	Significance with control wood
5	NS
10	NS
15	S
25	S
50	S
100	NS
150	NS
200	S
250	S
400	S

For each concentration, in total 9 values obtained from three independent experiments were used for the calculation. S indicate significant and NS not significant differences. The results of the individual experiments are presented in Tables 3, 4 and 5 in the appendix.

6.4.4 FTIR-spectroscopy

FTIR spectroscopy was used to check the binding of SC3 protein to the beech wood surface. ATR-FTIR spectra of the SC3-hydrophobin treated beech wood showed an increase in the intensity of the peak associated with the amide I band (at 1640 cm^{-1} wave number region) of the spectra in a concentration dependent manner (Fig. 10).

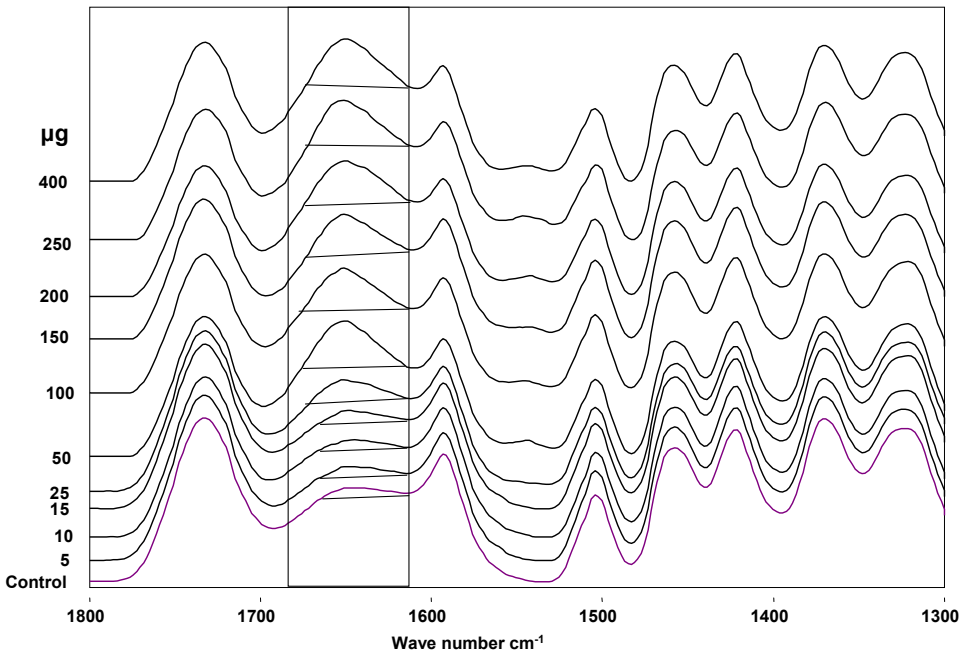

Fig. 10 ATR-FTIR spectra of *S. commune* hydrophobin SC3-treated beech wood. The intensity of the peak associated with the amide I band (as indicated by the horizontal line inserted into the spectra to elucidate the increase in the respective peak) of proteins increased with an increase in the concentration of the SC3 hydrophobin. Each spectrum represents vector-normalized, baseline-corrected averages of at least nine replicate spectra.

6.5 Discussion

Hydrophobins as small highly stable with an amphiphatic character are of interest as coating material due to their non-toxic (they are consumed by humans as parts of edible mushrooms since ancient days) and environment friendly nature. Hydrophobins such as SC3 from *S. commune* are secreted by the hyphal tips, cover the hyphae in form of a one-layered protein film and mediate the attachment of the hyphae to the solid supports (Wessels 1997, Wösten et al. 1994). During self-assembly of the firstly free proteins into films, the hydrophobins adopt a rodlet structure. Hydrophobin protein rodlets are about 10 nm thick. Since films of hydrophobins are amphiphatic, the hydrophilic surface of the hyphae renders hydrophobic (Wösten et al. 1993, Wösten et al. 1994, Wessels 1997). Moreover, the amounts of hydrophobins required to coat the surfaces are extremely in low quantities (Wessels 1997). These surprising features of the hydrophobins together with their function to mediate adhesion to surfaces has found a special interest in biotechnology, e.g. in tissue adhering on Teflon surfaces for medical purposes or in stabilization of foams in the food industry

(Scholtmeijer et al. 2001). Further, the adsorption of hydrophobins to the surfaces is possible at room temperature which helps in easy handling of the experimental setup and in applications (Wösten et al. 1994 and present study). To the authors opinion it might also be possible that hydrophobins are used in combination with other coating materials, for example in order to reduce at least the percentage of other chemical required for a specific coating, particularly if toxic, expensive and/or from non-renewable resources. Precedent uses of applying hydrophobins in combination with other surfactants are however not yet reported.

Hydrophobin coatings on various hydrophobic (Teflon, parafilm, polyethylene, polymethylmeta-acrylate, Nylon, insect cuticles, oil droplets) and hydrophilic (glass, cellulose) materials have been tested previously for their ability to change the respective surfaces from hydrophobic to hydrophilic or, respectively, from hydrophilic to hydrophobic (Hektor & Scholtmeijer 2005, Linder et al. 2005, Scholtmeijer et al. 2001, Wessels 1997, Wösten et al. 1994). The results for Teflon and for glass presented in this study confine the literature reports. Similarly, the results obtained with the Form-war film were as expected from behavior of similar materials. There were however no previous studies of hydrophobin coatings on wood materials. In this study, for the first time, adsorption of the SC3 hydrophobin on beech wood has successfully been tested. In the present study, 15 µg SC3 could bring a significant change in the wettability of wood surface for a beech wood block being 1 x 0.7 x 0.5 cm^3 in size.

Transport of water takes place from one part of a plant to another under capillary suction at cell walls of xylem cells, such as tracheids, vessels, etc. (Sperry 2003). Xylem vessels in wood of deciduous trees are formed from elongated cells called vessel elements having enlarged lumen diameters through intense inflated growth of the cell walls. Cell walls in between vessel elements will degrade during the process of vessel formation, giving rise to large tubes. On maturity, vessels have relatively large lumens, lack cytoplasmic content and have lignified secondary cell walls (Zwieniecki and Holbrook 2000). The size of various pores inside the wood such as tracheid lumina, pits in between vessels and cell wall pores range about 10, 2, and 0.01 µm, respectively (Baines et al. 1983, Sperry 2003). Pores play an important role

for the transport of water whilst the lignification of the secondary cell walls help to resist the pressure put on them during the water transport (Banks and Levy 1980, Baines et al. 1983, Fahlén 2005, Zwieniecki and Holbrook 2000). For the water transport, the inner surface of the vessels has to allow contacts to the water through adhesion. In consequence, the inner surface of the vessels has to have a certain hydrophilic character (Canny 2000). For wood in applications, this property can be a serious problem since wood taking up water is prone to attack e.g. by various types of fungi (white rot, brown rot, soft rot; see Schwarze 2000 and Schmidt 2006 for excellent overviews on fungal wood decay). To overcome problems in applications due to wettability of wood, various types of measures are applied to reduce wettability heat treatments, oil treatments, chemical treatments and combinations thereof (for examples from the literature see Sailer et al. 2000, Podgorski et al. 2000, Petrissans et al. 2003, Petric et al. 2007, Xie et al, 2006, Gao and Li 2007). There are however disadvantages in such treatments. Heat-treatment for example can cause changes in visual properties (colour) and in static properties (Sailer et al. 2000). Chemicals are often toxic (Mai and Militz 2007), despite that they are often also expensive and come usually from non-renewable resources.

Application of the non-toxic hydrophobins that are easily produced in fungal culture could overcome some of the problems related to currently perform wood modification techniques. Because hydrophobin protein rodlets are about 10 nm thick, it was assumed that hydrophobins are able to intrude not only the wood vessels and the pits between the vessels, but migrates also into the smallest pores inside the wood structure. This feature turned out to be very helpful in changing the nature of interior parts of the wood. For example as shown in this work, the vessel elements could be made hydrophobic by coating with hydrophobins. This phenomenon can bring a potential application of hydrophobins in wood technology, in particular in the field of wood modification.

Wessels (1997), Wösten et al. (1994) and Scholtmeijer et al. (2001) described that 1 to 1.5 mg of SC3 can coat 1 m^2 of Teflon surface. The present study with the solid beech wood indicated that 2.6 mg of SC3 is required to change the nature of 1 m^2 surface area of beech wood vessels. Wood is very non-homogeneous (Gardner et al.

1991), it is likely that more protein might be attached to the rough surfaces of wood vessels than to smooth surfaces such as Teflon. Although for industrial applications higher amounts of SC3 are needed, our results at the small scale indicate that low amounts of SC3 hydrophobin could bring drastic changes in the wettability of wood material. However, there is not only the possibility to reduce the wettability of wood by application of low amounts of hydrophobin but also the possibility of increasing wettability by application of higher amounts of hydrophobin (see Fig. 9). This latter possibility might be for example of interest when through should be transferred into the wood with the help of watery solutions. Further studies have to reveal whether after such treatment, another round of hydrophobin application can render the wood again more hydrophobic.

6.6 References

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6.7 Appendix to chapter 6

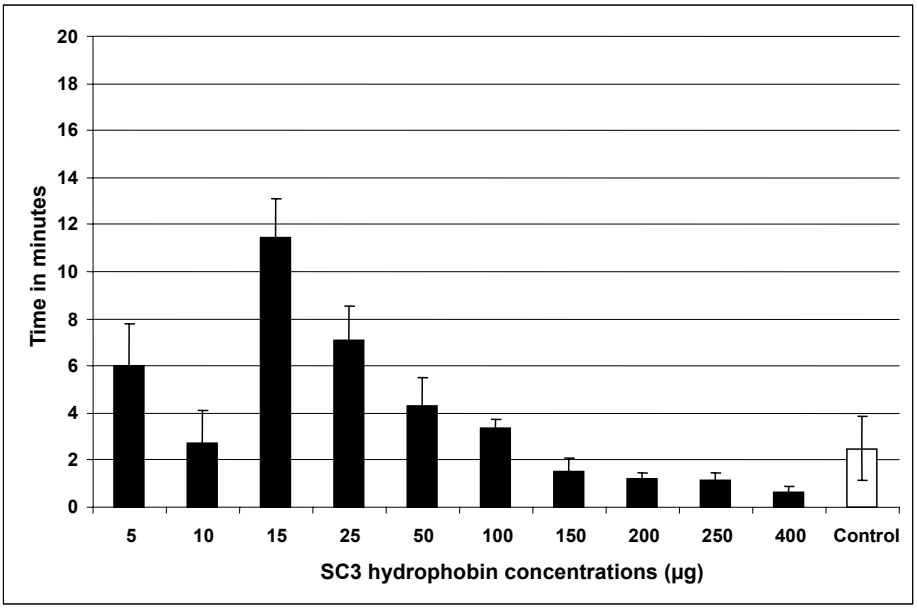

Fig. 11 Measurement of time required for the absorption of 5 µl water by the SC3 hydrophobin treated beech wood (Experiment 1).

Table 3 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of SC3-hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 1).

SC3 hydrophobin concentration (µg)	Significance with control wood
5	S
10	NS
15	S
25	S
50	S
100	NS
150	NS
200	NS
250	NS
400	S

For each concentration, in total 3 values were used for the calculation. S indicate significant and NS not significant differences.

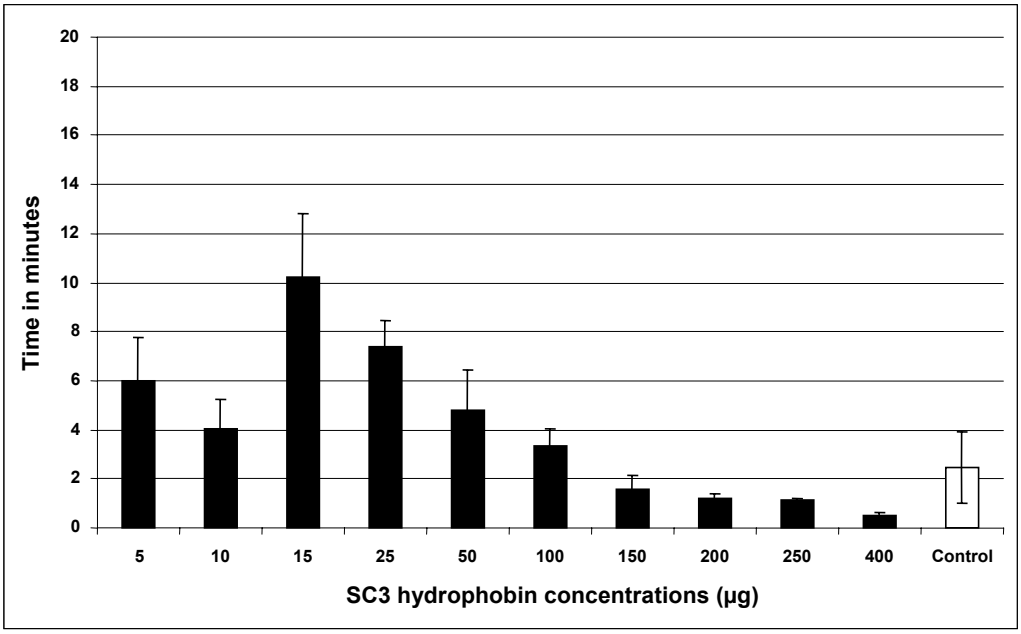

Fig. 12 Measurement of time required for the absorption of 5 µl water by the SC3 hydrophobin treated beech wood (Experiment 2).

Table 4 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of SC3-hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 2).

SC3 hydrophobin concentration (µg)	Significance with control wood
5	NS
10	NS
15	S
25	S
50	S
100	NS
150	NS
200	NS
250	NS
400	S

For each concentration, in total 3 values were used for the calculation. S indicate significant and NS not significant differences.

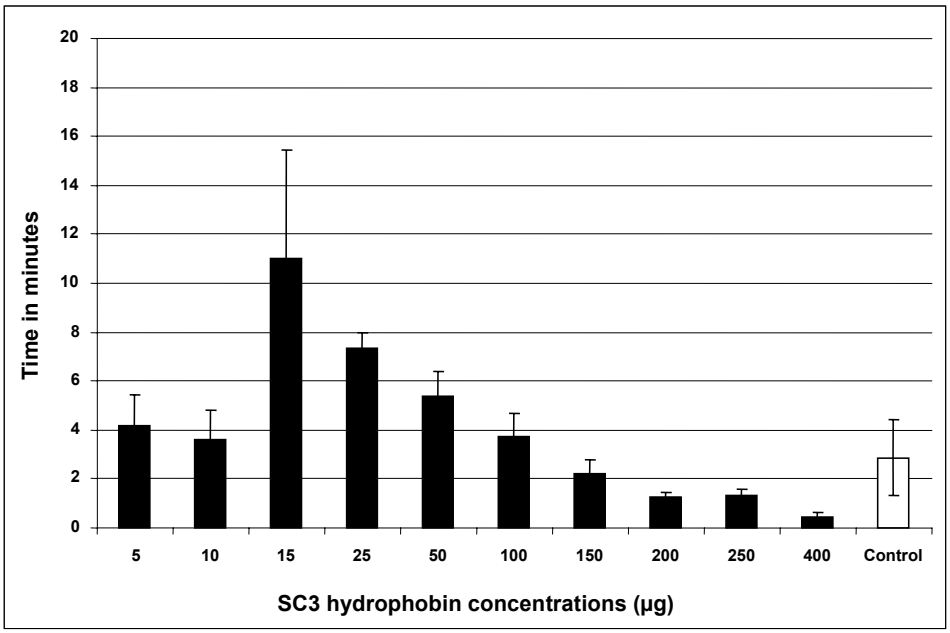

Fig. 13 Measurement of time required for the absorption of 5 µl water by the SC3 hydrophobin treated beech wood (Experiment 3).

Table 5 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of SC3-hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 1).

SC3 hydrophobin concentration (µg)	Significance with control wood
5	NS
10	NS
15	S
25	S
50	S
100	NS
150	NS
200	NS
250	NS
400	S

For each concentration, in total 3 values were used for the calculation. S indicate significant and NS not significant differences.

Chapter 7

Class I hydrophobins from *C. cinerea* and *P. ostreatus* in wood technology

7. Class I hydrophobins from *C. cinerea* and *P. ostreatus* in wood technology

7.1 Abstract

The surface activities of class I hydrophobins from *Coprinopsis cinerea* and *Pleurotus ostreatus* have been tested on wood. Beech wood turned either hydrophilic or hydrophobic, depending on the concentration of hydrophobins applied. Lower concentrations (15, 25, 50 μg/ml) of hydrophobins from *C. cinerea* and *P. ostreatus* resulted in hydrophobic behavior and higher concentrations (100 to 400 μg/ml) resulted in hydrophilic behavior of beech wood. Compared to the untreated control wood, ATR-FTIR spectra of the *C. cinerea* and *P. ostreatus* hydrophobin-treated beech wood showed an increase in the intensity of the peak associated with the amide I band of the protein part of the spectra in a concentration dependent manner, indicating the binding of the hydrophobin to the wood polymers.

7.2 Introduction

SC3, a class I hydrophobin from *S. commune* shown in chapter 6 to coat wood under changing its wettability is until now the best studied hydrophobin (Wösten 2001). Hydrophobins from other basidiomycetes also belong to class I although there is little sequence conservation between different hydrophobins (Walser et al. 2003). Amongst the class I hydrophobins from basidiomycetes are hydrophobins from *Coprinopsis cinerea* (Ăsgeirsdóttir et al 1997, Velagapudi 2006) and *Pleurotus ostreatus* (Ăsgeirsdóttir et al. 1998). Hydrophobins from *C. cinerea* and *P. ostreatus* were classified as class I hydrophobins, since they are highly insoluble in the aqueous solutions like hot SDS. In the vegetative mycelium of wild type monokaryon *C. cinerea* JV6, two genes for hydrophobins have been described in the literature namely for proteins CoH1 and CoH2 that are sized about 10 kD CoH1 has been purified from fungal cultures (Ăsgeirsdóttir et al 1997). However, recently it was reported that CoH2 is not expressed in the vegetative mycelium of *C. cinerea* (Velagapudi 2006). The vegetative mycelium of *P. ostreatus* N001 has also been shown to express two hydrophobins, namely POH2 and POH3. Based on the amino acid sequence homology, these hydrophobins are relatively similar to SC3 of *S. commune*. Omitting the variable N-termini of the hydrophobins, the homology between SC3 and POH2 as well as POH3 is 65.4% and that between CoH1 and POH2/POH3 is 69.5% and 64.6% respectively. The high percentages of homology between the hydrophobins of *S. commune*, *C. cinerea* and *P. ostreatus* suggest that these class I hydrophobins can partially substitute each other in function (Ăsgeirsdóttir et al. 1998, Wösten 2001, Walser et al. 2003).

In this chapter, the surface activity of the class I hydrophobins from *C. cinerea* and *P. ostreatus* on beech wood have been tested to check whether there is functional similarity among class I hydrophobins from different fungi in wood coating.

7.3 Materials and methods

7.3.1 Fungal strains

The monokaryotic *C. cinerea* strain JV6 was from the institute`s own collection and *P. ostreatus* N001 (dikaryon) was kindly provide by Prof. Ramirez, Universida Publica de Navarra, Pamplona, Spain.

7.3.2 Growth conditions

The strain *C. cinerea* JV6 was grown on YMG/T (4 g yeast, 10 g malt extract, 4 g glucose and 100 mg tryptophan, 1 liter water; Granado et al. 1997) medium at 37^{o}C in the dark and *P. ostreatus* N001 on SMY (10 g of sucrose, 10 g of malt extract, 4 g of yeast extract, 1 liter of H_2O, pH 5.6; Peñas et al. 2002) medium at 28^{o}C in darkness. Pre-cultures were prepared on 1% agar medium. Petri dish cultures were incubated for 7 days. Blocks of agar (1 cm^3) were cut from the edge of the cultures and used as inoculums for the pre-cultures in 500 ml Erlenmeyer flasks containing 100 ml liquid medium. Standing cultures were incubated for 10 days. Main cultures were inoculated with 10 ml homogenized mycelial macerate from the pre-cultures in 100 ml liquid medium.

7.3.3 Culture harvest

Harvest of cultures was done after cultivating standing main cultures for 7 days at 28^{o}C and 37^{o}C for *P. ostreatus* and *C. cinerea* respectively. The mycelium was separated from the culture medium with a nylon mesh (0.3 mm pore size) in a funnel connected to vacuum. The respective scrapped mycelium was collected into a Falcon tube (SARSTED AG & Co, Nümbrecht, Germany) and freeze-dried in a lyophilisator. Lyophilized mycelium was finely grinded by using mortar and pestle in the presence of liquid nitrogen.

7.3.4 Purification of hydrophobin from *C. cinerea* and *P. ostreatus* aerial hyphae

Hydrophobins were purified from the aerial hyphae as described by Wösten et al. (1993) and Ăsgeirsdóttir et al. (1998). Non-covalently bound lipids were removed from the mycelium by extracting with chloroform/methanol (2:1 [v/v]) for 5 times. The solutions were centrifuged for 10 minutes at 4000 rpm and the resulting pellet was extracted twice with hot 2% (w/v) SDS, 0.05 M Tris/Hcl, pH 6.8, at 100^{o}C for 10 min. After further centrifugation for 10 min at 4000 rpm, the resulting pellet was washed eight times with water and freeze-dried. The freeze-dried material was dissociated by sonicating (3 times, 30 seconds each) in presence of TFA (Tri-fluoro acetic acid, 200 ul/mg of freeze dried material) at 4^{o}C. The suspension was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected and dried.

The dried material was re-suspended in 60% ethanol (1 mg/ml), 0.1 M Tris, pH 8.0 in a 50 ml Falcon tube (SARSTED AG & Co, Nümbrecht, Germany) that was tightly closed and left overnight at room temperature. The suspension was centrifuged and the supernatant was dialyzed (Molecular porous membrane, Mol wt cut off 3.5 kD, SPECTRA/POR, SERVA, Heidelberg, Germany) against three changes of water for 60 min each time. The solution was bubbled with compressed air (with a needle connected to the tube from air supplying device) and the foam was collected and freeze-dried in a lyophilisator to get the powdered pellet form of hydrophobin proteins from the mycelium of *Coprinopsis* and *Pleurotus*, respectively. Protein was quantified by Bradford reagent according to the manufacturer's protocol (Pierce, Rockford, IL, USA). For subsequent usage, 4 mg of SC3 powder was solubilized in 10 ml of water.

7.3.5 Analytical procedures

1D-SDS-PAGE was performed according to Laemmli (1970). A 12 x 12 cm discontinuous 15 % acryl amide gel was used with 0.025 M Tris base, pH 8.3 as electrode buffer. The purified hydrophobin sample was mixed with sample buffer containing 0.06 M Tris-cl, pH 6.8, 10% glycerol, 0.025% bromophenol blue. Samples were reduced with 2β-mercapto-ethanol to 5% of total volume and then cooked at 98^{o}C and eventually cooled to room temperature. 10 µg samples with 1 µg/µl protein was loaded on the gel and run at 10-15^{o}C with a constant current of 20 mA. Gels were fixed overnight in 30% methanol with 12% acetic acid, 0.05% formaldehyde and stained with silver as described by Blum et al. (1987). The stained gel was scanned at 300 dpi resolution (Microtek, TMA, Hannover, Germany).

7.3.6 Coating beech wood with hydrophobins of *Coprinopsis* and *Pleurotus*

Beech wood blocks (0.7 x 0.5 x 1 cm^3, longitudinal to the axis x radial to the axis x tangential to the axis) with the cross-sectioned fibers facing downwards were immersed in 1 ml solution containing hydrophobin in glass tubes (1.5 cm in Ø, 10 cm in length) which were tightly closed. 5, 10, 15, 25, 50, 100, 150, 200, 250 and 400 µg/ml concentrations of hydrophobins were used. Note that the solutions with 5 and 10 µg/ml were produced by pipetting with a 20 µl pipette 12.5 and 25 µl respectively, of 4 mg/10 ml hydrophobin solution into water, in order to give a final volume of 1 ml

hydrophobins solution. Higher concentrations were prepared by pipetting with either a 200 µl or an 1000 µl pipette appropriate volumes of the original hydrophobin solution of 4 mg/10 ml into water to give a final volume of 1 ml with the required final concentration of hydrophobins. Wood blocks immersed in water without hydrophobin were used as controls. Three replicate wood blocks were used for each concentration for the hydrophobins of *Coprinopsis* and *Pleurotus*, respectively and incubated at room temperature for 24 hrs without disturbing. After incubation, wood blocks were taken out onto a glass plate with forceps and oven dried at 70^{o}C for 2 days. Two independent set of experiments were performed, thereby using wood blocks from the same batch whereas the hydrophobin solutions were each time newly prepared from aliquots of the *C. cinerea* and *P. ostreatus* hydrophobin powder, respectively.

7.3.7 Water absorption measurements

After drying, the 0.7 x 0.5 x 1 cm^3-sized wood blocks were placed into a glass Petri-dish, 5 µl of water was placed onto the tangential section of the wood blocks (as shown in Fig. 7 and Fig. 8 in chapter 6 of this thesis) and the Petri-dish was closed with the lid. The time for the absorption of the water by the wood was noted with a stop watch.

7.3.8 FTIR-spectroscopy

7.3.8.1 Purified hydrophobin from *C. cinerea* and *P. ostreatus*

About 0.5 mg purified hydrophobins from *C. cinerea* and *P. ostreatus* were used for the measurement. The hydrophobin powder was pressed onto the crystal with a concave press tip (specified for materials such as powders, pellets and irregular shaped solids as described by the manufacturer, Dura SamplIR) where the infrared beam in the FTIR-measurements is attenuated at the interface between the powder and the crystal. FTIR absorption spectra of hydrophobin were recorded (conditions: 32 scans at 4 cm^{-1} resolution) with an FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an Attenuated Total Reflection (the infrared beam is attenuated and totally reflected) unit (Dura Sampl IR). Six replicate measurements were performed for each of *C. cinerea* and *P. ostreatus* hydrophobins. The spectra were vector-normalized, baseline-corrected (rubber band method, 64

points as described in the OPUS version 5.0 software, Bruker, Germany) and averaged.

7.3.8.2 Control beech wood, *C. cinerea* and *P. ostreatus* hydrophobins treated beech wood

Beech wood blocks that were used for the water absorption measurements were oven dried at 70 ^{o}C for 2 days and kept in the FTIR measurement room at 19^{o}C for one day. FTIR absorption spectra of wood blocks were recorded with an FTIR spectrometer Equinox 55 (Bruker Optics, Ettlingen, Germany) combined with an Attenuated Total Reflection (the infrared beam is attenuated and totally reflected) unit (Dura Sampl IR). Conditions: 32 scans at 4 cm $^{-1}$ resolution. The wood blocks were pressed onto the crystal with a flat press tip (specified for materials such as wood blocks, paper etc. as described by the manufacturer, Dura SamplIR) where the infrared beam is attenuated at the interface between the wood block and the crystal. At least 3 replicate measurements were made for each replicate wood block of each concentration of hydrophobin treated wood blocks.

7.3.8.3 FTIR-spectral analysis

Spectral data were evaluated using OPUS version 5.0 software (Bruker, Germany). All FTIR spectra from purified hydrophobins and wood blocks treated with the different hydrophobin concentrations were vector-normalized, base-line corrected and averaged. Base line correction was made by the rubber band method using 64 base line points as described in the OPUS version 5.0 software, Bruker, Germany.

7.3.8.4 Statistical analysis

Statistics was performed by using SPSS software release 9.0.0, standard version (SPSS Inc., Chicago, USA). Data sets were compared using LSD test up to 95% confidence level ($p \leq 0.05$).

7.4 Results

7.4.1 Purified hydrophobins from vegetative mycelium of *Coprinopsis* and *Pleurotus*

About 0.3 % (w/w, 1600 µg hydrophobin from 0.5 g of mycelium) and 0.1 % (w/w, 600 µg hydrophobin from 0.5 g of mycelium) of hydrophobin was obtained from *Coprinopsis* and *Pleurotus* vegetative mycelium, respectively (Fig. 1). The finally obtained dried hydrophobins differed in structure. The hydrophobins from *P. ostreatus* were obtained as flat, yellowish coloured pellets whereas the hydrophobins from *C. cinerea* were obtained as powder as previously the SC3 hydrophobin from *S. commune*. The different appearance of the purified hydrophobins suggested that the *P. ostreatus* sample was probably of lower purity. When dissolving in water the solution of *P. ostreatus* hydrophobin was turbid whereas that of *C. cinerea* was clear, supporting this assumption. The molecular weight and purity of the protein was confirmed by SDS-PAGE. Two bands below 10 kD were observed for *C. cinerea* JV6 and two bands were also observed in case of *P. ostreatus* N001, one at 9 kD and another a faint band at 17 kD (Fig. 2). In the sample of the *P. ostreatus* hydrophobin, a background smear was visible, again indicating a lower purity of these hydrophobins. FTIR analysis of *C. cinerea* and *P. ostreatus* hydrophobin revealed a number of absorption peaks that are formed due to amide vibrations of the peptide group in the proteins (Fig. 3, 4 and Tables 1, 2). The spectra of the *C. cinerea* hydrophobins resembled that of *S. commune* SC3 spectra (compare Fig. 4 in chapter 6) whereas that of *P. ostreatus* was less fine in resolution (Fig. 4), supporting the view that there were differences in purity between the different protein samples.

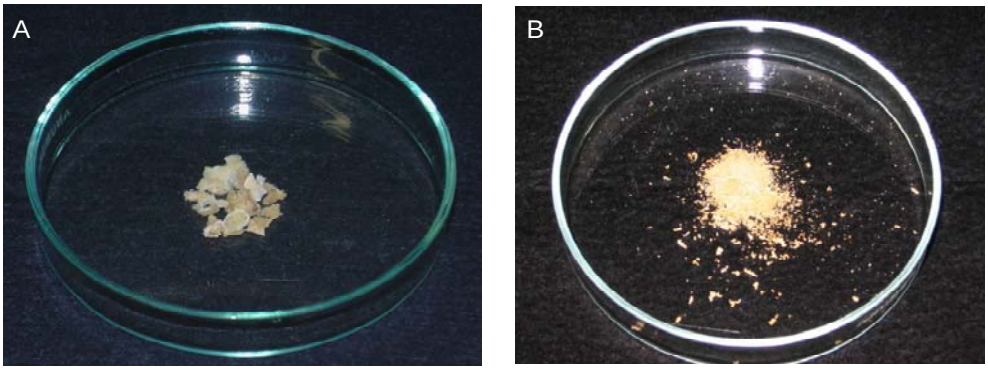

Fig. 1 Purified hydrophobins from the mycelium *P. ostreatus* N001 (A) and *C. cinerea* JV6 (B). In case of *P. ostreatus*, the hydrophobins were obtained as flat, yellowish pellets whereas hydrophobins of *C. cinerea* was laso obtained as yellow colour but in the form of powder as previously the SC3 hydrophobins from *S. commune* (compare Fig. 2 in chapter 6).

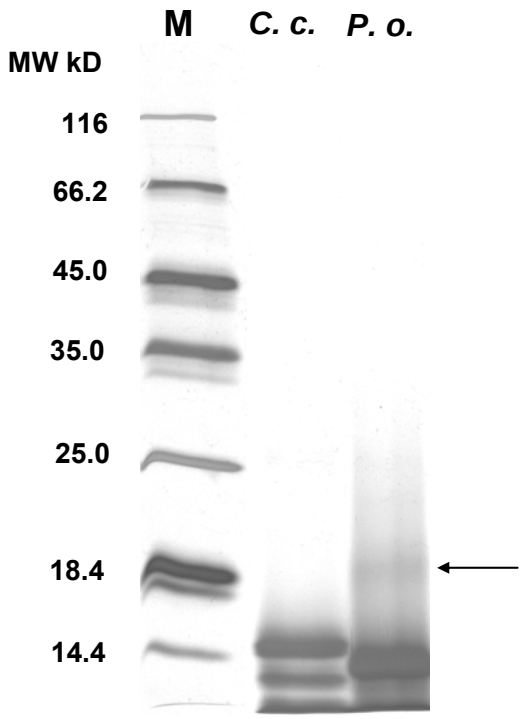

Fig. 2 Silver stained 1D-SDS-PAGE of purified hydrophobins from the vegetative mycelium of *C. cinerea* JV6 (*C. c.*) and *P. ostreatus* N001 (*P. o.*). A faint band in the *P. ostreatus* sample at 17 kD is indicated by arrow. Molecular weight markers are given at the left.

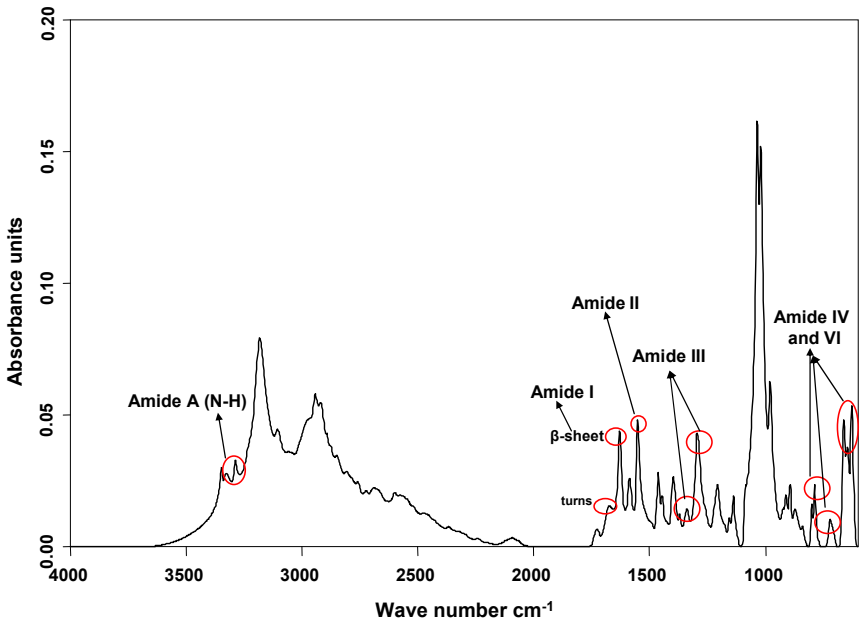

Fig. 3 ATR-FTIR spectra of purified *C. cinerea* hydrophobins. The Spectrum shown was obtained from vector-normalized, baseline-corrected and averaged 6 replicate spectra.

Table 1 Amide vibrations of the peptide group **(Fig. 3)** in the absorption bands in the FTIR spectra of *C. cinerea* hydrophobin.

P-eak position (cm^{-1})	Band	Assignment
3289	Amide A	NH stretch, in resonance with amide II overtone
3186, 2931 and 1587	unknown	
1675 and 1630	Amide I, ß-sheet	Mainly CO stretching, slightly coupled with CN stretching, CCN deformation, NH bending
1549	Amide II	NH bending coupled with CN stretching
1465 and 1400	unknown	
1340 and 1293	Amide III	NH bending and CN stretching
1205, 1144, 1029 and 900	unknown	
789 and 720	Amide VI	Out-of-plane NH bending
659 and 629	Amide IV	OCN bending, coupled with other modes

Peaks were assigned according to Jung 2000.

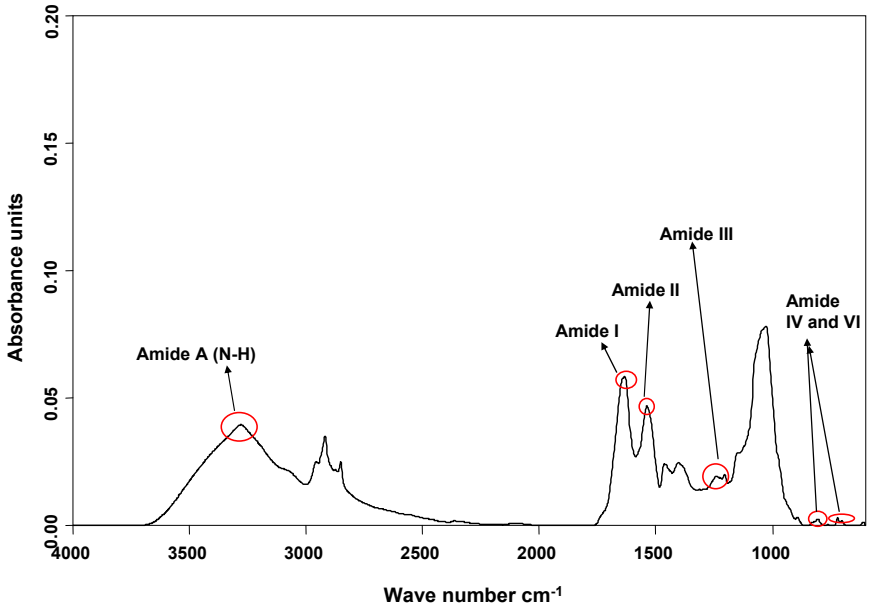

Fig. 4 ATR-FTIR spectra of purified *P. ostreatus* hydrophobins. The spectrum shown was obtained from vector-normalized, baseline-corrected and averaged 6 replicate spectra.

Table 2 Amide vibrations of the peptide group **(Fig. 4)** in the absorption bands in the FTIR spectra of *P. ostreatus* hydrophobin.

Peak position (cm^{-1})	Band	Assignment
3282	Amide A	NH stretch, in resonance with amide II overtone
2919 and 2854	unknown	
1636	Amide I, ß-sheet	Mainly CO stretching, slightly coupled with CN stretching, CCN deformation, NH bending
1541	Amide II	NH bending coupled with CN stretching
1460 and 1392	unknown	
1239	Amide III	NH bending and CN stretching
1033	unknown	
800, 720 and 705	Amide IV and VI	Out-of-plane NH bending, OCN bending, coupled with other modes

Peaks were assigned according to Jung 2000.

7.4.2 Hydrophobin activity on beech wood surface

Beech wood samples were treated with purified hydrophobin solutions of *C. cinerea* and *P. ostreatus* as described in the methods section. The surface activity of beech wood blocks changed upon treatment with *C. cinerea* and *P. ostreatus* hydrophobins (Fig. 5, 6, 7, 8 and Tables 3, 4), when compared with the control wood (wood treated only with water). The change in surface activity was dependent on hydrophobin concentration. Lower hydrophobin concentrations (below 100 µg total hydrophobin) resulted in lower hydrophilic behavior of the wood blocks and higher concentrations (100 to 400 µg total hydrophobin) resulted in higher hydrophilicity of the wood compared to the control wood (Fig. 9 and 10). Compared between the different sources of hydrophobins, the results with *P. ostreatus* were more variable, possibly again since the hydrophobins were not as pure (compare Fig. 10 and Fig. 15 in the

attachment). Because of this variability, no average values were calculated from the two experiments with the *P. ostreatus* hydrophobins. The average values of the two experiments performed with *C. cinerea* hydrophobins are found in the attachment. Also there was a relatively high general fine variation between the two experiments giving rise to larger standard deviation bars (Fig. 14 in the attachment), low hydrophobin concentrations of around 15 µg caused an immense increase in hydrophobicity, whereas higher concentrations (200 to 400) increased the wettability of the wood.

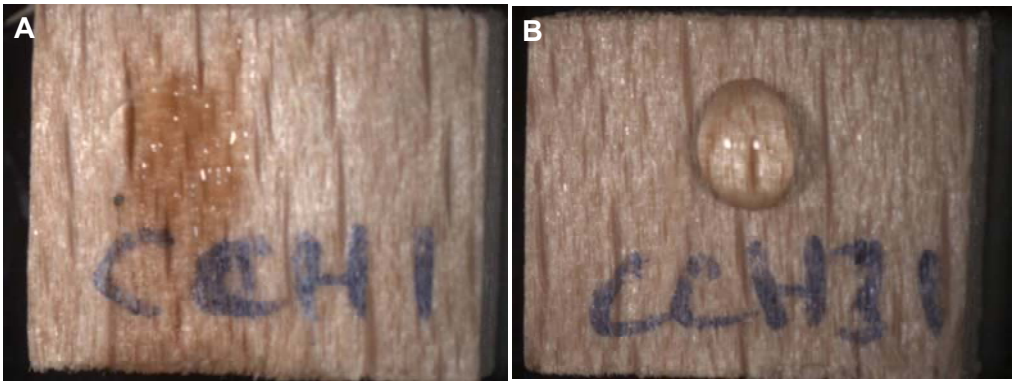

Fig. 5 Effect of treatment of beech wood with *C. cinerea* hydrophobin on water uptake of the wood. *C. cinerea* hydrophobin treated wood (A) and control wood (B). Photographs were taken 1 minute after placing 5 µl water on the wood blocks. 400 µg of *C. cinerea* hydrophobin in 1 ml water was used for the treatment.

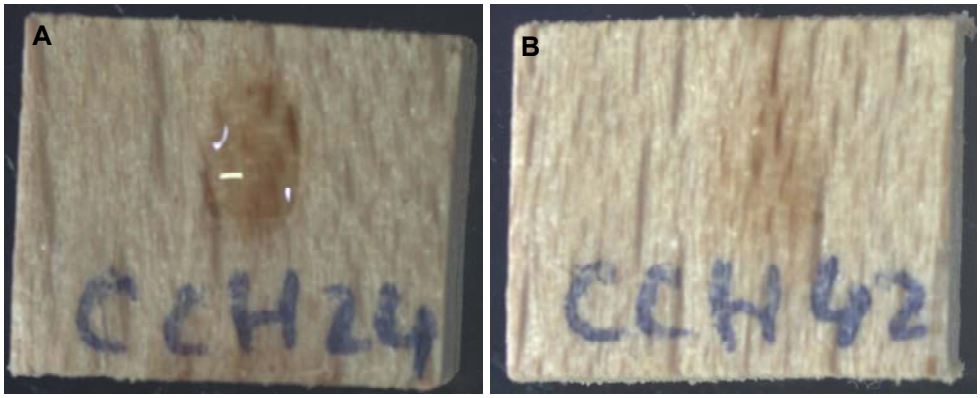

Fig. 6 Effect of treatment of beech wood with *C. cinerea* hydrophobin on water uptake of the wood. *C. cinerea* hydrophobin treated wood (A) and control wood (B).

Photographs were taken after 10 minutes after placing 5 µl water on the wood blocks. 15 µg of *C. cinerea* hydrophobin in 1 ml water was used for the treatment.

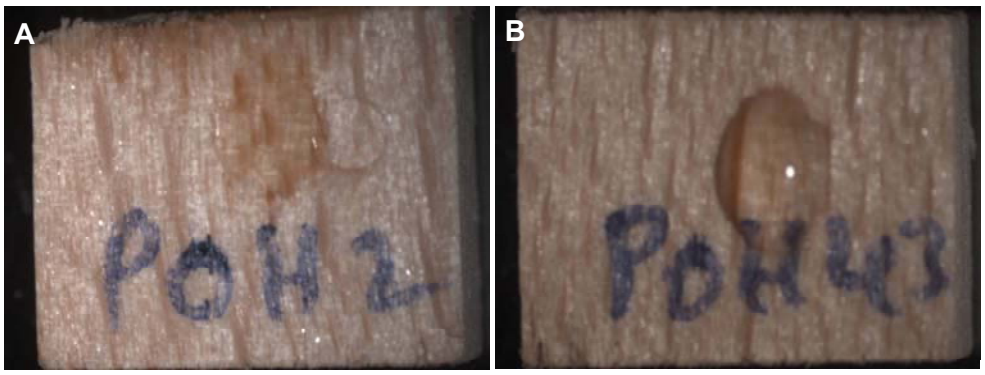

Fig. 7 Effect of treatment of beech wood with *P. ostreatus* hydrophobin on water uptake of the wood. *P. ostreatus* hydrophobin treated wood (A) and control wood (B). Photographs were taken 3 minutes after placing 5 µl water on the wood blocks. 400 µg of *P. ostreatus* hydrophobin in 1 ml water was used for the treatment.

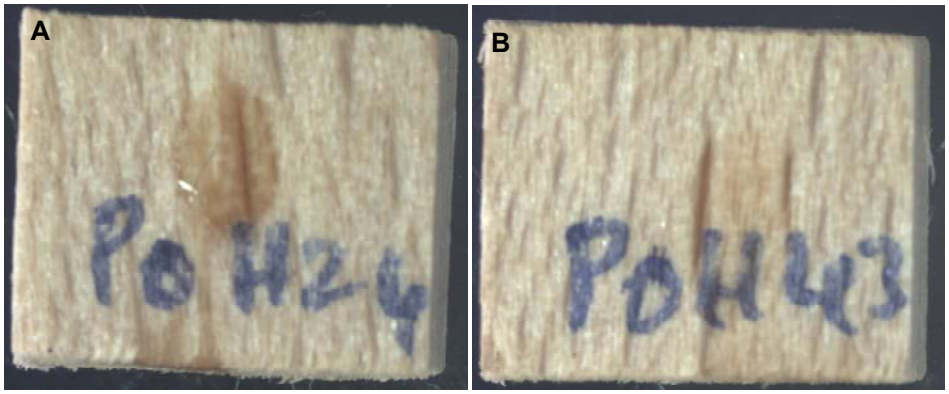

Fig. 8 Effect of treatment of beech wood with *P. ostreatus* hydrophobin on water uptake of the wood. *P. ostreatus* hydrophobin treated wood (A) and control wood (B). Photographs were taken 10 minutes after placing 5 µl water on the wood blocks.15 µg of *P. ostreatus* hydrophobin in 1 ml water was used for the treatment.

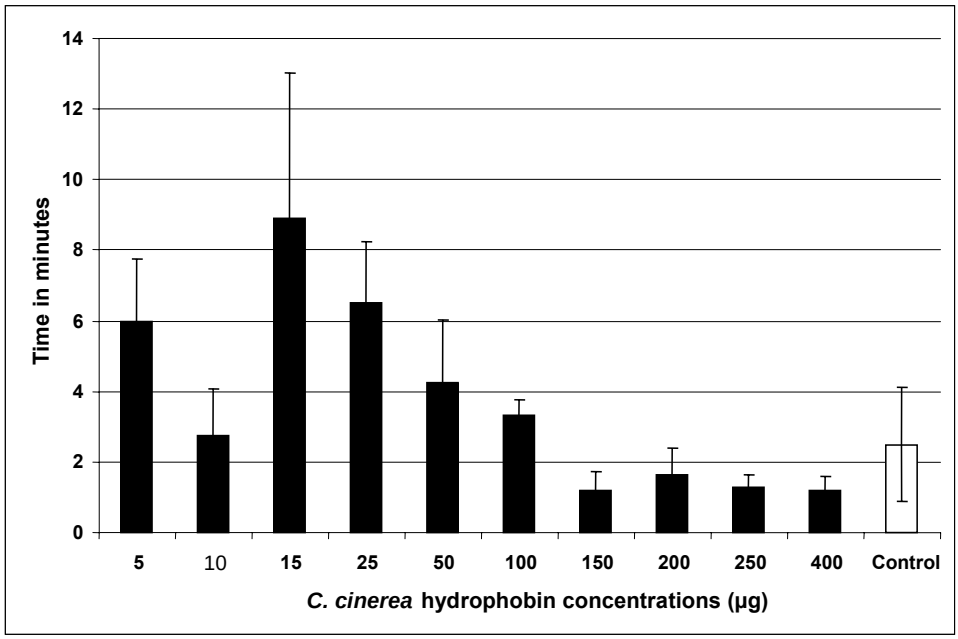

Fig. 9 Measurement of time required for the absorption of 5 µl water by the *C. cinerea* hydrophobin treated beech wood. Note, the jump in the results between the 10 µg and the 15 µg *C. cinerea* hydrophobin treatment might be due a fine error caused by a pipette change during the experiment (see material and methods). Values were averages of 3 values from experiment 1. The results of the experiment 2 and the combined experiments are presented in Fig. 13 and 14 in the appendix.

Table 3 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *C. cinerea* hydrophobin-treated beech wood in comparison with the untreated control beech wood (Experiment 1).

***C. cinerea* hydrophobin concentration (µg)**	**Significance with control wood**
5	S
10	NS
15	S
25	S
50	NS
100	NS
150	NS
200	NS
250	NS
400	NS

For each concentration total 3 values from experiment 1 are used for the calculation. S indicate significant and NS not significant. Statistical data of experiment 2 and the combined result of experiment 1 and 2 are provided in the appendix (see Tables 5 and 6).

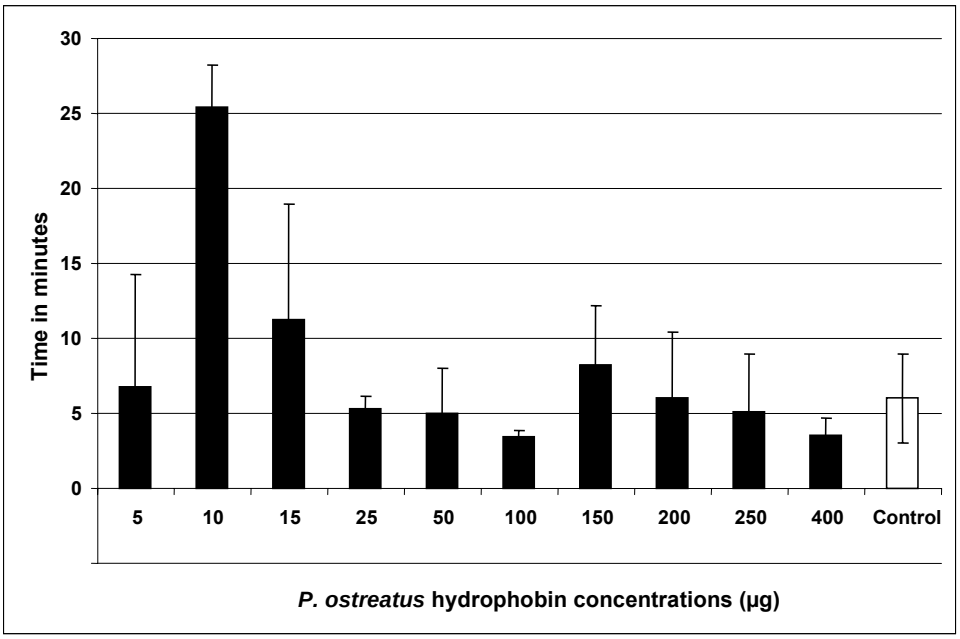

Fig. 10 Measurement of time required for the absorption of 5 µl water by the *P. ostreatus* hydrophobin treated beech wood. Note, the jump in the results between the 10 µg and the 5 µg *P. ostreatus* hydrophobin treatment might be due a fine error caused by a pipette change during the experiment (see material and methods). Values were averages of 3 values experiment 1. The results of the experiment 2 are presented in Fig. 15 in the appendix.

Table 4 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *P. ostreatus* hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 1).

***P. ostreatus* hydrophobin concentration (µg)**	**Significance with control wood**
5	NS
10	S
15	NS
25	NS
50	NS
100	NS
150	NS
200	NS
250	NS
400	NS

For each concentration total 3 values from experiment 1 are used for the calculation. S indicate significant and NS not significant. Statistical data of experiment 2 and the combined result of experiment 1 and 2 are provided in the appendix (see Tables 7 and 8).

7.4.3 FTIR-spectroscopy

ATR-FTIR spectra of the *C. cinerea* and *P. ostreatus* hydrophobin-treated beech wood showed an increase in the intensity of the peak associated with the amide I band (at 1640 cm^{-1} wave number region) of the spectra in a concentration dependent manner (Fig. 11 & 12). In case of the spectra from the *C. cinerea* hydrophobin-treated wood of concentrations 15 to 150 µg total hydrophobin, within one broad peak, in the region of 1615 to 1640 cm^{-1} there were small peaks observed which might be the α-helix and β-sheet structural forms of the hydrophobins on the beech wood surface. It could also be possible that in the lower concentrations the distribution of the hydrophobin is not uniform whereas in the higher concentrations the hydrophobin binding to the wood could be more uniform.

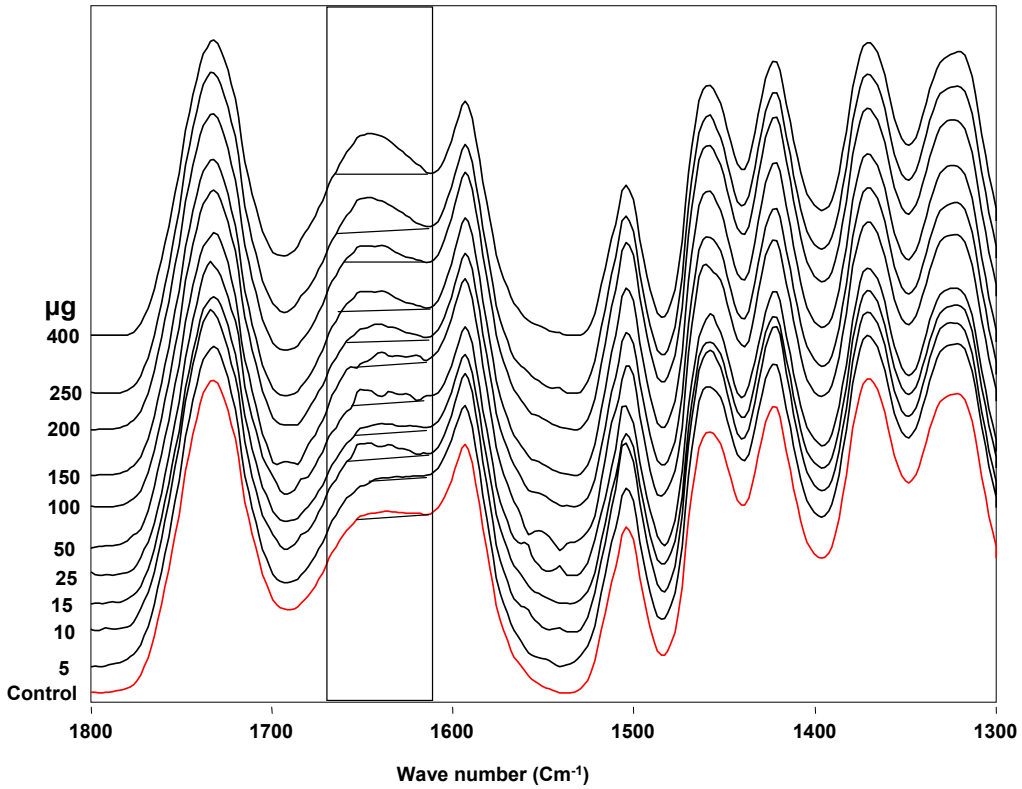

Fig. 11 ATR-FTIR spectra of *C. cinerea* hydrophobin treated beech wood. The intensity of the peak associated with amide I band (as indicated by the horizontal line inserted into the spectra to elucidate the increase in the respective peak) increased with increase in the concentration of the *C. cinerea* hydrophobin. Each spectrum represents the average of at least 9 vector-normalized, baseline-corrected.

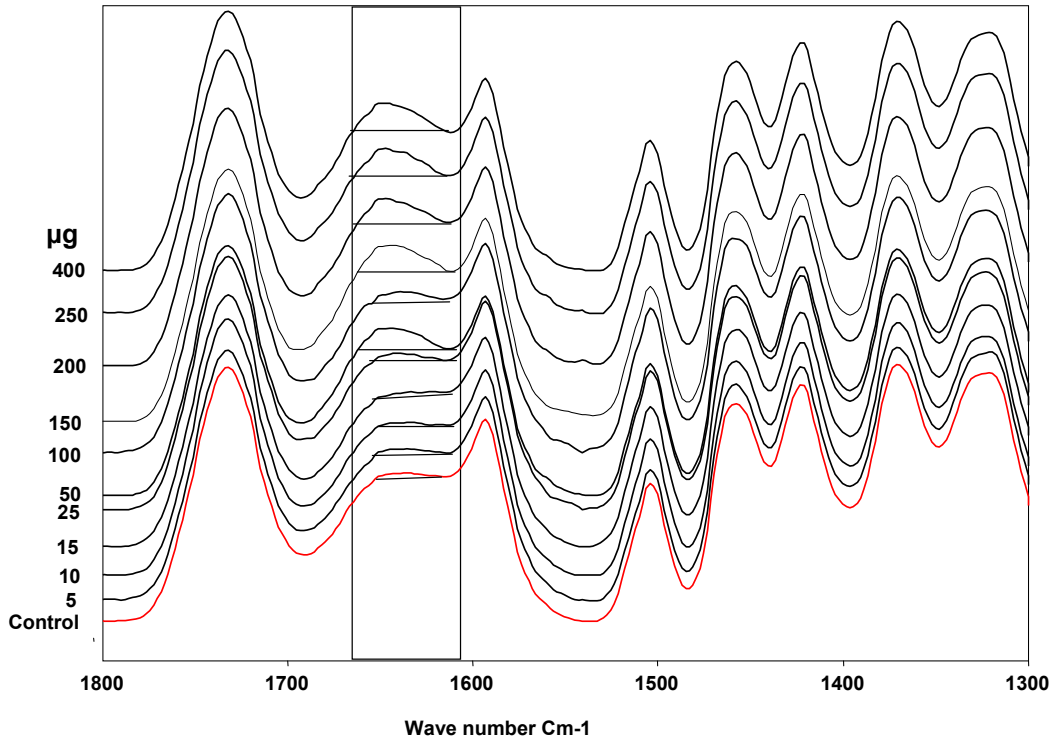

Fig. 12 ATR-FTIR spectra of *P. ostreatus* hydrophobin-treated beech wood. The intensity of the peak associated with amide I band (as indicated by the horizontal line inserted into the spectra to elucidate the increase in the respective peak) increased with increase in the concentration of the *P. ostreatus* hydrophobin. Each spectrum represents the average of atleast 9 vector-normalized, baseline-corrected.

7.5 Discussion

In this chapter, the surface activity of class I hydrophobins from *C. cinerea* and *P. ostreatus* were tested on their abilities of coating solid beech wood as previously done with the class I hydrophobin SC3 from *S. commune* (see chapter 6 of this thesis). Overall, the data suggest that the behavioral pattern of all class I hydrophobins were similar. Lower concentrations of hydrophobins (~15 µg/ml) resulted in an increase of hydrophobicity and higher concentrations (~400 µg/ml) in an increase of wettability. However, it should thereby also be noted that in case of *P. ostreatus* hydrophobin-treated wood samples the effect was not very demarking from the control wood samples (see Fig. 10 and Fig. 15 in the appendix to this chapter) as was observed in cases of SC3 of *S. commune* and of *C. cinerea* hydrophobin-treated wood. This effect might be due to not further defined impurities present in the *P. ostreatus* sample.

Previously, hydrophobins were tested to coat surfaces such as glass and Teflon in experiments in which the hydrophobins were able to change the surfaces hydrophilic to hydrophobic and vice versa (Wösten et al. 1994, present study). Since wood is a non-homogeneous material (Gardner et al. 1991) and the cell walls consist of both hydrophobic and hydrophilic components (Dean 1978, Uraki et al. 2006), the surface activity the hydrophobins conferred to the wood (hydrophobicity or hydrophilicity) seemed to be very much influenced by the actual hydrophobin concentration used (Fig. 9 and 10). The data from FTIR spectroscopy in which there was increase in the intensity of the peak associated with the amide I band supports that the hydrophobin activity on wood was concentration dependent (Fig. 11 and 12). The results indicate that the hydrophobins could bind to the wood as they do with other materials (de Vocht et al. 1998, Janssen et al. 2004, Linder et al. 2005, see chapter 6 of this thesis). However, the splitting of the amide I band in wood samples treated with lower concentrations of *C. cinerea* (Fig. 11) proteins suggest that different protein configurations might be present in these samples. Different protein conformations as folding intermediates have been described in the literature (Wösten and de Vocht 2000) that could explain the FTIR results.

Since there are both hydrophobic (e.g. lignin) and hydrophilic (e.g. cellulose and hemicellulose) components in wood (Dean 1978, Uraki et al. 2006), it is currently not known to which components hydrophobins are binding, possibly to both. It is also not known whether the hydrophobins can enter the cell walls. It would therefore be an interesting task to study where the hydrophobins are specifically located within the wood – just on the inner surface of the vessels or also within the cell-walls and to which wood polymeric component the hydrophobins are binding. Binding to the hygroscopic cellulose and hemicelluloses should render these hydrophobic which would explain the results of the treatments with the low hydrophobin concentrations. Binding to the hydrophobic lignin should render them hydrophilic which would explain the results with the higher hydrophobin concentrations. In chapter 6 of this thesis it was reported that, in order to obtain a high hydrophobic reaction on the hydrophilic vessel surface, about twice as much hydrophobin was required to coat the total inner vessel surface than that what is reported to be needed to cover a comparable surface area of Teflon (Wessels 1997). Since the estimation on the vessels

surfaces can by nature of the three-dimensional structure of the wood and the irregular overall vessel lumen structure be only very rough, the required amount determined for wood and Teflon coating is probably indeed very similar. Taking this for granted, higher concentrations might either form a secondary hydrophobin layer on the first layer but with opposing surfaces resulting in higher wettability of the wood. Alternatively, higher concentrations of hydrophobins might penetrate the wood cell walls making these more hygroscopic by making the hydrophilic character of the lignin. For both possibilities, experimental support is currently lacking. However, a simple mathematical addition of hydrophobin concentrations to two-fold, three-fold, four-fold, etc. does not go along with repetitive changing between hydrophobic and hydrophilic situations in the results of the laboratory experiments. Instead, the hydrophobicity decreased constantly with increasing hydrophobin concentrations (Fig. 9 and Fig. 10), arguing rather for hydropobins migrating at higher concentrations more into the cell walls, thereby interacting with the lignin and reducing the hydrophobicity of the cell walls.

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Appendix to chapter 7

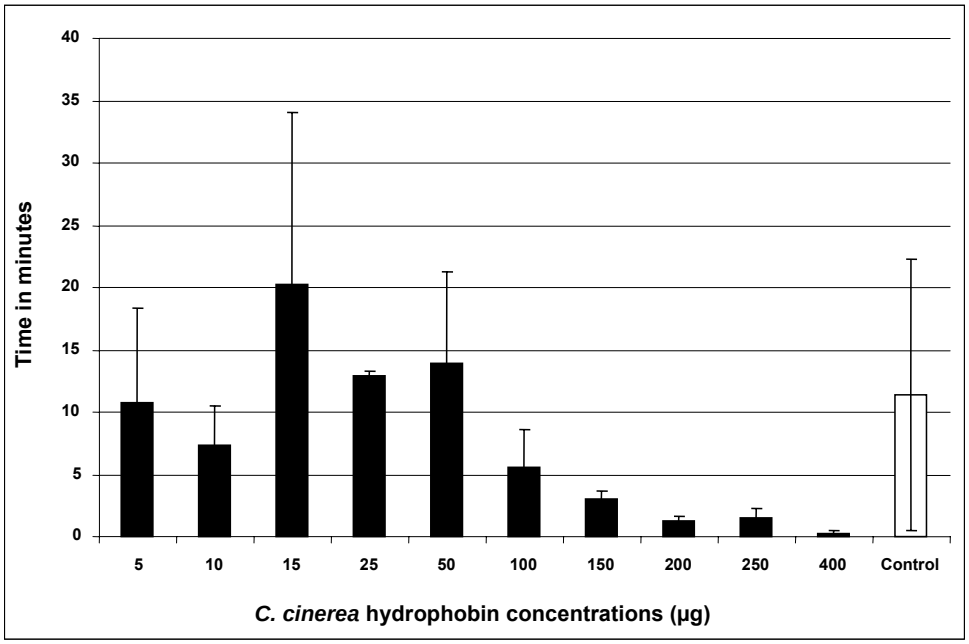

Fig. 13 Measurement of time required for the absorption of 5 µl water by the *C. cinerea* hydrophobin treated beech wood (Experiment 2).

Table 5 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *C. cinerea* hydrophobin-treated beech wood in comparison with the untreated control beech wood (Experiment 2).

***C. cinerea* hydrophobin concentration (µg)**	**Significance with control wood**
5	NS
10	NS
15	NS
25	NS
50	NS
100	NS
150	NS
200	NS
250	NS
400	S

For each concentration total 3 values from experiment 2 are used for the calculation. S indicate significant and NS not significant.

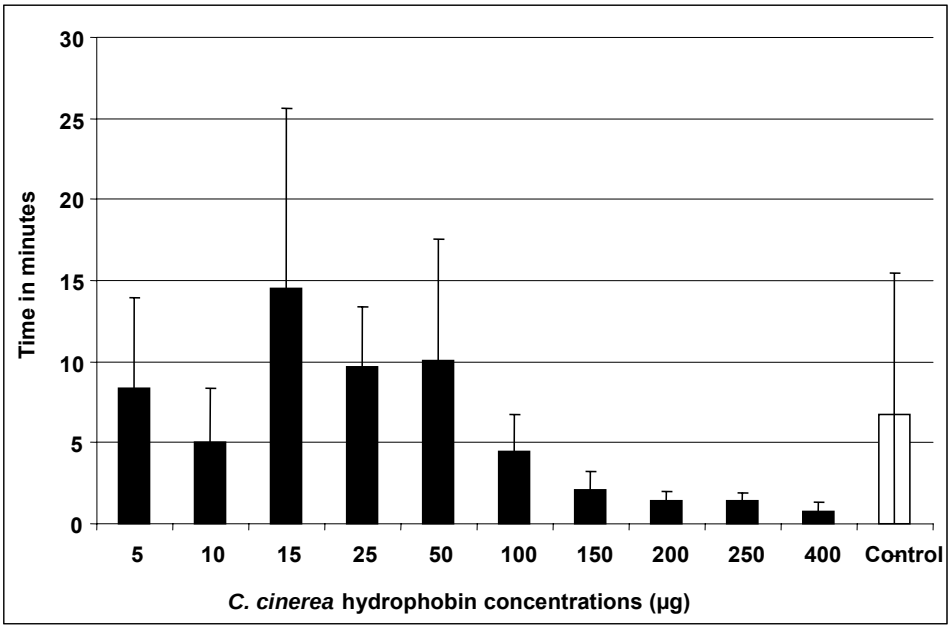

Fig. 14 Measurement of time required for the absorption of 5 µl water by the *C. cinerea* hydrophobin treated beech wood (Experiment 1 and 2).

Table 6 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *C. cinerea* hydrophobin-treated beech wood in comparison with the untreated control beech wood (Experiment 1 and 2).

***C. cinerea* hydrophobin concentration (µg)**	**Significance with control wood**
5	NS
10	NS
15	S
25	NS
50	NS
100	NS
150	NS
200	NS
250	NS
400	S

For each concentration total 6 values obtained from two independent experiments are used for the calculation. S indicate significant and NS not significant.

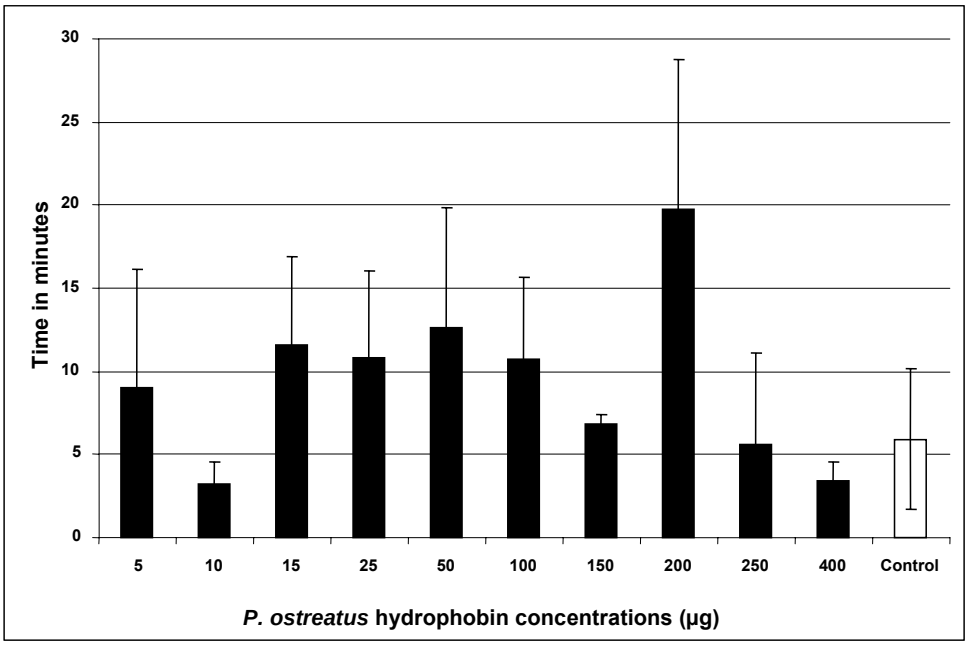

Fig. 15 Measurement of time required for the absorption of 5 µl water by the *P. ostreatus* hydrophobin treated beech wood (Experiment 2).

Table 7 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *P. ostreatus* hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 2).

***P. ostreatus* hydrophobin concentration (µg)**	**Significance with control wood**
5	NS
10	NS
15	NS
25	NS
50	S
100	NS
150	NS
200	S
250	NS
400	NS

For each concentration total 3 values from experiment 2 are used for the calculation. S indicate significant and NS not significant.

Table 8 Statistical data (calculated by LSD comparison of SPSS) indicating significant differences ($p \leq 0.05$) in the time of water absorption by different concentration of *P. ostreatus* hydrophobin treated beech wood in comparison with the untreated control beech wood (Experiment 1 and 2).

***P. ostreatus* hydrophobin concentration (µg)**	**Significance with control wood**
5	NS
10	S
15	S
25	NS
50	NS
100	NS
150	NS
200	S
250	NS
400	NS

For each concentration total 3 values from experiment 2 are used for the calculation. S indicate significant and NS not significant.

Chapter 8

General discussion:

Hydrophobins in wood biology and biotechnology

8. General discussion: Hydrophobins in wood biology and biotechnology

8.1 Introduction

Bioactive compounds such as sterols, phenols, glycoproteins and polysaccharides produced by a fungus during various stages of growth and development play an important role in the fungal life cycle (Shimizu et al 2003). The basidiomycete fungus *Schizophyllum commune*, the split-gill mushroom, is a species shown to produce various active substances/metabolites during its growth (Wessels 1965, 1979). This fungus is well known by the discovery of small secreted proteins called hydrophobins having an amphiphatic character. These small proteins are reported to perform various biological functions in the fungal life cycle (Wessels 1997). For example, the SC3 hydrophobin of *S. commune* is known to be involved in the formation of aerial hyphae by coating the hyphae with self-assembled layers of hydrophobin monomers and thereby making their surfaces hydrophobic (Wösten 2001). The SC4 hydrophobin in contrast coats the air channels within fruiting bodies thereby avoiding water filling in the fruiting bodies and preventing them from collapse (van Wetter et al. 2000). There can be different numbers of hydrophobins in a single fungus performing divergent functions (Wösten 2001, Walser et al. 2003, Velagapudi 2006). Other examples of fungal hydrophobins from our laboratory involves the basidiomycete fungus *Coprinopsis cinerea* which has 34 different hydrophobin genes whose functional analysis is in progress (Peddireddi et al. 2005, Velagapudi 2006). In this thesis work, the primary focus was to characterize the possible roles of hydrophobins in wood colonization and decay processes and to check the biotechnological applications of these surface active-proteins in wood technology.

8.2 Why to use *S. commune* for this project

S. commune as a widely distributed cosmopolitan basidiomycete fungus is used as a model fungus for studying mating types and fungal development in the basidiomycetes (Raper et al. 1958, Raper and Fowler 2004). *S. commune* was selected as a model organism for this study due to the existence of various hydrophobin mutant strains (van Wetter et al. 1996, Lugones et al. 2004, de Jong 2006). Several

hydrophobins have been described in *S. commune*, of which SC3 from the vegetative mycelium of the fungus is the best characterized until now. Deletion of the *Sc3* gene from the fungus results in lack of formation of a hydrophobin coat on aerial hyphae which, in consequence, remain hydrophilic. Another secreted protein called SC15 (not a hydrophobin since they do not contain the conserved 8 cysteines that are typical for the hydrophobins) has been described which helps the fungus in attachment to surfaces in the absence of SC3 hydrophobin. Deletion of the gene for the SC15 protein in a strain with a functional *Sc3* gene has however no obvious phenotype. In this study, deletion mutants of the genes for the SC3 hydrophobin and for the SC15 protein were both used to study their potential influence in wood colonization and wood decay processes.

8.3 *S. commune* in natural environments, interaction with other fungi and possible role of hydrophobins in combat interactions

In nature, the widely distributed fungus *S. commune* is usually found growing on dead branches mostly of deciduous trees and less often also on branches of coniferous tress. In some instances, it is also acting as pathogen on weakened living trees (Latham 1970, present study). In this study, we reported *S. commune* growing on a living *Juglans ailantifolia* tree (Peddireddi et al. 2005). On the same tree, fruiting bodies of another basidiomycete fungus *Trametes hirsuta*, were observed on a branch infected also by *S. commune*. To check the interaction of *S. commune* with other fungal species, we have isolated both these fungi and used them in laboratory combat tests. Dual inoculations of the isolated strains of *S. commune* ScJa 1 and *T. hirsuta* ThJa 1 on agar medium resulted in a deadlock situation as a mutual exclusion reaction in which neither species is capable of taking dominance over the other. The deadlock reaction eventually was followed by the replacement of *S. commune* by *T. hirsuta* (see chapter 2 of this thesis).

Further studies in this direction were performed in order to test the involvement of hydrophobins in the fungal interactions. Thereby, we used the co-isogenic, well characterized monokaryotic and dikaryotic laboratory *S. commune* strains 4-39, 4-40 and 4-39 x 4-40 and their corresponding *Sc3* hydrophobin mutants and paired them against the isolated strain *T. hirsuta* ThJa 1 upon which their antagonistic behaviour

was studied. All *S. commune* wild-type strains showed first a barrier formation and a blue pigmentation at the interaction zone against *T. hirsuta* ThJa 1 and they were subsequently replaced by *T. hirsuta* ThJa 1. The monokaryotic hydrophobin mutant strains also showed a similar pattern. However, there was no pigmentation and only insignificant barrier formation by the dikaryotic *Sc3* hydrophobin mutant. Since pigmentation and barrier formation by the hydrophobin mutant monokaryons occurred, this suggest that, in principle, the SC3 hydrophobin is not necessary for defense reactions against other fungi (see chapter 2 of this thesis).

8.4 Wood colonization and decay ability of *S. commune* and effect of hydrophobins in protein secretion

Schizophyllum commune found growing in nature on fallen trunks and branches is regarded as a white rot fungus but strains of the fungus showed in most of the instances in the laboratory a low ability to degrade wood (Humar et al 2001, 2002, Nilsson & Daniel 1983, this study). In nature, it can also act as a pathogen on living tress (present study). In our study we have used wood (un-decayed) from the same tree (*Juglans ailantifolia*) where it was found acting as an opportunistic pathogen and also wood from other natural substrates of this fungus such as beech (*Fagus sylvatica*) and pine (*Pinus sylvestris*) to test the decay ability of this fungus (see chapter 3 and 5 of this thesis). Further to this, several hydrophobin mutant strains and SC15 mutant strains were also studied to define a possible involvement of the hydrophobin proteins in the wood colonization and decay processes (see chapter 3 and 5 of this thesis). The mutants were also able to enter the wood as the wild type strains and there was no significant difference in the decay caused by the wild type and mutant strains (Chapter 3 of this thesis). Interestingly as determined by FTIR analysis, there were some differences in the mycelial protein and polysaccharide contents of SC3 hydrophobin mutants compared with other strains when growing in and on wood (see chapter 5 of this thesis). A more obvious pattern of differences in the mycelial protein and polysaccharide contents was observed when the fungi were grown in the liquid cultures (see chapter 4 of this thesis). The qualitative differences in the FTIR spectra amongst the wild type and mutant strains harvested from liquid cultures and also 2D electrophoresis data on proteins produced by a *S. commune* wild type strain and a SC3 hydrophobin mutant (Fig. 1) support the hypothesis that there are differences in the

type and quantity of the protein/metabolites secreted by the wild type and hydrophobin mutant strains (see chapter 4 and appendix to this chapter). Generally, when using solid wood blocks, wood decay by *S. commune* strains was low in wood from deciduous trees (3-9% in *J. ailantifolia* wood, 1-3% in beech, 1-5% in birch) or there was no wood decay (0-0.5%) in pine wood (see chapter 3 of this thesis). The limited wood decay ability of *S. commune* found on *J. ailantifolia*, beech and birch wood confirms a preference of the fungus for wood of deciduous trees. The results also confirm earlier reports by other researchers (Schmidt and Liese 1980, Schirp et al. 2003) that usually on the decay ability of *S. commune* strains is low. Further results from this thesis indicated however that *S. commune* can decay wood to a greater extent when provided in saw dust form (see chapter 3 of this thesis). Since nutrients were added to the sawdust, it is currently not clear, whether a better aeration due to a larger surface for fungal colonization helped the fungus to attack the wood or whether supply of nutrients (such as nitrogen source) in higher concentration induced production of enzymes required for wood decay or whether both together were the reason for the increase in decay values. In any case, no obvious differences in wood decay were encountered in the behaviour of *S. commune* wild type strains, hydrophobin mutants and SC15 mutants. Accordingly, the SC3 hydrophobin and the SC15 protein are not necessary for *S. commune* to enter and degrade the wood although deletion of the *Sc3* hydrophobin gene obviously affects the secretome of *S. commune* (Fig. 1 and see previous report on effects on protein secretion by deletion of the *Sc3* gene from the fungal genome as reported by van Wetter et al. 2000 and Talbot et al. 1996). There are obvious differences in visual proteins secreted by the wild type strain 4-39 and the SC3 hydrophobin mutant strain ΔSC3 4-39 as indicated by presence or absence of individual spots in 2D-gels or by strong differences in intensities of protein spots (Fig. 1). Currently it is however not known whether secreted enzymes acting in wood decay are affected. Since the sequence of the genome of *S. commune* will soon be released by the JGI (Joint Genome Institute, http://www.jgi.doe.gov/index.html), identification of secreted proteins by the wild type and the mutant strain can soon be addressed.

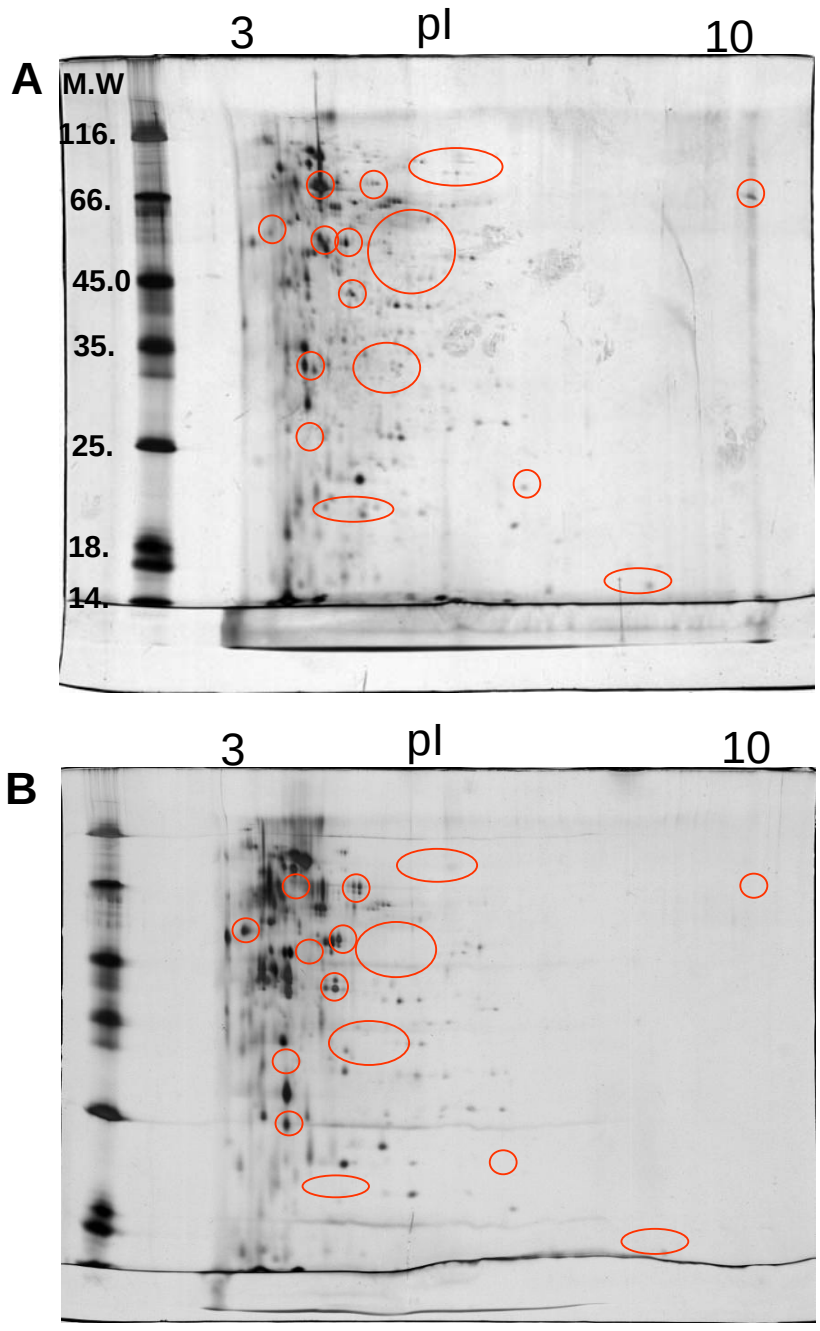

Fig. 1 2D-gel electrophoresis comparison of the protein contents in culture supernatants of the *S. commune* wild type 4-39 (A) and the *Sc3* hydrophobin knock out mutant *ΔSc3* 4-39 (B) grown in standing culture in liquid *S. commune* minimal medium (Dons et al. 1979) at 25 °C in continuous light. The amount of protein loaded per lane was about 100 µg (further experimental details are given in the attachment). Encircled in the photos of the 2D gels are areas where obvious differences between the two strains are seen in the presence or absence or in the intensities of protein spots. For the correct interpretation of the results it is important to know that in the experiment the wild-type monokaryon 4-39 showed an unusual slow growth

phenotype compared to other strains grown in parallel (see details in the attachment to the discussion). It is possible that the frequent *thn* mutation arised in the strain that leads to a change in growth pattern (thin hyphal mycelium, Fowler and Mitton 2000). Since this was not further enquired at the end of the experiment, it has to be confirmed in the future, whether the differences in protein spots seen in the two 2D gels are indeed differences between a healthy wild type strain and the co-isogenic ΔSC3 hydrophobin mutant strain.

8.5 Application of hydrophobins in wood technology

Hyrophobins are the most surface active proteins known and can be used for changing the nature of surfaces (Wösten and de Vocht 2000). Hydrophobic surfaces can be changed to hydrophilic and hydrophilic surfaces can be changed to hydrophobic (Wessels 1997). In this study, the surface activity of the SC3 hydrophobin from *S. commune* has been tested on hydrophobic and hydrophilic materials. Hydrophobic Teflon and form-war film turned hydrophilic and hydrophilic glass turned hydrophobic on coating with SC3 hydrophobin (see chapter 6 of this thesis). The SC3 protein was also used as a coating on solid beech wood. Beech wood turned both hydrophilic and hydrophobic, depending on the concentration of SC3 hydrophobin used. Lower concentrations (50, 25, 15 μg/ml) of SC3 resulted in hydrophobic behavior and higher concentrations (100 to 400 μg/ml) resulted in hydrophilic behavior of beech wood. To examine whether other hydrophobins show a similar behavior on the wood surface, hydrophobins from *Coprinopsis cinerea* and *Pleurotus ostreatus* were isolated and used to coat on beech wood (see chapter 7 of this thesis). In principle, hydrophobins from *S. commune*, *C. cinerea* and *P. ostreatus* behaved in a similar manner. The binding of the different hydrophobins to the solid wood surfaces was analysed with FTIR spectroscopy. Compared to the control wood ATR-FTIR spectra of the hydrophobin-treated beech wood showed an increase in the intensity of the peak associated with the amide I band of the protein part of the spectra. This increase occurred in a concentration dependent manner of the hydrophobins indicating the binding of the hydrophobins to the wood. An interesting question to solve is to what the hydrophobins will bind. The cell wall of wood vessels consists of cellulose, hemicellulose and lignin (Zwieniecki and Holbrook 2000). Attaching the hydrophobin films to the hydrophilic cellulose microfibrils of the inner S_3 layer of the cell walls (Fahlén 2005) should give the vessels inner surface then a hydrophobic nature, an assumption that is in agreement with the results obtained when using hydrophobin

solutions of low concentration for coating the wood (chapter 6 and see also Fig. 2). Why is it than that the effect of making the wood hydrophobic is reversed when applying higher concentrations of hydrophobins to the wood? As discussed in detail in chapter 6 and 7 of this thesis, by the small size of the hydrophobins, which are about 110 amino acids in length (Wösten 2001, Walser et al. 2003, Velagapudi 2006) and which, when assembled, adopt a rodlet structure of 10 nm in size (Wösten et al. 1994, Wessels 1997), there is the possibility that the proteins enter through small pores into the cell walls of the wood vessels. Upon entering, they will come in contact with hydrophobic lignin. When interacting with this, the hydrophobic character of the lignin should be turned into hydrophilic – which is the effect we see when we use high concentrations of hydrophobins (see chapter 6 and 7 of this thesis).

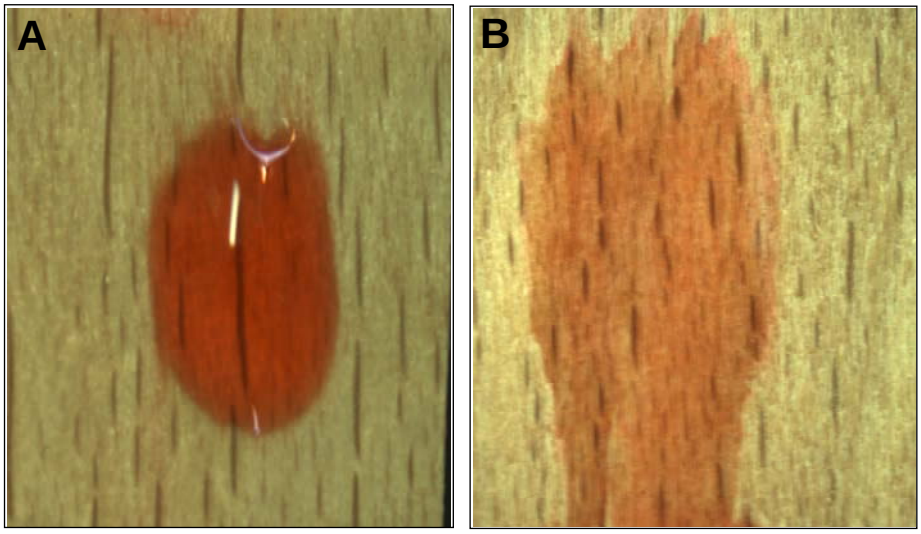

Fig. 2 Beech wood blocks were treated with water (A) or a solution of SC3 hydrophobin (3 ml solution 1mg/ml in concentration) (B) as described in chapter 6 of this thesis. Subsequently, 10 µl of a solution of red food colouring was pipetted onto the wood and the wood blocks were photographed after 3 minutes.

8.6 Some general conclusions from this thesis work regarding the function of hydrophobins

1. The SC3 hydrophobin and the interacting SC15 protein are not necessary for *S. commune* to enter into wood and *S. commune* strains are able to decay the wood

to a low level irrespectively of whether being a wild type strain or a hydrophobin mutant strain.

2. There were significant differences in the secretome of a *S. commune* wild type and a hydrophobin mutant strain in standing liquid cultures, i.e. deletion of the Sc3 hydrophobin gene affects the secretome.
3. Accordingly, *S. commune* wild type strains and hydrophobin mutants could be discriminated while growing on the surface of beech wood.
4. When growing within wood, differences between the strains were not as obvious as when growing on the wood surface.
5. *S. commune* was able to interact in competitive manner with another, fungal white rot species, *T. hirsuta*, in similar pattern irrespectively whether being a monokaryotic wild type strain or a monokaryotic hydrophobin mutant strain, whereas a dikaryotic hydrophobin mutant strain showed a less strong defense reaction compared to a wild type dikaryotic strain.
6. Class I hydrophobins from *S. commune*, *C. cinerea* and *P. ostreatus* were successfully applied in coating of wood.
7. Effects of hydrophobin application to wood depended on protein concentration – low hydrophobin concentrations (15 µg) resulted in making the wood hydrophobic, high hydrophobin concentrations (400 µg) in making the wood more hydrophilic.

8.7 Future outlook

1. In this PhD work, the general ability of hydrophobins in application in wood coatings was tested. From this work, it became clear that the hydrophobins are able to bind to the wood components. Currently it is not known to which component of wood the hydrophobins are binding. An interesting task would therefore be to elucidate to which component exactly the hydrophobins are binding. Once knowing this, one can further try to optimize the long term stability of the protein on the wood which would open up interesting possibilities of the usage of hydrophobins for products of the wood market. A special interest for applications for example could be in the production of wood windows that need to resist high humidity why appropriate coatings of best protecting properties

particularly also for joineries are searched for (Amburgey and Johnson 1979, Ahola et al. 1999, Alblas and Kattenis 2002, de Meijer 2002, Grull et al. 2004).

2. For economical applications, large amounts of cheaply produced hydrophobins are required. Hydrophobins are naturally the most produced proteins by the fungi with yields up to 60 mg/l of culture medium (Scholtmeijer et al. 2001). However, the handling of these proteins-isolation from culture medium and/or mycelium, purification into powdery form and subsequent solubilization-is quite difficult, and requires a lot of expertise in order to regularly obtain high amounts of proteins (own experience, R. Velagapudi and U. Kües, personal communication, Linder et al. 2005, Walser et al. 2003). Often, after harvesting and purification, the proteins do not solve well in water or they quickly aggregate again, possibly by impurities that still will be present in the samples. If the latter happens, the proteins will not anymore be suitable for coating experiments. Thus, better methods for protein purification, solubilization and storage promoting better stability in the monomeric form need to be established.
3. With better purification, solubilization and storage techniques, there is the possibility to overexpress hydrophobins e.g. in suitable fungal hosts to obtain even higher protein yields. Previous studies in this direction using the bacterium *Escherichia coli* and the yeast *Hansenula polymorpha* were not successful in obtaining higher yields of the proteins (Scholtmeijer et al. 2001). However, if overproduction finally works, this can bring a drastic change in the application of hydrophobins not only in the wood technology as demonstrated in this study, but also in several other fields of economical importance (e.g. in food technology, medicine, pharmacy, nanotechnology, etc).
4. In experiments of basic research in this and other studies, *S. commune* was shown to be a mild wood rot fungus in the temperate regions but it is regarded as a severe wood destroyer in the tropical regions (Schmidt and Liese 1980). It would be interesting to study the different enzymes for wood decay that are produced by strains of this fungus from different parts of the world. One should test the ability of the individual enzymes as well as combinations of different enzymes for their ability to degrade different wood polymers in order to understand why *S. commune* behaves differently in the different parts of the world.

5. In this study, in case of *C. cinerea* and *P. ostreatus*, there were more than one hydrophobin used for the coating of the wood. Neglecting the negative effects caused likely by the impurities present in the *P. ostreatus* hydrophobin sample used the combination of different hydrophobins of similar or different biological functions in this study did not reveal principal differences in the coating of wood. The effects observed on wood could however be different with application of other hydrophobins and other combinations. To understand this aspect better, different hydrophobins coming from the same species should be separated and their function individually and in combination tested [for example by production in an ascomycetous yeast that do not possess hydrophobin such as *Saccharomyces cerevisiae* (Dujon et al. 1994, Goffeau et al. 1997, Mewes et al. 1997, Winzeler and Davis 1997)].

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8.9 Appendix to the General discussion:

8.9.1 Effects of deleting the SC3 hydrophobin gene and/or the protein SC15 gene from the genome of *S. commune* on growth of the fungus

8.9.2 Introduction

SC3 is the only hydrophobin expressed and secreted in monokaryons of *S. commune* and it is known to influence protein secretion by the fungus (van Wetter et al. 2000, Talbot et al. 1996, present study). It also influences the surface hydrophobicity of fungal hyphae by forming an amphiphatic film over the fungal cell walls with the hydrophobic surface targeted to the outside and the hydrophilic surface targeted to the cell walls (Wösten 2001). In its function to supply a hydrophobic surface to fungal cells, hydrophobin SC3 is supported in an unknown manner by another small secreted protein, SC15. It is so far not known, whether SC15 also helps in protein secretion. A *ΔSc15* mutant and a double mutant lacking both a functional *Sc3* gene and a functional *Sc15* gene were therefore also included in this study.

Ergosterol is the principal sterol of the fungal cell membranes (Beauvais & Latge 2001, Piovano et al 2005). The ergosterol ($C_{28}H_{44}O$; Mol wt: 396.66 g) content in the fungal mycelium is usually considered to be the measure of biomass as it is present in all the cells as a component of cell membranes (in equal amounts) (Charcosset and Chauvet 2001, Dawson-Andoh 2002, Pasanen et al 1998, Nakayama et al 2001). Free sterols in the plasma membrane play an important role in fluidity and permeability of the membranes thereby affecting the activities of membrane-bound proteins (Parks 1995, Shobayashi et al 2005). This study was performed to check further whether the SC3 hydrophobin mutation causes differences in the quality and quantity of protein secreted by *S. commune* by analyzing the SC3 hydrophobin mutant *ΔSc3* 4-39 and its co-isogenic wild type strain 4-39. The ergosterol content of the *S. commune* wild type strain and the co-isogenic mutant strain have been measured to whether lack of hydrophobins affects the permeability regulation of the membranes.

8.10.1 Methods and materials

8.10.1.1 Fungal strains

S. commune wild type strain 4-39 (*MAT*A41 *MAT*B41, CBS 341.81), the co-isogenic *ΔSc3* hydrophobin mutant Δ*Sc3 4-39*, the co-isogenic *ΔSc15* mutant Δ*Sc15 4-39* and the *Sc3Sc15* double mutant Δ*Sc3*Δ*Sc15 MATA41 MATB43* were kindly provided by Prof. Wösten, The Netherlands.

8.10.1.2 Growth conditions

All strains were grown in liquid standing cultures at 25 ^{o}C in continuous light. Strains were cultivated on *S. commune* minimal medium (20 g glucose, 1.5 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$ x $7H_2O$, 1 g yeast extract, 0.12 mg thiamine-HCl, 0.1 mg pyridoxine HCl, 0.005 mg biotin, 0.2 mg $CuSO_4$ x $5H_2O$, 0.08 mg $MnCl_2$ x $4H_2O$, 0.4 mg cobaltous chloride hexahydrate, 1.2 mg calcium nitrate tetrahydrate per 1 liter H_2O; Dons et al. 1979). Pre-cultures were prepared on 1% agar medium. Petri dish cultures were incubated for 7 days. Blocks of agar (1 cm^3) were cut from the edge of the fungal colonies and used as inoculums for the pre-cultures in 500 ml Erlenmeyer flasks containing 100 ml liquid medium and incubated for 10 days. Main cultures were inoculated with 10 ml homogenized mycelial macerate from the pre-cultures in 100 ml liquid *S. commune* minimal medium. Three replicates were used for each day of harvest from each strain.

8.10.1.3 Culture harvest

Cultures were harvested on day 3, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, 19, 21, 23 and 25 of cultivation. Mycelium was separated from the supernatant with a nylon mesh (0.3mm pore size) through a Büchner funnel connected to a vacuum pump. The scrapped mycelia were collected into 50 ml Falcon tubes (SARSTED AG & Co, Nümbrecht, Germany) and freeze-dried in a lyophilisator and the dry weight of the biomass determined. Culture supernatants were collected separately into other 50 ml Falcon tubes and used for protein measurements. Protein was quantified by using Bradford reagent according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

8.10.1.4 Ergosterol measurements

8.10.1.4.1 Sample preparation for ergosterol

The mycelium from each replicate was taken into a 100 ml pyrex bottle (PYREX, Paris, France). About 50 ml of 10% KOH in methanol and 200 ppm or 0.001g of 2, 6-di-tetra-butyl 4-methyl phenol were added. 250 µg of cholesterol was then added and the samples were incubated (hydrolyzed) in a water bath for 8 hours at 50 ^{o}C. Subsequently, samples were cooled down to room temperature. An aliquot of 6 or 7 ml per sample was taken into a fresh 10 ml glass reagent tube (PYREX) and centrifuged at 2000 rpm, 4 ^{o}C for 15 min. 4 ml supernatant was taken into a fresh 10 ml glass tube (PYREX) and 2 ml n-hexane was added and mixed well with the samples. 4 ml of distilled water was added and samples were shaken vigorously for 30 min after which they were centrifuged at 2000 rpm at 4 ^{o}C for 15 min. 1 ml n-hexane phase (upper phase) was taken into an Eppendorf tube (SARSTED AG & Co, Nümbrecht, Germany), dried in a speed-vac (Eppendorf, Concentrator 5301, Hamburg, Germany) and stored at -20 ^{o}C until further use for GC-MS analysis.

8.10.1.4.2 Quantitative analysis of ergosterol by GC-MS

Ergosterol has been determined according to Nielsen & Madsen (2000) with slight modifications. Samples were dissolved in 50 µl pyridine (PIERCE, Rockford, USA) and 50 µl BSTFA [(N,O-bis (Trimethylsilyl) trifluoro acetamide, PIERCE)] in an Eppendorf tube and allowed to react at room temperature for 30 min and subsequently the mixture was evaporated to dryness in the speed-vac (Eppendorf Concentrator 5301). The pellet was redissolved in 150 µl toluene and the resulting solution was transferred into a 100-200 µl limited conical glass container that is placed in a 300 µl glass vial (12x32 mm, clear, Alltech GROM GmbH, Rottenburg-Hailfingen, Germany). The glass vial was tightly closed with a rubber cap and a metal ring and fitted into the GC auto sampler for sample injection.

A gas-chromatography system (Agilent technologies 6890N Network, USA) fitted with a high temperature ion source was used for the determination of ergosterol. The system was controlled with a PC containing chemstation software (G1701DA MSD Chemstation, Agilent 5973 Network MS coupled to 6890N GC system, Agilent 7683 series, Aligent, USA) automatic sample injector was used to inject samples of 1.0 µl with split ratio 40:1 at 320 ^{o}C. Separation was performed on a 30 m (length) x 0.25 mm (ID), 0.25 µm film thickness, DB-5 ms capillary column (Agilent 122-5532,

Agilent, Santa Clara, California, USA) with the following temperature program: initial trap 80 ^{o}C for 1 min, then 30 ^{o}C/min to 260 ^{o}C, then 7 ^{o}C/min to 320 ^{o}C holding 3.5 min to a final run of 22.26 min. An Agilent 5973 network mass selective detector has been used. Tune settings were as follows: Ion source temperature of 300^{o}C, electron multiplier at 200 V and 3 ion tune of 69, 218.9 and 502.

Cholesterol (10 μg/ml) was used as an internal standard (I.S.). An ergosterol spectrum standard curve for quantifying was obtained by using 200, 100, 50, 10, 2, 0.5, 0.1 and 0.01 μg/ml ergosterol. From the spectra data files, the peak area of cholesterol and ergosterol were derived from the coordinates m/z 329 and m/z 363, respectively, by using the automated peak detection and integration part of the chemstation software.

8.10.1.5 2D Electrophoresis

8.10.1.5.1 Precipitation of protein samples from culture supernatants with TCA

Supernatants were separated from fungal mycelium with a nylon mesh (0.3 mm pore size) from 7 day old cultures of *S. commune* wild type *4-39* and of the hydrophobin mutant Δ*Sc3 4-39*. Samples were centrifuged in 50 ml Falcon tubes at 20,000 rpm for at least 30 min to remove polysaccharides and other impurities. The solutions were decanted into fresh 50 ml Falcon tubes. TCA stock solution (45.4 ml water to 100 g TCA, kept at 4^{o}C) was added in amounts of 1/10 of the sample volumes, and the samples were vortexed and kept on ice overnight. After protein precipitation, samples were centrifuged at 4000 rpm for 30 min. The protein pellets were dispersed as much as possible with 3 ml of wash solution [80% acetone/20% Tris-Hcl (50 mM, pH 7.5) at 4^{o}C], by stirring or by sonication for a few seconds. The pellets were again centrifuged at 4000 rpm for 10 min and the pH of the supernatants were determined. The washing process with acetone/Tris-Hcl was repeated until the pHs of the supernatants were similar to the pH of the acetone-Tris stock solution (pH 7.5). Pellets were finally dispersed with 3 ml of 100% ice-cold acetone and incubated at -20^{o}C for 30 min. The pellets were then centrifuged at 4000 rpm for 10 min and the pHs of the supernatants were determined (usually, the pH should be between 5 and 6). The remaining pellets were air dried at room temperature.

8.10.1.5.2 First dimension of 2D-PAGE

The air dried pellets were dissolved in 100 µl rehydration buffer without DTT and without bromo-phenol blue [8 M urea, 4 % (w/v) CHAPS, 0.5 % IPG buffer (Amersham Biosciences, New Jersey, USA)]. A 4 µl sample was separated for measuring the amount of protein. The remaining sample was used for 2D-PAGE, dissolved in rehydration buffer [(with 1.25 ml bromophenol blue), 3.5 mg DTT and 125 µl ethylene glycol)] and thoroughly mixed. Sample solutions containing approximately 100 µg protein were mixed with already prepared rehydration buffer and made up to a final volume of 250 µl. The protein solutions were loaded onto 13 cm IPG-strips that were of a pH range 3-10 (Amersham Biosciences, New Jersey, USA). The IPG-strips of 13 cm length were placed onto the ceramic holders. The samples were then rehydrated and subsequently focused in an Ettan IPGphor isoelectric focusing machine (Amersham, New Jersey, USA) for a total of 40,000 Vhr at 15°C with the following settings: 50 µA per strip with rehydration (12 hr, 20 V, step and hold), 150 V (1hr), 200 V (1hr), 500 V (1hr), 1,000 Vhr, 8,000 V (gradient, 1 hr), 8000 V (focusing to 40,000 Vh), all according to the protocol of the manufacture.

In the 2nd dimension, proteins were separated on 12% PAGE (Poly Acryl amide gel Electrophoresis) using Electrophoresis unit (Biometra, Göttingen, Germany) together with a protein marker (Precision Protein Marker, 14–116 kDa, Fermentas, St. Leon-Rot, Germany). Proteins were separated at 15°C for 3 hrs in two steps: Step-1: 20 mA constant for 30 min (low voltage is applied for short time to allow the migration of proteins from the IPG strip onto the gels), step-2: 35 mA constant for approximately two and a half hours. The gels were then overnight fixed in fixation solution containing 30% methanol with 12% acetic acid and 0.5% formaldehyde and subsequently stained with silver (Blum et al. 1987). Stained gels were scanned at 300 dpi resolution (Microtek, TMA 1600, Hannover, Germany).

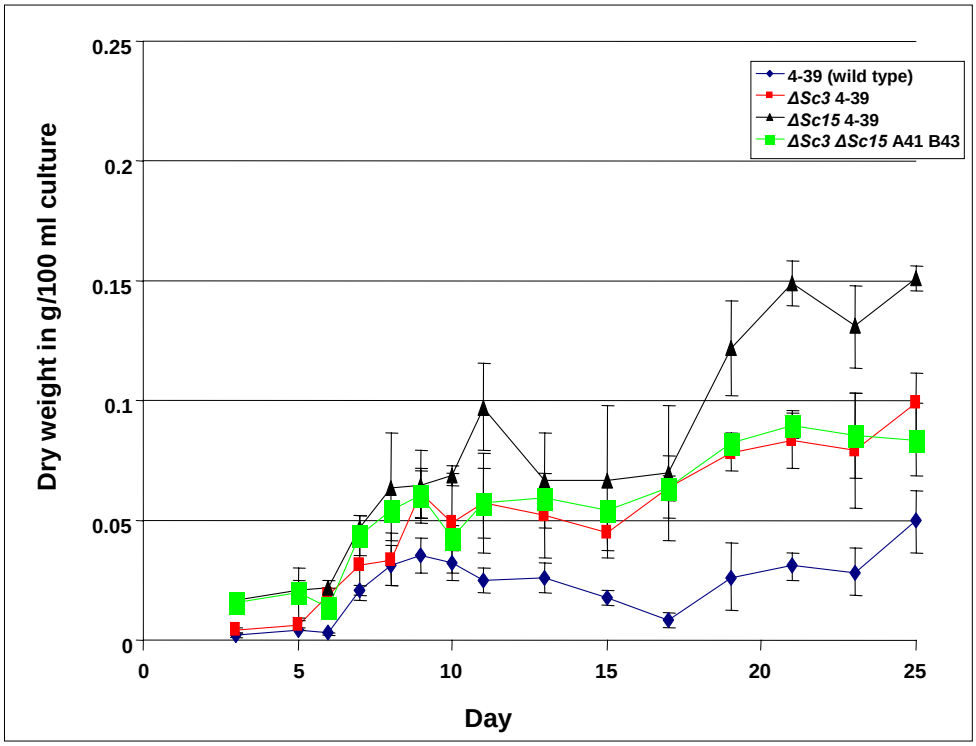
8.10.2 Results

8.10.2.1 Biomass production

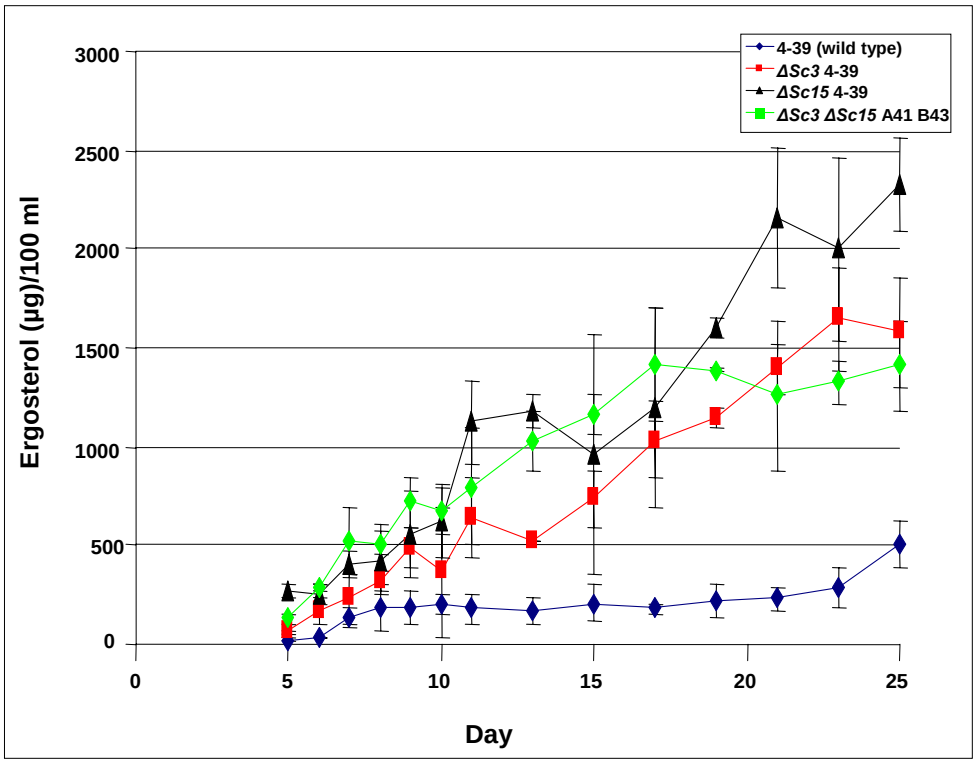
The biomass and the ergosterol content of the mycelia and the protein concentrations in the cultures supernatants were determined over the time for standing *S. commune* minimal medium liquid cultures of the *S. commune* wild type strain 4-39 and the

co-isogenic *S. commune* mutant strains *ΔSc3* 4-39, and *ΔSc15* 4-39 and the double mutant *ΔSc3ΔSc15 MAT*A41*MAT*B43. Of the four tested strains, the *ΔSc15* mutant had the highest biomass production and, unexpectedly, the wild type monokaryon 4-39 showed the lowest biomass (Fig. 3A). The two mutants lacking a functional SC3 hydrophobin gene had an alike biomass production to each other that was lower than that of the *ΔSc15* mutant. A similar order of strains were observed upon determination of the ergosterol contents in the biomass per 100 ml culture (Fig. 3B) and the protein amounts in the culture supernatant (Fig. 3C). When calculating the relative amounts of ergosterol to biomass (Fig. 3D), respectively the relative amount of protein in the supernatant to biomass (Fig. 3E), it became clear that the ergosterol values were too variable to allow any deeper conclusions on the effect of the hydrophobin and the SC15 deletions on permeability regulation of the membranes. In contrast, the relations between the amounts of proteins present in the supernatants to the biomass produced revealed in most instances similar values for all strains with no drastic variations.

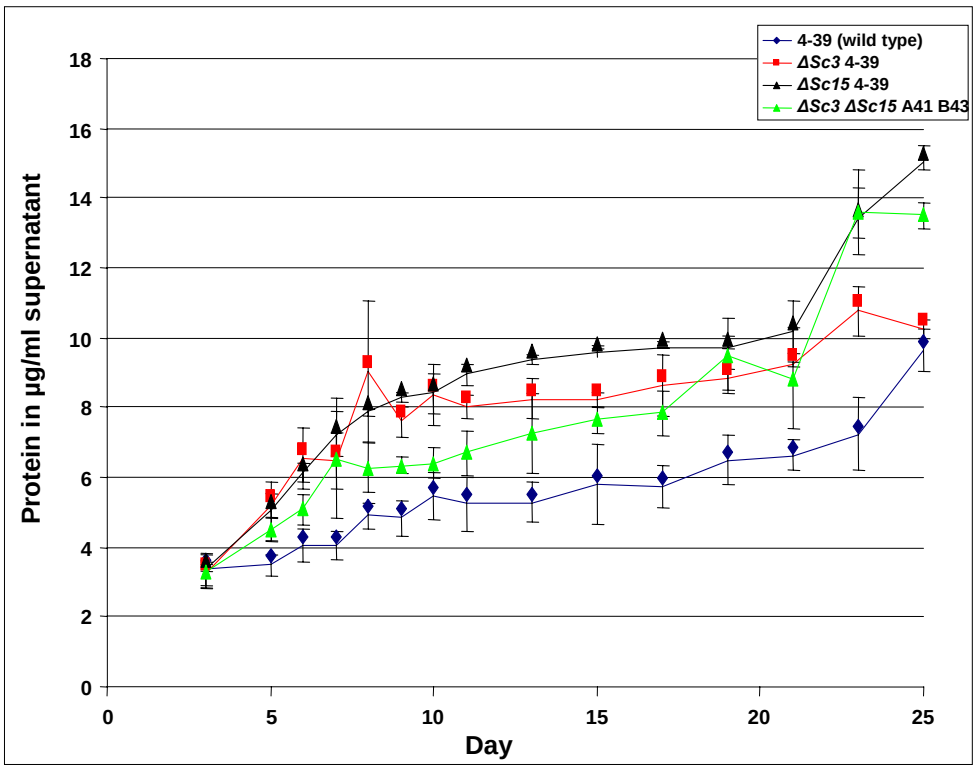
3A



3B


4-39 (wild type)
ΔSc3 4-39
ΔSc15 4-39
ΔSc3 ΔSc15 A41 B43
Ergosterol (µg)/100 ml
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Day

3C


4-39 (wild type)
ΔSc3 4-39
ΔSc15 4-39
ΔSc3 ΔSc15 A41 B43
Protein in µg/ml supernatant
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
Day

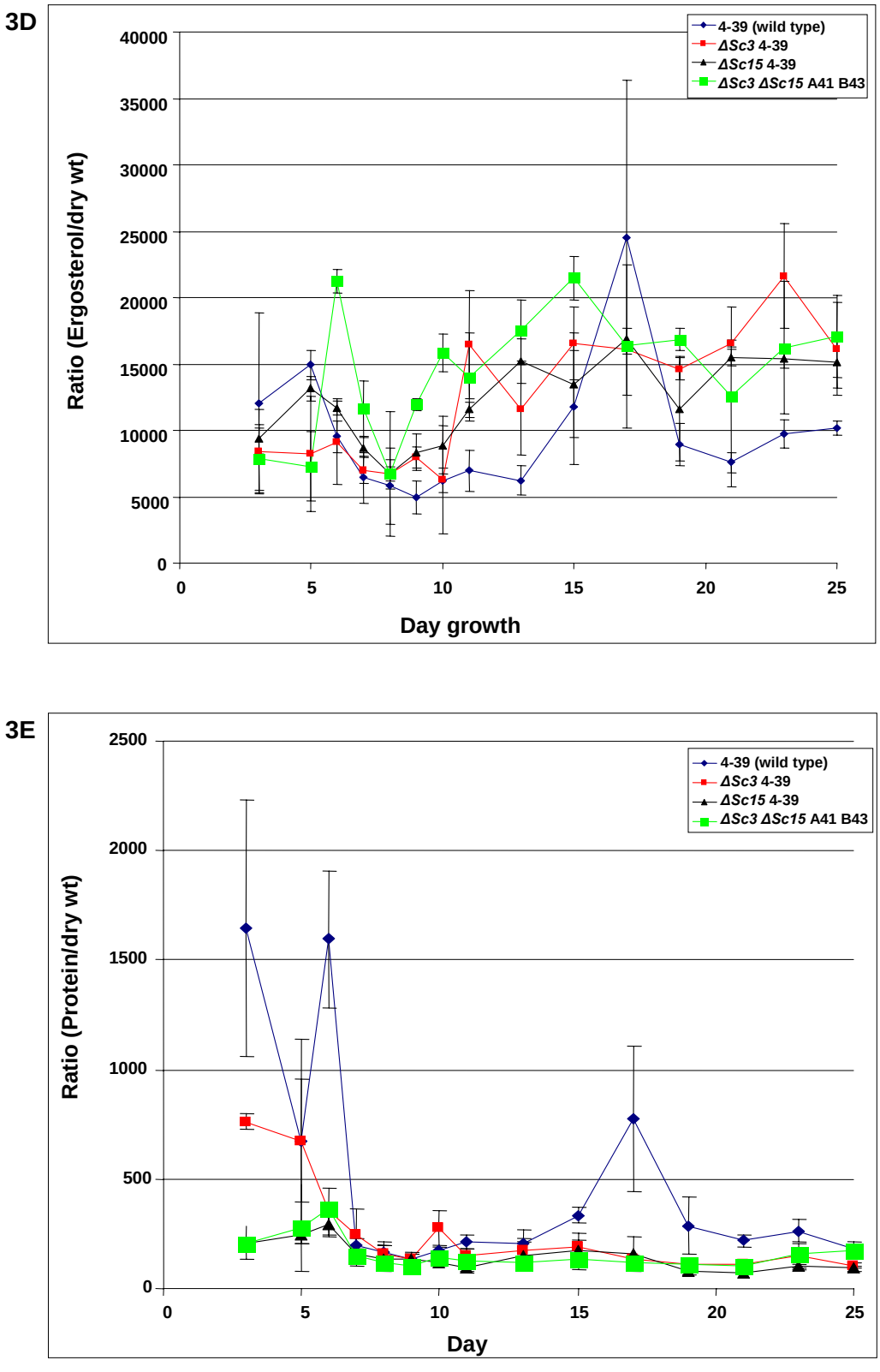


Fig. 3 Comparison of *S. commune* wild type strain *4-39*, its coisogenic SC3 mutant *ΔSc3* 4-39, the SC15 mutant *ΔSc15* 4-39, the SC3 SC15 mutant *ΔSc3 ΔSc15 MAT*A41*MAT*B43 of dry

weight in grams per 100 ml liquid culture (3A), Ergosterol (µg) per 100 ml culture medium (3B), Protein (µg) per ml culture medium (3C), Ratio of Ergosterol to biomass (3D), Ratio of Protein to biomass (3E).

8.10.2.2 Protein gel electrophoresis

Proteins from day 7 of cultivation of the wild type strain 4-39 and the SC3 hydrophobin mutant strain were collected and analysed by 2D-gel electrophoresis (see Fig. 1 in the general discussion of chapter 8). The data from the 2D gel electrophoresis showed that the proteins secreted into the culture supernatant by the wild type and by the Sc3 knockout mutant are mostly in the acidic range. There were several spots that appeared in the typical low molecular weight range of hydrophobins. By visual inspection of the gels, it appeared that a number of protein spots were specific to the wild type strain and the others specific to mutant strain. Also, there were more stronger spots in the mutant than in the wild type strain (Fig. 1).

8.10.3 Further conclusions

Care has however to be taken not to over interpret the data presented in this attachment. The wild type strain 4-39 showed a surprisingly poor growth during this experiment, a growth behaviour alike to the SC15 mutant strain *ΔSc15* 4-39 was instead expected for the wild type strain. In the literature, it has been repeatedly been reported that *S. commune* strains were prone in high frequencies to a transposon-induced mutant *thn* that regularly occurs during growth of the fungus and causes by unknown reasons a growth advantage compared to the normal wild type growth. The *thn* mutation has a serious effect on the appearance of the mycelium of the fungus - it causes a thin growth by producing thin hyphal growth (Fowler and Mitton 2000). Since in the experiment presented here the strains were not further analysed for their mycelial morphology, the comparison of protein production between the wild type and the SC3 hydrophobin mutant strain by 2D-analysis has to be repeated in the future to exclude the possibility that the differences seen were not due to new genetic defects that accumulated in one of the strains. With such a new analysis, it would be interesting to define by proteomic techniques the specific proteins that differ between the two strains.

The data that are presented in this appendix appear to show that hydrophobins likely affect the secretome of *S. commune*. The lack of current availability of the genome sequence of *S. commune* has limited our protein work to go-on further. Thus, the interesting and challenging task to identify the proteins that are expressed/secreted or not secreted in the absence of specific hydrophobin genes should soon be possible to address. This can be effectively performed when once the genome sequence is available. The genome of the fungus is currently in progress of sequencing at the JGI (Joint Genome Institute, http://www.jgi.doe.gov/index.html).

Curriculum vitae

Personal profile

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Education

Ph. D (Since April 2003)	:	Georg-August-University of Göttingen, Germany
Thesis title	:	"Hydrophobins in wood biology and technology"
M. Sc (1998-2000)	:	Chemistry, Devi Ahilya University, Indore, Madhya Pradesh, India
B. Sc. (1995-1998)	:	Botany, Zoology and Chemistry, Acharya Nagarjuna University, Andhra Pradesh, India

Work Experience

Senior Research Fellow (08.2001 - 02.2003)	:	National Agricultural Technology Project (World Bank Sponsored) of Indian Council of Agricultural Research, A.N.G.R. Agricultural University, India. Project title: "Evaluation of locally available feeds and fodders, improvement in their quality and formulation of economic and complete rations for ruminants in irrigated cotton based production system" Principal Investigator : Prof. Dr. G.V. Krishna Reddy

Research Assistant: (06.2000 -07.2001)	Dept. of Chemistry, Siddhartha Engineering College Vijayawada, India Project title: "Analysis of various water samples for different analytical parameters" Supervisor : Prof. Dr. Sarath Babu Mogallapu
Tutor in Chemistry: Part-time (09.2001- 06.2002)	Sri Krishnaveni Junior Science College, Vijayawada, India

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